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The Clinical Application of a Novel Ristocetin-Independent von Willebrand Factor Activity Assay

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# The Clinical Application of a Novel Ristocetin-Independent von Willebrand Factor Activity Assay

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An abstract of
A thesis submitted to the Faculty of the
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## Abstract

The Clinical Application of a Novel Ristocetin-Independent von Willebrand Factor Activity Assay

By Stefanie DiGiandomenico, M.D., M.S.

*Background:* Von Willebrand disease (VWD) and acquired von Willebrand syndrome (AVWS) are bleeding disorders that can result in significant morbidity and mortality. Initial screening includes measuring von Willebrand factor (VWF) activity. Current gold standard activity assays, ristocetin cofactor (VWF:RCo) and glycoprotein IbM (VWF:GPIbM), have limitations that can hinder accurate diagnosis. The novel glycoprotein Ib nanobody (GPIbNab) assay may mitigate these diagnostic challenges, but its clinical application remains unexplored.

*Objective:* To assess concordance of GPIbNab with gold standard activity assays in individuals with and without VWD or AVWS.

*Methods:* This was a cross-sectional study of subjects who were evaluated for a bleeding disorder or have a diagnosis of VWD or AVWS. Performance characteristics of the GPIbNab assay (GPIbNab:6C11 and GPIbNab:6D12) were assessed. All subjects presented with a historic diagnosis, or lack thereof, based on gold standard activity assays as determined by the local treating physician. Subjects were then assigned a diagnosis based on GPIbNab and VWF antigen (VWF:Ag). Criteria for diagnosis of VWD or AVWS was a GPIbNab or VWF:Ag level <50 IU/dL. A receiver operating characteristic curve determined the optimal cutpoint for GPIbNab activity to VWF:Ag ratio. Pearson correlation analysis measured concordance between assays.

*Results:* 91 samples from 82 individuals undergoing evaluation for VWD and 21 samples from 17 individuals undergoing evaluation for AVWS were collected from August 2021 to September 2023. *VWD Cohort*: There were 39 diagnoses of type 1 VWD, 12 of type 2 VWD, 4 of VWD of unclear type, and 2 historic diagnoses of VWD. Twenty-five patients did not have a diagnosis of VWD. There were statistically significant, positive correlations between GPIbNab:6D12 and VWF:GPIbM ($r$=0.80) and VWF:RCo ($r$=0.71), and between GPIbNab:6C11 and VWF:GPIbM ($r$=0.78) and VWF:RCo ($r$=0.51). *AVWS Cohort*: 10 of 17 subjects had a diagnosis of AVWS. There were statistically significant, positive correlations between VWF:GPIbM and GPIbNab:6D12 (r=0.88) and VWF:GPIbM and GPIbNab:6C11 (r=0.89). There were moderately positive correlations between both GPIbNab assays and VWF:RCo, though these results were not statistically significant.

*Conclusion:* The novel GPIbNab assay strongly correlates with VWF:GPIbM in the diagnosis of VWD and AVWS, supporting its potential as a valuable diagnostic tool.

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## A. BACKGROUND

### Von Willebrand Disease and Acquired Von Willebrand Syndrome

Von Willebrand disease (VWD) is the most common inherited bleeding disorder, with a prevalence estimated between 0.1% and 1% of the population[1,2]. VWD is caused by qualitative or quantitative defects in von Willebrand factor (VWF). VWF bridges platelets to sites of endothelial injury and binds factor VIII, thus playing a critical role in the initiation of primary hemostasis[3]. VWF circulates in the body as multimers, varying in size from 500 to 20,000 kDa. The most active forms of VWF are high molecular weight multimers (HMWM)[4].

VWD is classified into three major types according to underlying defect. Types 1 and 3 result from a deficiency in VWF, with mild to moderate deficiency defining type 1 and severe deficiency characterizing type 3. A distinct subtype of type 1, type 1C, is associated with accelerated clearance of VWF from circulation. Type 2 VWD, on the other hand, is a qualitative disorder resulting from dysfunctional VWF. It is further divided into four variants: 2A, 2B, 2M, and 2N. Both types 2A and 2M involve impaired VWF binding to platelets, but type 2A is distinguished by a reduction in HMWM, whereas these multimers remain intact in type 2M. Type 2B is caused by a gain-of-function mutation in VWF that increases binding of VWF to platelets. In type 2N, defecting VWF binding to factor VIII leads to low factor VIII levels[5]. Type 1 is the most common form of VWD, accounting for 70-80% of cases, whereas type 3 is the least common, occurring in less than 5% of cases[6].

Mild mucocutaneous bleeding is often the defining feature for VWD, though bleeding severity can range from minimal to life-threatening. Type of VWD, age, gender, and VWF activity levels can impact bleeding phenotype[5]. Validated bleeding questionnaires, such as the International Society for Thrombosis and Haemostasis bleeding assessment tool (ISTH-BAT), may help identify persons with significant bleeding diatheses and can act as screening devices for bleeding disorders such as VWD[6,7].

In contrast to inherited VWD, acquired von Willebrand syndrome (AVWS) is rare, and is predominantly seen in older individuals; AVWS is often associated with hematologic disorders, malignancy, autoimmunity, or cardiovascular disorders[8-10]. The pathophysiology of AVWS generally involves increased clearance or degradation of VWF, resulting in functional defects or deficiency. The specific mechanism by which this is achieved is dependent on the underlying condition. For example, mechanical stress from cardiac valvulopathies can cause sheering of HMWM, whereas increased clearance of VWF is observed in lymphoproliferative diseases due to selective adsorption of HMWM on malignant cells[11,12]. Like VWD, mild mucocutaneous bleeding is most common manifestation; however, bleeding severity can vary based on the associated disease, with more severe bleeding observed in individuals with lymphoproliferative disorders[11,13].

Despite the prevalence of VWD and the potential for devastating complications in either the inherited or acquired disorder, the accurate diagnosis of these entities remains challenging.

## Diagnosing VWD and AVWS

VWD is diagnosed by an evaluation of bleeding history and supportive laboratory data. The initial screening for VWD and AVWS typically includes measuring VWF antigen (VWF:Ag) levels, factor VIII activity, and VWF activity. The ristocetin cofactor assay (VWF:RCo) and glycoprotein IbM assay (VWF:GPIbM) are considered gold standards for measuring VWF activity.

Recent guidelines from the American Society of Hematology, ISTH, National Bleeding Disorders Foundation (formerly National Hemophilia Foundation), and World Federation of Hemophilia, define diagnosis of VWD as either VWD level <30 IU/dL regardless of bleeding history or <50 IU/dL with a history of bleeding. To further delineate between type 1 and type 2 VWD, VWF activity to VWF:Ag ratio is calculated. A ratio of greater that 0.7 is consistent with type 1, whereas a ratio less than 0.7 is indicative of type 2. The cutoff point of <0.7 was chosen to confirm type 2 VWD based on pooled sensitivity of 0.90 from six observational studies. The sensitivity for the previous cut-point of <0.5 was 0.58 to 0.79[14]. Second-tier testing, including genetic testing, may be pursued as needed to reach a diagnosis[14]. In AVWS, VWF activity levels are usually reduced, whereas VWF:Ag levels can be normal or mildly low. VWF multimers are often abnormal[15].

*VWF:RCo*

VWF:RCo is a platelet-dependent assay that measures GPIb receptor binding to VWF[16]. Although it is a commonly used and widely available test, its utility is limited by its high coefficient of variation, estimated to be between 40 and 50%, and poor accuracy in defining activity levels less than 10-20%[16-19]. Sensitivity estimates range from 0.83 to 1.00[20]. Additionally, it is susceptible to common sequence variations in the A1 domain of the *VWF* gene, such as the D1472H polymorphism in the C-terminal autoinhibitory module of VWF, which can lead to falsely low VWF:RCo levels. One study reported that the D1472H polymorphism was present in 63% of African Americans, compared to just 17% of Caucasians[21]. This variation can result in discrepant results between ethnic groups and a misdiagnosis of VWD[21].

*VWF:GPIbM*

VWF:GPIbM is a ristocetin-independent activity assay that leverages gain-of-function mutations to create a modified GPIbα receptor that can spontaneously bind VWF[22]. In a meta-analysis assessing sensitivity of VWF:GPIbM across 2 studies, range was 0.62 to 0.82[20]. Unlike VWF:RCo, it is unaffected by common sequence variations in the VWF gene[21]. However, few centers possess the technology to run this assay, and it may incur a higher cost relative to VWF:RCo[23]. In addition, falsely high levels may be seen in individuals with type 2B VWD, likely related to the gain of GPIbα function inherent to the VWF:GPIBM assay[23,24].

**A Novel Activity Assay**

Nanobody-triggered glycoprotein Ib binding assay (GPIbNab) is a novel activity platform that uses nanobodies rather than ristocetin to activate VWF[25]. Nanobodies are the heavy chain fragments of antibodies and are engineered to bind specific epitopes[26]. This technology can be leveraged to measure platelet-binding activity of VWF[25].

The A1 domain of VWF is the primary site for platelet binding[27]. When activated, it can bind the ligand-binding domain of the GPIbα receptor on platelets, ultimately leading to clot formation. Flanking either side of the A1 domain are two autoinhibitory modules, the C-terminal autoinhibitory module (C-AIM) and the N-terminal autoinhibitory module (N-AIM)[28]. When the autoinhibitory modules adopt a closed conformation, the A1 domain is inactive. However, when these modules are in an open conformation, the A1 domain becomes activated. GPIbNab targets specific residues in the N-AIM, inducing an open conformation of the autoinhibitory modules and thus activating the A1 domain. Binding of the A1 domain to the recombinant GPIbα ligand binding domain is measured by ELISA and reported as the activity level[25].

*Preclinical Results*

In preclinical studies, commercially available VWD samples were used to evaluate GPIbNab's performance. In type 1 VWD samples, GPIbNab activity levels were decreased, similar to VWF:Ag levels. Compared to type 1 VWD samples, the GPIbNab to VWF:Ag ratio was significantly decreased in type 2 VWD samples. In type 3 VWD samples, GPIbNab activity was undetectable. The presence or absence of the D1472H polymorphism had no effect on nanobody binding. Additionally, GPIbNab can distinguish between high and low proportions of HMWM by differentiating platelet-

binding activity of VWF multimers, a feature not described with either the VWF:RCo or VWF:GPIbM assays[29].

While this early data is promising, whether GPIbNab has the same diagnostic ability as our current gold standard assays in the clinical setting is unknown. This study aims to characterize the test performance of GPIbNab and assess its concordance with VWF:GPIbM and VWF:RCo in individuals with and without VWD and AVWS. We hypothesize that GPIbNab will correlate positively with gold standard assays and exhibit high sensitivity and specificity for diagnosing VWD and AVWS. Additionally, we will compare GPIbNab activity levels across different racial and ethnic groups, hypothesizing no significant differences in average activity levels when stratified by these categories.

## B. METHODS

### Study Aims

1. Describe test characteristics, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for GPIbNab in the diagnosis of VWD.
2. Evaluate concordance between GPIbNab, VWF:GPIbM, and VWF:RCo activity assays in individuals with and without VWD by assessing correlation and comparing average activity values.
3. Compare average GPIbNab activity levels between individuals with and without VWD, stratified by (1) race and (2) ethnicity.
4. Evaluate concordance between GPIbNab, VWF:GPIbM, and VWF:RCo activity assays in individuals with and without AVWS.

### Study Design, Setting, and Study Population

This was a cross-sectional study conducted at Children's Healthcare of Atlanta and Emory University. Samples were collected between August 2021 and September 2023. Our study population included pediatric and adult patients who have undergone a bleeding disorder evaluation or have a diagnosis of VWD or AVWS at Children's Healthcare of Atlanta or Emory University. Pediatric samples were obtained through an IRB-approved VWD Registry Program that is funded through Hemophilia of Georgia

and is active within the Hemophilia of Georgia Center for Bleeding and Clotting Disorders and Children's Healthcare of Atlanta. Adult samples were obtained through an IRB-approved biorepository at Emory University. Samples were excluded if there was no VWF activity, either VWF:RCo or VWF:GPIbM, available. Relevant patient variables, including age, sex, race, ethnicity, baseline VWD/AVWS labs, blood group, and genetic testing to assess the presence of D1472H polymorphism, was extracted through medical chart review. Bleeding scores from the validated International Society on Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH-BAT) was used to quantify bleeding severity at diagnosis[6].

**Sample Collection**

Specimens for the biorepository at Emory University are collected as part of routine clinical testing. The VWD Registry Program collects patient samples at the time of enrollment with additional samples collected annually if otherwise indicated by clinical need. Blood is drawn via venipuncture into blue-topped sodium citrate tubes and centrifuged. Plasma is then separated from cells and frozen in aliquots within four hours of collection. Aliquots not required for clinical testing are stored at -80°C until all testing is complete, after which they are de-identified and preserved for long-term storage at -80°C in the repository. Before use in VWF assays, samples are rapidly thawed in a 37°C water bath.

## Laboratory Assays

*GPIbNab Assay*

Recombinant ligand-binding domain of GPIbα was produced from transfected Expi293F cells, and VWF-activating nanobody expression occurred via E. coli and was purified using gel filtration chromatography and Ni-affinity chromatography following established methods[25].

Platelet GPIb binding to VWF was assessed using the GPIbNab assay as previously described[29]. 96-well ELISA microplates (Corning) were coated first with 1.0 μg /ml anti-GPIbα monoclonal antibody and then with 1.0 μg /ml ligand-binding domain. Activating nanobody at indicated concentrations was then added. A horseradish peroxidase labeled goat anti-rabbit IgG antibody (Santa Cruz Biotechnology) and polyclonal anti-VWF antibody (Dako) were used to probe bound VWF. Bound VWF was then detected via hydrolysis of 3,3',5,5'-tetramethylbenzidine.

*VWF:Ag Assay*

VWF:Ag was measured with ELISA using polyclonal antibody for capture and horseradish peroxidase-conjugated anti-human VWF antibody for detection (Dako)[29].

*VWF:RCo Assay*

VWF:RCo was measured with commercial BC von Willebrand reagent (Siemens Healtchare diagnostics). Veronal Buffer was added to patient plasma, followed by ristocetin. Change in turbidity was used to assess platelet agglutination[30].

*VWF:GPIbM Assay*

VWF:GPIbM was measured via the INNOVANCE VWF Ac reagent (Siemens Healthcare Diagnostics) on a Sysmex analyzer. Veronal buffer was added to patient plasma, followed by heterophilic blocking reagent, recombinant GPIb, and anti-GPIb antibody-coated polystyrene particles. Change in turbidity was used to assess platelet agglutination [30].

## Diagnosis

Prior to initiation of the study, all patients had a historic diagnosis – or lack thereof – based on gold standard activity assays as assessed by the local treating physician. Each subject was then assigned a separate diagnosis based on GPIbNab and VWF:Ag values. Criteria for diagnosis of VWD or AVWS will be GPIbNab or VWF:Ag level <50 IU/dL. A receiver operating curve was used to determine optimal cut-point for GPIbNab activity to VWF:Ag ratio. Sensitivity was prioritized. These cut-points were then used to differentiate between type 1 and type 2 VWD.

## Statistical Analysis

Sensitivity, specificity, PPV, and NPV were calculated to assess test characteristics. True positives, false negatives, true negatives, and false positives were based on historic diagnoses assigned by local treating physicians using gold standard

activity assays. Correlation amongst assays was measured using Pearson correlation analysis. Comparisons in activity levels between assays were assessed by Dunnett's method. Bonferroni adjustment for multiple comparisons was used if there are more than one pair of comparisons. VWF:RCo levels <10 (the lower limit of detection) were assigned a value of 5. Statistical significance will be set at a p-value of <0.05.

## C. RESULTS

### VWD Cohort

Ninety-one samples from 82 individuals undergoing VWD evaluations were collected from August 2021 to September 2023. There were 39 (47.6%) diagnoses of type 1 VWD, 12 (14.6%) of type 2 VWD, 4 (4.9%) of VWD of unclear type (type 1 versus type 2), and 2 (2.4%) historic diagnoses of VWD. 25 (30.5%) patients did not have a diagnosis of VWD (Table 1). There were 91 evaluable GPIbNab:6D12 and GPIbNab:6C11 values, 47 VWF:GPIbM values, and 25 VWF:RCo values. 2 of the 25 VWF:RCo values were <10 IU/dL and therefore assigned a value of 5 IU/dL. The lowest limit of detection for GPIbNab:6D12 was 6.1 IU/dL and for GPIbNab:6C11 was 3.4 IU/dL.

Median age was 20.0 years (range 3.6 – 79.4). Sixty-seven percent (n=55) of subjects were female. For those who reported race and ethnicity, 26.8% (n=22) identified as Black or African American, 58.5% (n=48) identified as White or Caucasian, 8.5% (n=7) identified as Hispanic, and 47.6% (n=39) identified as non-Hispanic (Table 2).

We first constructed a receiver operator curve to determine optimal cutoff points for GPIbNab to VWF:Ag ratio. For GPIbNab:6D12, the optimal cutoff point was 0.649, which yielded a sensitivity and specificity of .96 and .31, respectively, for distinguishing between type 1 and type 2 VWD (Figure 1). The optimal cutoff point for GPIbNab:6C11 was 0.525. The sensitivity with this cutoff point was 0.71, and the specificity was 0.69 (Figure 2).

We then calculated test characteristics for GPIbNab in diagnosing VWD versus no VWD. Sensitivity and specificity of GPIbNab:6D12 for diagnosing VWD versus no VWD was 0.79 and 0.76, respectively. PPV was 0.87 and NPV was 0.65 (Table 3). GPIbNab:6C11 yielded a sensitivity of 0.89 and specificity of 0.52, with PPV of 0.78 and NPV of 0.71 (Table 4).

Concordance between GPIbNab, VWF:RCo, and VWF:GPIbM was assessed next. We first compared median activity values, stratified by diagnosis. In samples without VWD (Figure 3), median activity values were 101.24 IU/dL for GPIbNab:6D12 (n= 27), 63.09 IU/dL for GPIbNab:6C11 (n=27), 50.5 IU/dL for VWF:RCo (n=2), and 102.46 IU/dL for VWF:GPIbM (n=25). There was a significant difference between VWF:GPIbM and GPIbNab:6C11 ($p = 0.001$). Comparison between VWF:GPIbM and GPIbNab:6D12 did not reach statistical significance (0.982). VWF:RCo only had 2 data points and was not included in the pairwise comparison. In samples with type 1 VWD (Figure 4), median activity values were 34.37 IU/dL for GPIbNab:6D12 (n= 44), 25.44 IU/dL for GPIbNab:6C11 (n=44), 36.5 IU/dL for VWF:RCo (n=20), and 30.00 IU/dL for VWF:GPIbM (n=14). There were no statistically significant differences between either nanobody and VWF:GPIbM or VWF:RCo. In subjects with type 2 VWD (Figure 5), median activity values were 34.22 IU/dL for GPIbNab:6D12 (n= 13), 24.45 IU/dL for GPIbNab:6C11 (n=13), 34.00 IU/dL for VWF:RCo (n=3), and 28.00 IU/dL for VWF:GPIbM (n=7). Again, there were no statistically significant differences between either nanobody and VWF:GPIbM or VWF:RCo in this cohort. VWF:RCo only had 2 data points and was not included in the pairwise comparison.

Pearson correlation analyses were then performed. There were positive, statistically significant correlations between 6D12 and VWF:GPIbM ($r$=0.80) and VWF:RCo ($r$=0.71), as well as between 6C11 and VWF:GPIbM ($r$=0.78) and VWF:RCo ($r$=0.51).

Next, we compared mean activity values in African American and Caucasian individuals with and without VWD (Table 5). The non-VWD group 10 samples from African American subjects and 12 samples from Caucasian subjects. The mean GPIbNab:6D12 activity level was 112.7 IU/dL in African Americans and 109.8 IU/dL in Caucasians (p=>0.99). For GPIbNab:6C11, the mean activity level in African Americas was 75.8 IU/dL and in Caucasians was 60.7 IU/dL (p=0.79). In the VWD group, there were 14 samples from African American subjects and 38 from Caucasian subjects. There was a significant difference between the mean GPIbNab:6D12 activity levels in African American and Caucasian subjects (60.9 IU/dL versus 33.9 IU/dL, p=0.01). This finding was not replicated with GPIbNab:6C11, where the mean activity level for African Americans was 33.8 IU/dL and for Caucasians was 25.8 IU/dL (p=0.38).

We also compared mean activity values in Hispanic and non-Hispanic subjects with and without VWD (Table 6). In the non-VWD group, there were 2 samples from individuals who identified as Hispanic and 20 from those who identified as non-Hispanic. The mean GPIbNab:6D12 activity level in Hispanic subjects was 54.2 IU/dL, whereas the mean activity level was 114.4 IU/dL in non-Hispanic subjects. This difference was not statistically significant (p=0.07). The VWD group included 4 Hispanic subjects and 23 non-Hispanic subjects. There were no statistically significant

differences seen between Hispanic and non-Hispanic individuals with either GPIbNab:6D12 (55.6 IU/dL versus 45.0 IU/dL, p=0.62) or GPIbNab:6C11 (37.7 IU/dL versus 27.3 IU/dL, p=0.30).

**AVWS Cohort**

Twenty-one samples from 17 individuals undergoing AVWS evaluations were collected from August 2021 to September 2023. Ten of the 17 individuals (59%) had a diagnosis of AVWS as assigned by a local treating provider. Of the 21 samples, there were 21 accompanying GPIbNab:6D12 and GPIbNab:6C11 values, 12 VWF:GPIbM values, and 8 VWF:RCo available. 2 of the 8 VWF:RCo values were below the lower limit of detection and were therefore assigned a value of 5. The lowest limit of detection was 4.9 for GPIbNab:6D12 and 4.03 for GPIbNab:6C11.

Median age was 59.8 years (range 18.2-90.2). There was a slight female predominance (52.9%). For those who identified their race or ethnicity, the majority of subjects were White or Caucasian (47.1%, n=8) and non-Hispanic (76.5%, n=13) (Table 7). In subjects with AVWS, the mean VWF:RCo was 23.6 IU/dL, the mean VWF:GPIbM was 42.8 IU/dL, the mean GPIbNab:6D12 was 59.4 IU/dL, and the mean GPIbNab:6C11 was 34.3 IU/dL. In those without AVWS, the mean VWF:GPIbM was 129 IU/dL, the mean GPIbNab:6D12 was 107.6 IU/dL, and the mean GPIbNab:6C11 was 74.2 IU/dL. There were no corresponding VWF:RCo values for samples that underwent evaluation for but did not have a diagnosis of AVWS.

There was strong, positive correlation between VWF:GPIbM and GPIbNab:6D12 ($r=0.88$, $p = 0.0002$) and between VWF:GPIbM and GPIbNab:6C11 ($r=0.89$, $p = 0.0001$). Both GPIbNab:6D12 ($r=0.55$) and GPIbNab:6C11 ($r=0.61$) demonstrated moderately positive correlation with VWF:RCo, though these results did not reach statistical significance (Table 8). The sensitivity of GPIbNab:6D12 for detecting AVWS was 0.50, and the specificity was 0.86. The sensitivity and specificity of GPIbNab:6C11 for detecting AVWS, on the other hand, was 0.64 and 0.57, respectively.

## D. DISCUSSION

We report the first description of the performance of GPIbNab, a novel nanobody-triggered VWF activity assay, in a clinical setting. We observed that in individuals with VWD or AVWS or undergoing an evaluation for one of these bleeding disorders, both GPIbNab:6D12 and GPIbNab:6C11 correlate well with the VWF:GPIbM assay.

In our VWD cohort, both activating nanobodies demonstrated statistically significant, strong correlation with VWF:GPIbM and moderate to strong correlation with VWF:RCo. The sensitivity of either activating nanobodies for diagnosing VWD was comparable to or better than the VWF:RCo and VWF: GPIbM assays[20], suggesting that the GPIbNab assay could function as an effective screening tool for VWD. GPIbNab:6C11 exhibited higher sensitivity than the GPIbNab:6D12 version of the assay, though at the expense of reduced specificity, which aligns with its consistently lower average activity levels compared to GPIbNab:6D12. This difference in activity is likely due to variations in the VWF binding sites targeted by each nanobody, though further investigation is ongoing. It may also explain why GPIbNab:6C11 has poorer correlation with VWF:RCo, as the latter assay has limited sensitivity for detecting activity at levels less than 10-20%[18]. Moving forward, exploring whether a diagnostic approach that combines both nanobodies would further improve sensitivity could be valuable.

A major limitation of the VWF:RCo assay is its susceptibility to common sequence variations in the A1 domain of the VWF gene. These variations can lead to a

decreased VWF:RCo to VWF:Ag ratio without indicating a true functional defect—a phenomenon that disproportionately affects African Americans[21]. This susceptibility raises concerns about potential overtreatment and increased medical costs for affected individuals. Our cohort lacked sufficient genetic data to directly assess how the GPIbNab assay performs in patients with or without these common variants. Instead, we compared average activity levels stratified by race. Notably, none of these comparisons revealed significantly lower activity levels in African Americans compared to Caucasians. These findings suggest that, in clinical settings, the GPIbNab assay may not be influenced by common sequence variations in the VWF gene, aligning with preclinical data. This may be because unlike VWF:RCo, which targets the C-AIM of the A1 domain, GPIbNab binds to residues in the N-AIM. As previously discussed, the C-AIM is susceptible *VWF* gene polymorphisms, such as the D1472H polymorphism.

Interestingly, among individuals with VWD, we observed that African American subjects had higher mean GPIbNab:6D12 activity levels compared to Caucasians. This finding is consistent with previous population-based studies showing increased average VWF activity in African Americans[31]. Notably, while GPIbNab:6D12 exhibited this pattern, GPIbNab:6C11 did not, which may reflect the relatively small study population. A larger cohort may be necessary to confirm these findings. Another possibility is that the two nanobodies behave differently because they bind to distinct epitopes.

Our study also found that the GPIbNab assay correlates well with the VWF:GPIbM assay in the AVWS cohort. However, despite this correlation, its sensitivity and specificity for diagnosing AVWS remained poor. The accurate diagnosis of this

disorder has long been challenged by preanalytical and individual variables, particularly with ristocetin-based activity assays[32]. The VWF:GPIbM assay has demonstrated improved sensitivity and specificity compared to the VWF:RCo assay in this population[33, 34]. A VWF activity-to-antigen ratio cutpoint of 0.8 has been suggested as a potential indicator of AVWS, though alternative thresholds such as 0.6, 0.7 and 0.83 have also been proposed[35-39]. In our study, sensitivity and specificity were determined based on diagnostic criteria of either VWF:Ag or GPIbNab levels <50 IU/dL. When adjusting the diagnostic threshold to include a GPIbNab-to-VWF:Ag ratio of <0.8, the sensitivity of GPIbNab:6C11 improved to 92%, though this resulted in reduced specificity for GPIbNab:6C11 and decreased sensitivity for GPIbNab:6D12. Future studies may benefit from constructing a receiver operating characteristic curve to establish the optimal cutoff point for GPIbNab in the diagnosis of AVWS.

Beyond its role in measuring VWF activity, GPIbNab may offer additional diagnostic value in two key ways. First, it can differentiate between high and low proportions of HMWM. Since loss of HMWM is associated with VWD and AVWS variants[36,40], this distinction could be clinically valuable. While multimer analysis remains the gold standard for detecting VWF structural abnormalities, it is time-intensive, operationally burdensome, and not widely available at all laboratories[37].

Second, adjusting the concentration of the activating nanobody may enhance diagnostic accuracy. Constraints in accurate activity quantification at very low levels can complicate disease classification, particularly in distinguishing between severe type 1 and type 3 VWD[41]. In our VWD and AVWS cohorts, the lowest limit of activity detection

was 4.9 IU/dL for GPIbNab:6D12 and 3.4 IU/dL for GPIbNab:6C11, compared to 10 IU/dL for VWF:RCo and 8 IU/dL for VWF:GPIbM. By manipulating nanobody concentrations in the GPIbNab assay, we may further improve diagnostic precision. A similar principle is applied in the diagnosis of hemophilia, where reflex assays are performed at low factor VIII and IX levels to enhance accuracy[42].

Our study has several limitations. First, we acknowledge the relatively small study population. A change in personnel prevented assessment of technician-to-technician variability. Thus, we included only samples processed by our initial technician. Second, the same cohort used to construct our receiver operating curve was also used to calculate assay sensitivity and specificity, introducing a risk of incorporation bias. Third, while all samples had associated GPIbNab:6D12 and GPIbNab:6C11 values, not all had corresponding VWF:RCo and VWF:GPIbM levels. This discrepancy arose partly from preference of VWF activity assay of the local treating physician. Additionally, during the study period, Emory University and Children's Healthcare of Atlanta began processing VWF:GPIbM on-site, leading to discontinuation of VWF:RCo as an activity assay option. Finally, we recognize that while VWF:GPIbM and VWF:RCo remain the gold standard activity assays, their inherent limitations may impact the accuracy of diagnoses against which we are comparing GPIbNab. Genetic testing is not the standard of care for most subtypes of VWD, thus most of the patients did not have it performed. Moving forward, incorporating a study population in which genetic testing has been performed to confirm diagnosis of VWD could provide a more reliable benchmark for assessing GPIbNab performance.

There are several promising directions for future research. First, GPIbNab must undergo thorough reproducibility and validation assessments, which will require a larger study population. Additionally, to enhance the external validity of this assay as a screening tool for VWD, it will be important to characterize GPIbNab in a healthy cohort of individuals who have not sought evaluation for a bleeding disorder. Further analyses exploring GPIbNab's performance across different subgroups will help refine its diagnostic utility for VWD and AVWS. For example, evaluating its accuracy in patients with and without common *VWF* gene sequence variations or across different underlying conditions of AVWS would be insightful. Moreover, investigating the correlation between bleeding phenotype and GPIbNab levels may further clarify its clinical significance.

In conclusion, the GPIbNab assay strongly correlates with the VWF:GPIbM assay in individuals diagnosed with VWD or AVWS, as well as those undergoing an evaluation for these disorders. In these populations, this novel assay may serve as an effective screening tool or become part of the battery of diagnostic assays used in identifying a VWF abnormality.

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Table 1: VWD Study Population Diagnoses

| **Diagnosis** | **N (%)** |
|---|---|
| Type 1 VWD | 39 (47.6) |
| Type 2 VWD | 12 (14.6) |
| Type 3 VWD | 0 (0) |
| Unknown Type[a] | 4 (4.9) |
| Other[b] | 2 (2.4) |
| No VWD | 25 (30.5) |

Abbreviations: VWD, von Willebrand Disease
a. Type 1 versus Type 2 VWD
b. Subjects who had previously been diagnosed with VWD but currently do not meet criteria for diagnosis

Table 2: VWD Study Population Demographic Characteristics

| **Characteristics** | **Total N = 82** |
|---|---|
| Age[a], Years | |
| Median (Range) | 20.0 (3.6 – 79.4) |
| Missing | 5 |
| Sex, N (%) | |
| Female | 55 (67.1) |
| Race, N (%) | |
| Back or African American | 22 (26.8) |
| White or Caucasian | 48 (58.5) |
| Other | 6 (7.3) |
| Missing | 6 (7.3) |
| Ethnicity, N (%) | |
| Hispanic | 7 (8.5) |
| Non-Hispanic | 39 (47.6) |
| Missing | 36 (43.9) |

a. Age at which GPIbNab sample was collected

Figure 1: Receiver Operator Curve to Determine Optimal GPIbNab:6D12 to VWF:Ag Cutoff Point

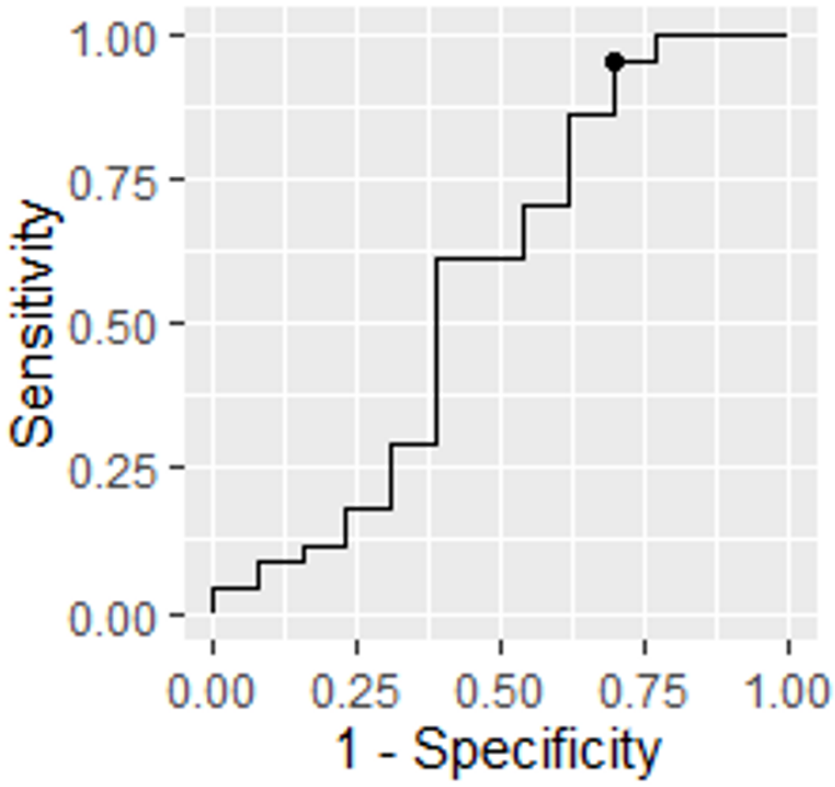


Figure 2: Receiver Operator Curve to Determine Optimal GPIbNab:6DC11 to VWF:Ag Cutoff Point

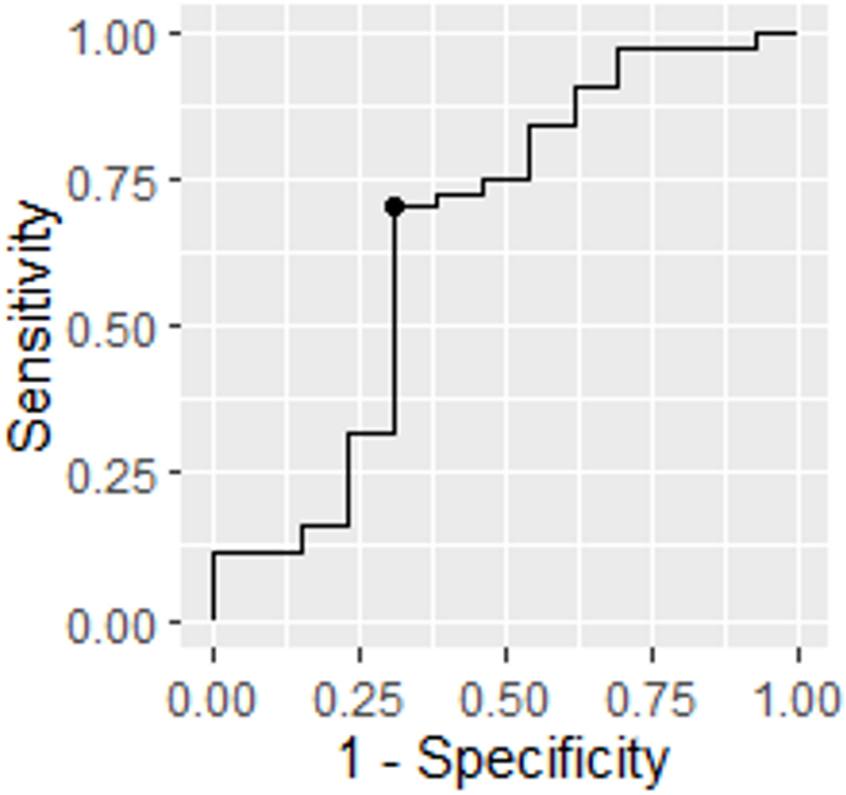


Table 3: Test Performance Characteristics of GPIbNab:6D12 in Predicting VWD versus No VWD

| | **Gold Standard Diagnosis** VWD | **Gold Standard Diagnosis** No VWD | **Total** | **Test Characteristics** |
|---|---|---|---|---|
| **GPIbNab: 6D12 Diagnosis** VWD | 46 | 7 | 53 | PPV = 0.87 |
| **GPIbNab: 6D12 Diagnosis** No VWD | 12 | 22 | 34 | NPV = 0.65 |
| **Total** | 58 | 29 | 87 | |
| **Test Characteristics** | Sensitivity = 0.79 | Specificity = 0.76 | | |

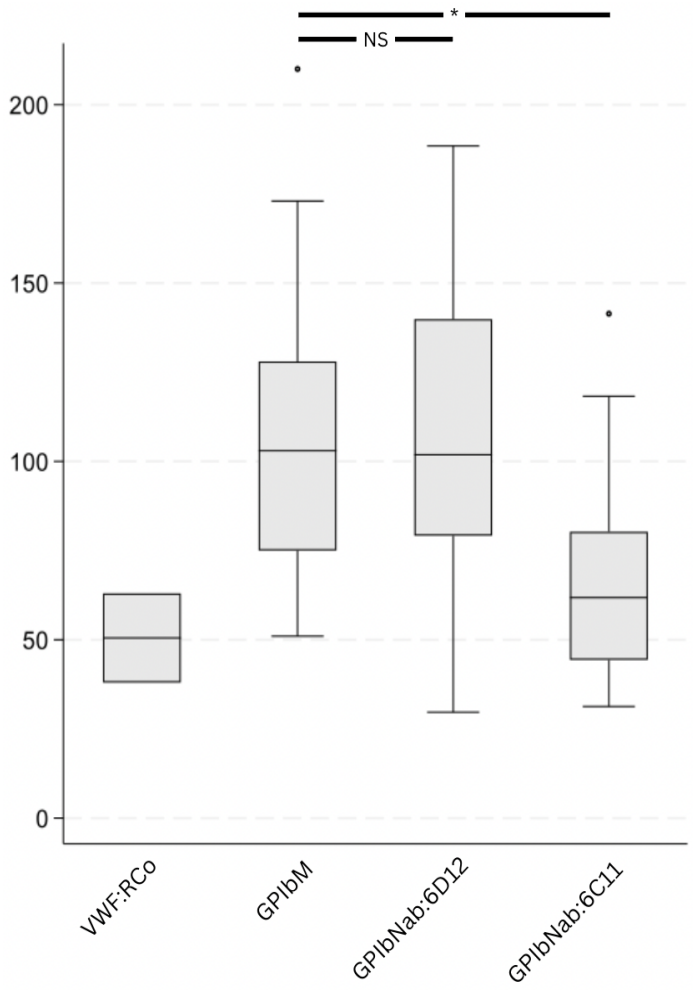
Abbreviations: VWD, von Willebrand Disease, PPV, Positive-Predictive Value, NPV, Negative Predictive Value

Table 4: Test Performance Characteristics of GPIbNab:6C11 in Predicting VWD versus No VWD

| | **Gold Standard Diagnosis** VWD | **Gold Standard Diagnosis** No VWD | **Total** | **Test Characteristics** |
|---|---|---|---|---|
| **GPIbNab: 6C11 Diagnosis** VWD | 51 | 14 | 65 | PPV = 0.78 |
| **GPIbNab: 6C11 Diagnosis** No VWD | 7 | 15 | 22 | NPV = 0.68 |
| **Total** | 58 | 29 | 87 | |
| **Test Characteristics** | Sensitivity = 0.88 | Specificity = 0.52 | | |

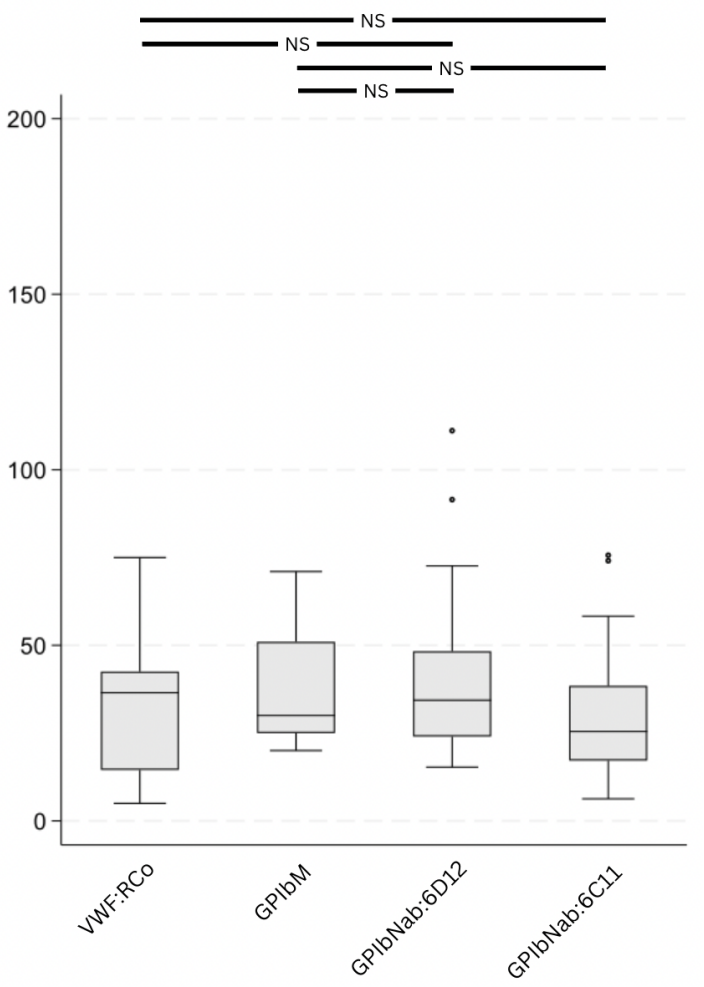
Abbreviations: VWD, von Willebrand Disease, PPV, Positive-Predictive Value, NPV, Negative Predictive Value

Figure 3: Comparison of Median Activity Assay Values in Subjects without VWD



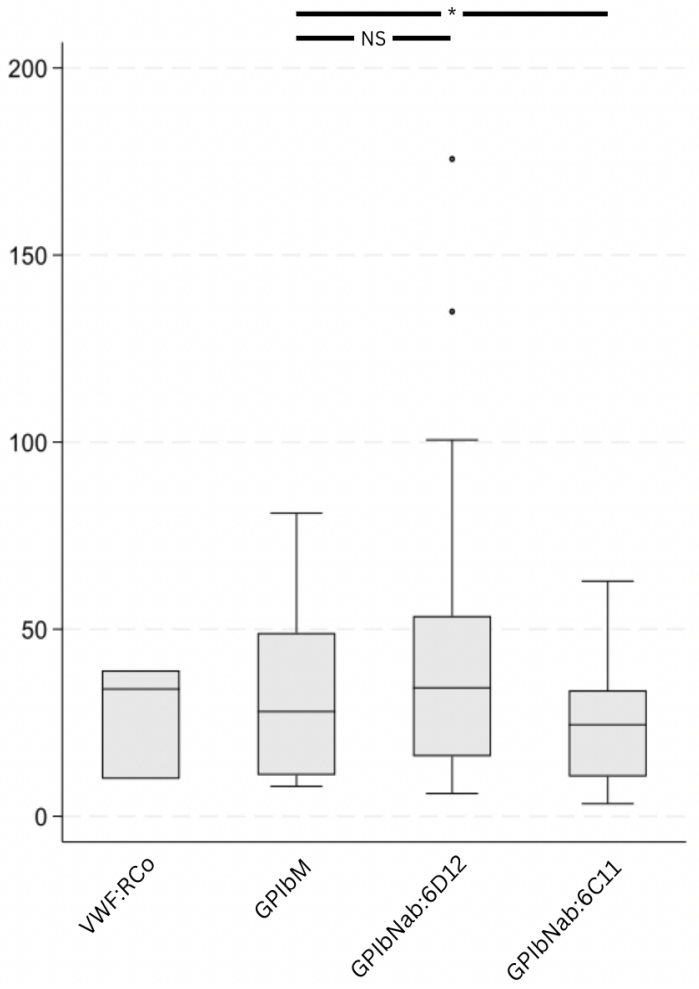

Abbreviations: NS, Not Significant

Figure 4: Comparison of Median Activity Assay Values in Subjects with Type 1 VWD




Abbreviations: NS, Not Significant

Figure 5: Comparison of Median Activity Assay Values in Subjects with Type 2 VWD




Abbreviations: NS, Not Significant, *, p value <0.05

Table 5: Mean GPIbNab Activity Level, Stratified by Diagnosis and Race

| | African American | Caucasian | P-value |
|---|---|---|---|
| **No VWD** | | | |
| N | 10 | 12 | |
| Mean Activity Level (IU/dL) | | | |
| GPIbNab:6D12 | 112.7 | 109.8 | >0.99 |
| GPIbNab:6C11 | 75.8 | 60.7 | 0.79 |
| **VWD** | | | |
| N | 14 | 38 | |
| Mean Activity Level (IU/dL) | | | |
| GPIbNab:6D12 | 60.9 | 33.9 | 0.01 |
| GPIbNab:6C11 | 33.8 | 25.9 | 0.38 |

Abbreviations: VWD, von Willebrand Disease

Table 6: Mean GPIbNab Activity Level, Stratified by Diagnosis and Ethnicity

| | Hispanic | Non-Hispanic | P-value |
|---|---|---|---|
| **No VWD** | | | |
| N | 2 | 20 | |
| Mean Activity Level (IU/dL) | | | |
| GPIbNab:6D12 | 54.2 | 114.4 | 0.07 |
| GPIbNab:6C11 | 36.4 | 66.3 | 0.12 |
| **VWD** | | | |
| N | 4 | 23 | |
| Mean Activity Level (IU/dL) | | | |
| GPIbNab:6D12 | 55.6 | 45.0 | 0.62 |
| GPIbNab:6C11 | 37.7 | 27.3 | 0.30 |

Abbreviations: VWD, von Willebrand Disease

Table 7: AVWS Study Population Demographic Characteristics

| **Characteristics** | **Total**<br>**N = 17** |
|---|---|
| Age[a], Years | |
| Median (Range) | 59.8 (18.2– 90.2) |
| Missing | 2 |
| Sex, N (%) | |
| Female | 9 (52.9) |
| Race, N (%) | |
| Back or African American | 4 (23.5) |
| White or Caucasian | 8 (47.1) |
| Other | 4 (23.5) |
| Missing | 1 (5.9) |
| Ethnicity, N (%) | |
| Hispanic | 3 (17.6) |
| Non-Hispanic | 13 (76.5) |
| Missing | 1 (5.9) |

a. Age at which GPIbNab sample was collected

Table 8: Correlation between VWF activity assays in AVWS Study Population

| **GPIbNab** | **GPIbM** | **VWF:RCo** |
|---|---|---|
| GPIbNab:6D12 | r=0.88 (p=0.0002) | r=0.55 (p=0.16) |
| GPIbNab:6C11 | r=0.89 (p=0.0001) | r=0.61 (p=0.10) |