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Active von Willebrand Factor in patients with a bleeding diathesis

Lisa N. van der Vorm [a,b,c], Dana Huskens [a,b,*], Lisa Florin [d], Pieter De Kesel [d], Mark Roest [a,b], Bas de Laat [a,b,c], Katrien M.J. Devreese [d,e]

[a] Cardiovascular Research Institute Maastricht, Maastricht University Medical Centre, Maastricht, the Netherlands
[b] Synapse Research Institute, Maastricht, the Netherlands
[c] Department of Clinical Chemistry and Haematology, Gelre Hospitals, Apeldoorn, the Netherlands
[d] Coagulation Laboratory, Department of Diagnostic Sciences, Ghent University, Ghent, Belgium
[e] Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium

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ABSTRACT

Introduction: Increased levels of circulating von Willebrand Factor (VWF) in its active, platelet-binding conformation have been implicated in the pathogenesis of several thrombotic conditions as well as bleeding conditions characterized by severe thrombocytopenia. However, it is unclear whether the proportion of activated VWF in the circulation also plays a role in patients with mild to moderate bleeding without thrombocytopenia.

Methods: Citrated plasma samples were collected from 145 patients with a bleeding diathesis with unknown cause. Active VWF levels were measured with an in-house developed ELISA assay. In addition, VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo) and (flow-cytometric) platelet-VWF binding (Plt:VWF) were determined.

Results: Active VWF levels were on average mildly, but not significantly, lowered in patients with a bleeding diathesis compared to the reference interval (especially in individuals with non-O blood groups). Active VWF was not significantly different for subjects with (median 107.4%, IQR 18.3) versus without (median 111.1%, IQR 32.3%) an increased bleeding score, nor between subjects with suspected VWD (median 104%, IQR 20.6%) versus other suspected causes of bleeding diathesis (median 111.7%, IQR 33.3%).

Conclusion: In this clinically heterogeneous population of patients with a mild bleeding phenotype, quantification of active VWF levels does not have added diagnostic value to VWF:Ag and VWF activity assays in the diagnosis of unexplained bleeding disorders.

Dear Editors,

Whereas the diagnosis of established bleeding disorders, such as haemophilia, is well defined, there are many patients with a mild to moderate bleeding diathesis (e.g. frequent epistaxis, menorrhagia, prolonged post-operative bleeding) of unknown cause, who pose a significant diagnostic challenge [1]. Following initial screening based on personal/family history, bleeding scores, full blood cell count and routine coagulation screening tests (e.g. PT and aPTT), these patients can be referred for further evaluation of clotting factor activity, platelet function testing (e.g. PFA-200®, light transmission aggregometry (LTA)) and von Willebrand factor (VWF) antigen/activity [2]. The diagnosis of von Willebrand Disease (VWD) is based on VWF antigen concentration (VWF:Ag) and VWF function tests, most commonly the VWF ristocetin cofactor activity (VWF:RCo) [3]. However, VWF:RCo assays suffer from high variability and may give falsely low VWF activities as a result of polymorphisms. A more recently developed assay uses recombinant GPIb fragments with two gain-of-function mutations that allow binding to VWF (VWF:GPIbM) [3]. Although this assay does not require ristocetin like the VWF:RCo assay, it is based on non-physiological binding of VWF to a mutant receptor.

Recently, we developed an immunosorbent assay, based on a variable heavy chain antibody fragment (VHH), to quantify active VWF. This is the haemostatically active fraction of VWF that is circulating *in vivo* in its platelet GP1bα-binding conformation. In a healthy study population, only a small proportion of VWF was in its active conformation [4]. Several pathological conditions have been associated with increased levels of active VWF, using an assay based on the same VHH for active VWF [5]. The spontaneous interaction between active VWF and platelets can tilt the haemostatic balance to either the prothrombotic (e.g. TTP and

* Corresponding author. Synapse Research Institute, Pastoor Habetsstraat 50, 6217 KM, Maastricht, the Netherlands.
E-mail address: d.huskens@thrombin.com (D. Huskens).

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HELLP syndrome, associated with on average 2 to 12-fold and 3-fold increased active VWF levels compared to healthy controls, respectively) or the bleeding (e.g. von Willebrand disease (VWD) type 2B) side [5]. In VWD type 2B, enhanced binding of platelets to active VWF (2–15 fold increased levels compared to healthy controls [5], due to gain-of-function mutations in the A1 domain) results in clearance of high-molecular-weight (HMW) VWF multimers and platelets, and consequently a bleeding phenotype.

However, it is unclear whether the proportion of activated VWF in the circulation also plays a role in patients with mild to moderate bleeding without thrombocytopenia. It can be hypothesized that, since individuals with relatively high circulating active VWF levels are more at risk for spontaneous platelet-aggregate formation, relatively low circulating active VWF levels may delay clot formation and hence contribute to a mild bleeding diathesis. Therefore, we investigated whether in a population of patients with a mild bleeding diathesis there were more patients with very low active VWF levels compared to a control population. In addition, we sought to identify cases in which active VWF levels may have additional diagnostic value over the commonly used VWF:Ag and VWF:RCo assays.

Between December 2014 and December 2017, patients with a mild bleeding diathesis of unknown cause were recruited for participation in the current study in the Ghent University Hospital in Belgium. All patients were referred by their haematologist for laboratory evaluation for diagnosis of a bleeding disorder. The types of bleeding seen in these patients were frequent hematomas (n = 23), epistaxis (n = 19), menorrhagia (n = 6), petechiae/ecchymoses (n = 9), heavy postpartum bleeding (n = 6), prolonged post-surgical bleeding (n = 10), gingival bleeding (n = 4) and combinations of these bleeding symptoms. For 13 patients there was a clinical suspicion of von Willebrand's disease (as depicted in Fig. 2B). The study was approved by the Ethical Committee of the Ghent University Hospital. Citrated whole blood was collected by antecubital venipuncture (BD Vacutainer, Becton Dickinson, Plymouth, UK). Platelet-poor plasma (PPP) was prepared and subsequently stored at −80 °C until further testing. Active VWF was quantified using an ELISA, based on a variable heavy chain antibody (VHH) specific for VWF in its GP1bα-binding conformation, as described previously [4]. Normal pooled plasma (NPP) was used as a standard in every plate and sample results were normalized (%) to NPP on the same plate. VWF antigen (VWF:Ag) and VWF:RCo were measured using the HemosIL® AcuStar VWF:Ag assay and the HemosIL® AcuStar VWF:RCo assay, respectively (IL-Werfen, Bedford, MA, USA). Platelet-VWF binding (Plt:VWF) was measured by flow cytometry, as described previously [4], and is expressed as median fluorescence intensity (MFI), corrected for the MFI of the control condition without ristocetin. The ISTH-BAT bleeding scores were determined by consulting the medical records, by one and the same person (clinical pathologist) to avoid bias, and were considered elevated if > 5 in women, >3 in men and >2 in children [6]. Associations between continuous variables were determined using the non-parametric Spearman's rank correlation coefficient. Differences in active VWF levels between two groups were compared using the non-parametric Mann-Whitney U test. Comparison of active VWF levels between three groups was performed using the non-parametric Kruskal-Wallis test. A p-value of 0.05 was considered statistically significant for all comparisons.

We included 145 patients (32 men, 89 women, 24 children) with a bleeding diathesis of unknown cause. The ISTH-BAT bleeding score was documented for 109 subjects (24 men, 68 women, 17 children), and was increased above the respective reference ranges [6] for 10 men, 12 women and 7 of the included children.

Active VWF levels in the entire study population (n = 145) correlated significantly with all other VWF parameters (except Plt:VWF binding with low ristocetin), with almost identical correlation coefficients to those previously found in a healthy population [4]. The strongest correlation (Spearman's r = 0.442, p < 0.001) existed between active VWF and VWF:Ag, followed by VWF:RCo (r = 0.399, p < 0.001) and Plt-VWF binding in the presence of a high (1.2 mg/mL) concentration of ristocetin (r = 0.386, p < 0.001).

In these subjects with a bleeding tendency, active VWF, VWF:Ag levels and VWF:RCo activity were relatively lower, albeit not statistically significant, compared to the previously determined reference intervals

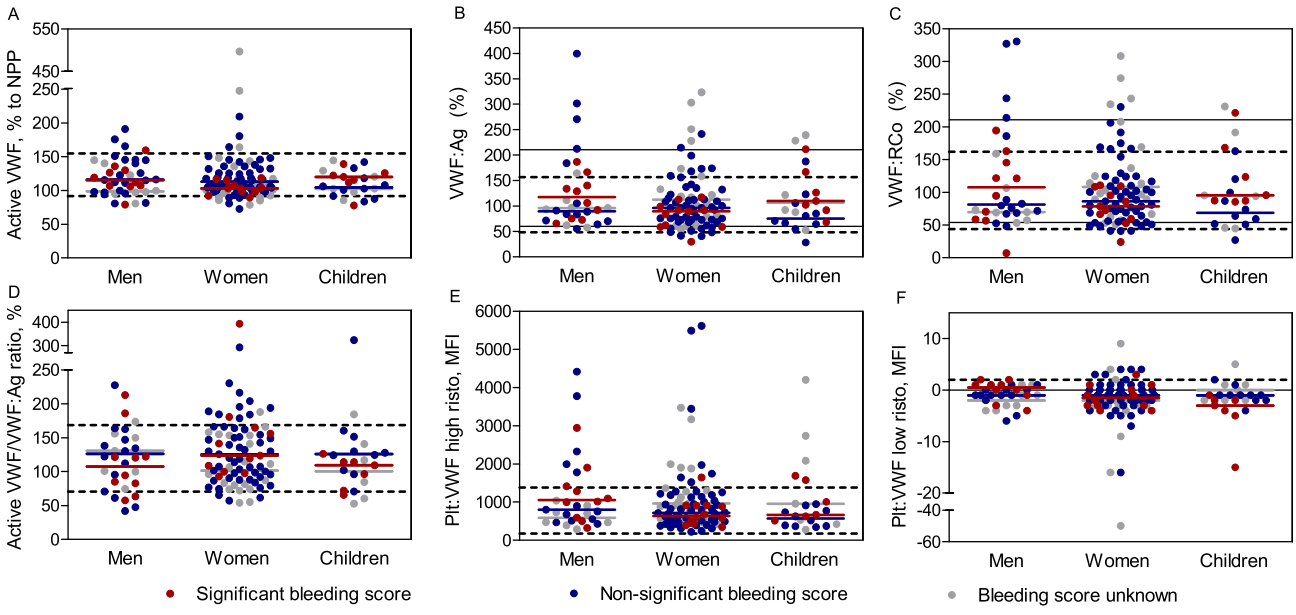

Fig. 1. VWF parameters in patients with a bleeding diathesis compared to reference values. Patients with a significant bleeding score according to the ISTH-BAT classification are depicted as red dots, those with a non-significant bleeding score as blue dots and individuals with an unknown bleeding score are depicted as grey dots. Reference ranges are depicted as dashed lines. For VWF:Ag and VWF:RCo, solid lines represent reference intervals for blood group non-O and dashed lines for blood group O. For Plt:VWF binding with high ristocetin the reference interval was previously determined in 56 healthy donors. For Plt:VWF binding with low ristocetin only the upper limit is depicted as a dashed line, the lower limit is not relevant. For both Plt:VWF with low and high ristocetin, MFI values were corrected for MFI values of an unstimulated (no ristocetin) control condition. The horizontal bars represent the medians, with the colour indicating the group based on bleeding score. Abbreviations: NPP, normal pooled plasma; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity; Plt:VWF, platelet to VWF binding; Risto, ristocetin. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

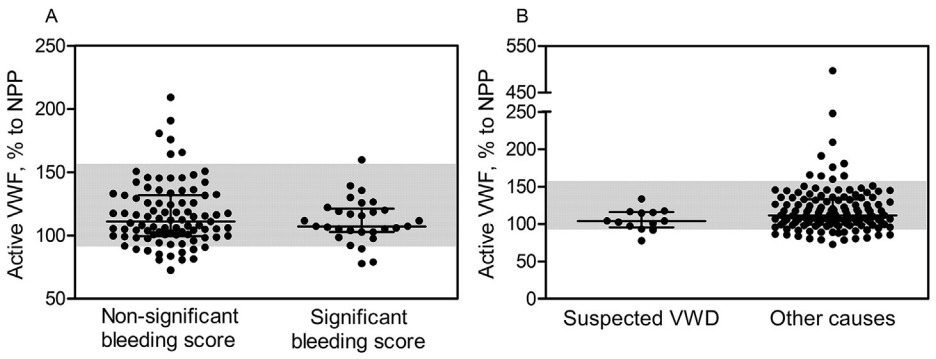

Fig. 2. Active VWF related to clinically significant bleeding and suspected VWD. (A) Active VWF is not different in subjects with significant bleeding (defined as >5 in women, >3 in men and >2 in children) and (B) not different in subjects with suspected von Willebrand disease versus other suspected causes of the bleeding diathesis. The grey area delineates the reference interval determined in a previous study in healthy individuals [4]. Horizontal bars represent the median. Abbreviations: NPP, normal pooled plasma; VWD, von Willebrand disease.

[7], especially the reference interval for blood group non-O individuals (Fig. 1A–D). Several individuals had higher, but none had lower Plt:VWF binding in response to ristocetin compared to the reference range in healthy controls (Fig. 1E and F). Red dots indicate individuals with a significant bleeding score. Active VWF was not significantly different for subjects with versus without an increased bleeding score (Fig. 2A), although it must be noted that the bleeding score does not always correlate optimally with clinical bleeding tendency [8].

Active VWF was not significantly different between subjects with suspected VWD versus other suspected causes of bleeding diathesis (Fig. 2B). In VWD 2B patients, bleeding is due to thrombocytopenia as a result of the clearance of platelet-VWF-aggregates. In the current study 31 patients had a platelet count below 150×10^9/L, of which three patients had a platelet count below 50×10^9/L. Only one of these three thrombocytopenic patients had active VWF levels above the reference range (209.2%), concurrently with mildly elevated VWF:Ag (241.6%) and normal VWF:RCo activity (206.4%), hence this thrombocytopenia is not due to VWD type 2B, but may rather be explained by a thrombocytopathy.

In one patient with a bleeding diathesis, active VWF levels were increased almost 5-fold (497%), in conjunction with substantially increased VWF:Ag (323.5%) and mildly elevated VWF:RCo (234.3%) and FVIII (200.0%) levels. The platelet count was normal (232×10^9/L). However, light transmission aggregometry (LTA) demonstrated a platelet ATP secretion defect and primary wave disaggregation upon stimulation with epinephrine. A second patient with strongly elevated active VWF levels (247.5%) had a similar pattern of increased VWF level and activity, and was also suspected for a platelet secretion defect. Thus, the bleeding symptoms in these patients can be explained by platelet secretion defects, but the cause of the high (active) VWF levels and activity in these patients remains unknown. However, since both were elderly patients (70 and 77 years) they may be (partly) attributed to comorbidities that affect endothelial VWF secretion and activation, in particular inflammatory conditions. Inflammation induces activation of the endothelium, resulting in excessive release of VWF into the circulation, whereas inflammation reduces the activity of the VWF-cleaving protease ADAMTS13, hence active VWF levels increase [9]. Unfortunately, no inflammatory markers (e.g. CRP) were measured to confirm this was the cause of the elevated active VWF levels.

One patient with an active VWF level that was not above the reference interval but relatively high (142%) compared to the low VWF:Ag (54.6%) and VWF:RCo (50.8%) was diagnosed with Henoch-Schonlein purpura. This is a rare inflammatory disease of the small blood vessels. The presence of abnormally large (and hence active) multimers has previously been described for patients with this disease [10].

On the other end of the spectrum, three patients with a bleeding tendency of unknown cause had active VWF levels below the lower limit of the reference interval (91.6%). All also had VWF:Ag (41.6–66.6%) and VWF:RCo (41.2–57.9%) on the lower end of their reference intervals with normal FVIII levels (69.1–86.2%) and platelet count (159–269 $\times 10^9$/L). Their bleeding symptoms were not more severe or distinct from those of the patients with increased active VWF, hence the decreased active VWF levels seem to have no clinical relevance in these patients.

In conclusion, in this highly heterogeneous population of individuals with a bleeding diathesis, active VWF levels were on average slightly, albeit not significantly, lower compared to healthy individuals. The diagnosis of mild bleeding disorders is very challenging, and in approximately half of the cases, the underlying cause is not found [1]. This study adds that, in this population, quantification of active VWF levels does not have added diagnostic value to VWF:Ag and VWF activity assays in the diagnosis of unexplained bleeding disorders.

Declaration of competing interest

The authors declare no conflict of interest.

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