Iranian Journal of Blood & Cancer

Iran J Blood Cancer, 2024, 16(2), 60-69
https://ijbc.ir

Review Article

Type 2N von Willebrand Disease: Overcoming Diagnostic Challenges for Accurate Diagnosis

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Citation Azari B, Ahmadinejad M. Type 2N von Willebrand Disease: overcoming Diagnostic challenges for accurate diagnosis. Iran J Blood Cancer. 2024 June 30;16(2):60-69.

doi 0

Article info:
Received: 20 Mar 2024
Accepted: 19 May 2024
Published: 30 June 2024

Keywords:
von Willebrand Factor
von Willebrand Disease
Hemophilia A

Abstract

Von Willebrand Factor (vWF) defects can cause von Willebrand Disease (vWD), which is known to be the most prevalent inherited bleeding disorder worldwide. According to the latest classifications, vWD is categorized into three main types. Types 1 and 3 are quantitative defects, while type 2 vWD is caused by qualitative abnormalities in vWF. Furthermore, ISTH classifies type 2 vWD is into four subtypes known as 2A, 2B, 2N, and 2M. Type 2N vWD is an uncommon type of vWD that is inherited in an autosomal recessive pattern. In this type, the binding capacity of vWF to Factor VIII (FVIII) is reduced, resulting in FVIII's shortened half-life in the patient's plasma. Due to the pathophysiology of Type 2N vWD, affected individuals exhibit signs and symptoms similar to those with mild to moderate hemophilia A. These symptoms include mucocutaneous bleeding or bleeding following trauma or surgery. Furthermore, the primary laboratory findings of affected individuals are comparable to those of hemophilia A patients, with Factor VIII levels ranging from 1 to 40 U/dL. It is crucial to differentiate these disorders for optimal treatment and accurate genetic counseling. Physicians may use a combination of clinical assessment, family history, bleeding scores, and laboratory tests to differentiate between the two disorders. Further genetic testing may be necessary to confirm the diagnosis and assess the risk of inheritance. This review outlines methods for diagnosing type 2N vWD and distinguishing it from hemophilia A, based on published papers and current guidelines.

1. INTRODUCTION

In 1924, Eric Adolf von Willebrand described von Willebrand disease (vWD) as a bleeding disorder with signs and symptoms like hemophilia A [1]. Nowadays, it is known to be the most common inherited bleeding disorder, which is caused by either qualitative or quantitative deficiencies of the von Willebrand Factor (vWF) [2, 3]. Studies estimate the prevalence of severe or symptomatic forms of vWD to be 1:10000, although reports indicate higher rates in more restricted population-based studies [4, 5]. Based on the type of abnormality, the International Society of Thrombosis and Hemostasis (ISTH), along with the American Society of Hematology (ASH) and the World Federation of Hemophilia (WFH), classify vWD into three types. In this

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[DOI: 10.61186/ijbc.16.2.60]

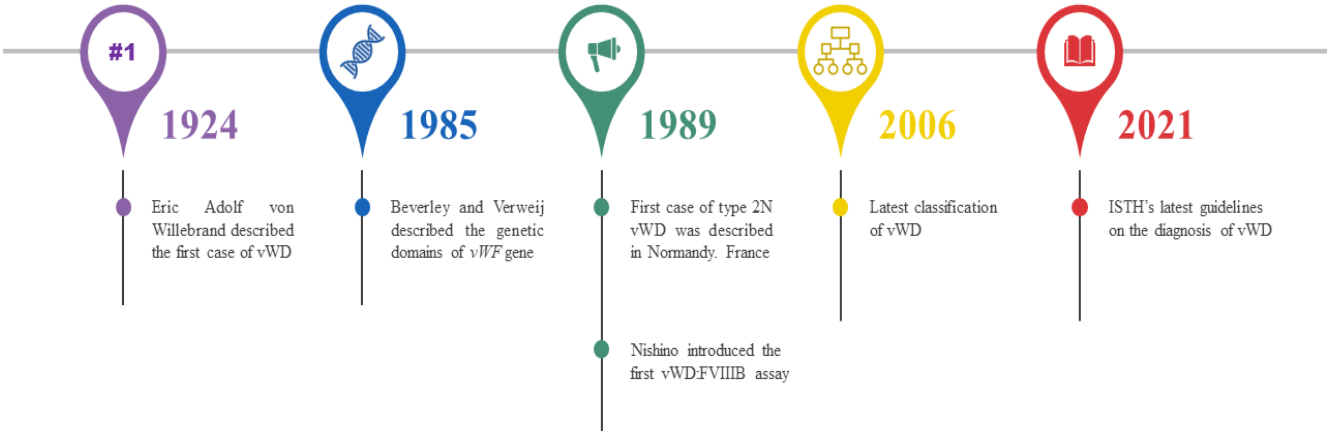

Figure 1. A brief timeline of key events in type 2N vWD.

classification, types 1 and 3 are categorized as quantitative defects, while type 2 is caused by a mutation in the *VWF* gene leading to a qualitative abnormality [2, 6]. Furthermore, type 2 vWD is divided into four subtypes known as 2A, 2B, 2N, and 2M [7]. Among these variants, type 2N vWD is an autosomal recessive disorder that impairs the binding capacity of vWF to FVIII. This results in low levels of FVIII in plasma that resembles mild to moderate hemophilia A [8]. Due to this resemblance, it is likely for type 2N vWD patients to be misdiagnosed with hemophilia A [9, 10]. Therefore, it is critical to accurately differentiate between these two disorders to provide appropriate genetic counseling and treatment. [2, 9, 10]. This abnormality was first reported 30 years ago in a patient with normal bleeding time (BT) but low levels of FVIII from Normandy, France; thus, it was named type 2N (Normandy) vWD [11, 12]. This review article discusses the challenges of diagnosing type 2N vWD accurately based on published articles and the most recent guidelines.

2. METHODS

To ensure proper diagnosis of type 2N vWD, accurate diagnostic methods must be used to distinguish it from hemophilia A. This review has referred to over 70 articles from PubMed, Medline, Scopus, and Google Scholar databases to investigate the latest diagnostic techniques. The investigation was performed using the following keywords: von Willebrand Factor, VWF gene, von Willebrand Disease, type 2N von Willebrand disease, laboratory findings, and vWF:FVIII binding assay. The search was limited to English articles and English-translated papers until 2023. A standardized data extraction form was used to extract the study design, patient characteristics, signs and symptoms of type 2N vWD, diagnosis criteria, and methods used to diagnose type 2N vWD and prevalence of causative type 2N vWD mutations. Through a narrative structure, the extracted data were synthesized to highlight the diagnostic challenges in type 2N vWD and provide ways to overcome them. The data were organized according to the diagnostic methods used to diagnose affected patients with type 2N vWD to show each method's strengths and limitations.

3. PATHOPHYSIOLOGY OF TYPE 2N VWD

Von Willebrand factor is a large multidomain glycoprotein encoded by *the VWF* gene on the short arm of chromosome 12 containing 52 exons [13-15]. With multiple domains represented as D1-D2-D'-D3-A1-A2-A3-D4-B-C1-C2-Ck, vWF has an essential role in primary and secondary hemostasis [14-16]. During the events of primary hemostasis, vWF tethers platelets to damaged subendothelial cells and accelerates platelet plug formation [17]. In its latter role, vWF interacts with FVIII in a way that prolongs FVIII's half-life in plasma [14, 18]. By binding to FVIII, this large molecule is vital in protecting FVIII against activated protein C and activation by factor IX [19, 20]. Moreover, vWF-bound FVIII regulates the immune recognition of this critical coagulation factor; therefore, it is crucial in protecting the exogenous FVIII from inhibitory antibodies that may develop in hemophilia A patients [21, 22]. Furthermore, vWF reduces the binding capacity of FVIII to receptors related to lipoproteins, which in turn increases the half-life of FVIII. [23]. This binding site ranges from D' to D3 domains of vWF, which is divided into the TIL'-E'-VWD3-C8_3-TIL3-E3 subdomains [24-26]. In a recent study, Shiltagh et al. suggested that FVIII binds to

[DOI: 10.61186/ijbc.16.2.60]

vWF mainly through the TIL'-E' (i.e. D') structure [27]. Any point mutations in exons 17 through 27 of *the VWF* gene, which encode these domains, can potentially compromise the bond between vWF and FVIII, leading to reduced levels of FVIII in plasma [8, 28]. Inheritance of two alleles with these mutations can lead to homozygous or compound heterozygous forms of type 2N vWD that can be presented with different clinical manifestations [29].

4. VWD TYPE 2N DIAGNOSIS AND ITS CHALLENGES

There is a high likelihood of misdiagnosis between type 2N vWD and mild to moderate hemophilia A in males and hemophilia A carriers in females due to their similar clinical manifestations and laboratory findings [10]. Signs and symptoms of type 2N vWD include mucocutaneous bleeding or bleeding following trauma or surgery. This bleeding can be seen in the gastrointestinal tract in more severe cases. Moreover, reports confirm postpartum hemorrhages in female patients following labor [30, 31]. Hemarthroses and muscle hematomas can be observed in affected patients like to those in mild to moderate hemophilia A [31, 32].

Diagnosis of type 2N vWD usually begins with laboratory screening tests for blood coagulation. Affected patients show normal PT with prolonged aPTT, followed by low levels of FVIII ranging from 1 to 40 U/dL. Moreover, primary laboratory findings indicate normal or subnormal rates for both vWF:Ag and vWF:RCo assays [33]. Due to these results, type 2N vWD patients have FVIII:C/vWF:Ag ratio values below 0.6 to 0.7 [9, 34-37]. These primary findings are almost identical to those found in hemophilia A patients; thus, there is a high potential for misdiagnosis of type 2N vWD based on primary laboratory findings [9, 10]. As a result of these similarities, ISTH suggests a diagnostic panel to differentiate vWD type 2N from hemophilia A. This panel comprises targeted genetic testing and a specific assay called vWF:FVIIIB, which gauges the binding capacity of vWD to FVIII. Furthermore, ISTH states that there is insufficient evidence to use genetic studies or vWF: FVIIIB assay for suspected patients. Therefore, the recommended approach is to use both tests during the diagnostic workup [2]. In addition, several studies indicated that both vWF:FVIIIB assay and genetic testing have an acceptable sensitivity [9, 36, 38-46].

3.1. Primary laboratory findings

Due to the heterogeneity of vWD, initial laboratory evaluation for this disease demands a battery of screening tests along with assays to assess the presence of fully functional vWF protein [29, 47, 48]. Therefore, the process of making an accurate diagnosis is time-consuming and requires patience from both individuals affected and their clinicians. [47, 48]. Coagulation screening tests are inexpensive assays that detect congenital or acquired alterations in primary and secondary hemostasis [49]. Bleeding time (BT), platelet count, prothrombin time (PT), and activated partial thromboplastin time (aPTT) are standard screening assays ordered by physicians [50]. BT and platelet count indicate qualitative and quantitative defects of platelets, respectively [51, 52]. Therefore, type 2N vWD patients exhibit normal values for both assays [5]. PT and PTT are simple tests for assessment of the coagulation pathway [14]. Both assays can assess the common coagulation pathway, which involves factors V, X, prothrombin, and fibrinogen. However, PT primarily evaluates the extrinsic pathway with factor VII as its key enzyme, while PTT is used to evaluate the intrinsic pathway, which includes factors XI, IX, and VIII [14, 47]. Due to the reduced levels of FVIII in type 2N vWD, affected individuals usually have normal PT with isolated prolonged PTT values [47]. Despite having accepted sensitivity, these tests show low specificity and reproducibility. Therefore, more specific assays were developed to diagnose and confirm bleeding disorders more efficiently [53].

The confirmatory assays are developed to increase the chance of accurate diagnosis [47]. These assays have higher sensitivity and specificity compared to coagulation screening tests and include vWF antigen (vWF:Ag), vWF:Ristocetin Co-factor (vWF:RCo), vWF:Glycoprotein-IbR (vWF:GPIbR), vWF:Glycoprotein-IbM (vWF:GPIbM), and factor FVIII coagulation (FVIII:C) assays [29, 47, 48].

The vWF:Ag test is a type of immune-based assay that is used to measure the levels of vWF antigen present in a patient's plasma. This test is performed using either an enzyme-linked immunosorbent assay (ELISA) method or an automated latex immunoassay. The ELISA method involves the use of antibodies that are specifically designed to bind to vWF antigens, which are then detected using an enzyme-linked secondary antibody. On the other hand, the automated latex immunoassay uses latex particles that have been coated with vWF antibodies, which will bind to vWF antigens present in the plasma sample. The amount of bound antibodies is then measured and used to determine the concentration of vWF antigen in the sample. [54]. vWF:Ag has acceptable reliability and reproducibility but does not assess vWF's function in patients. Thus, it requires additional assays to evaluate the functionality of vWF [29].

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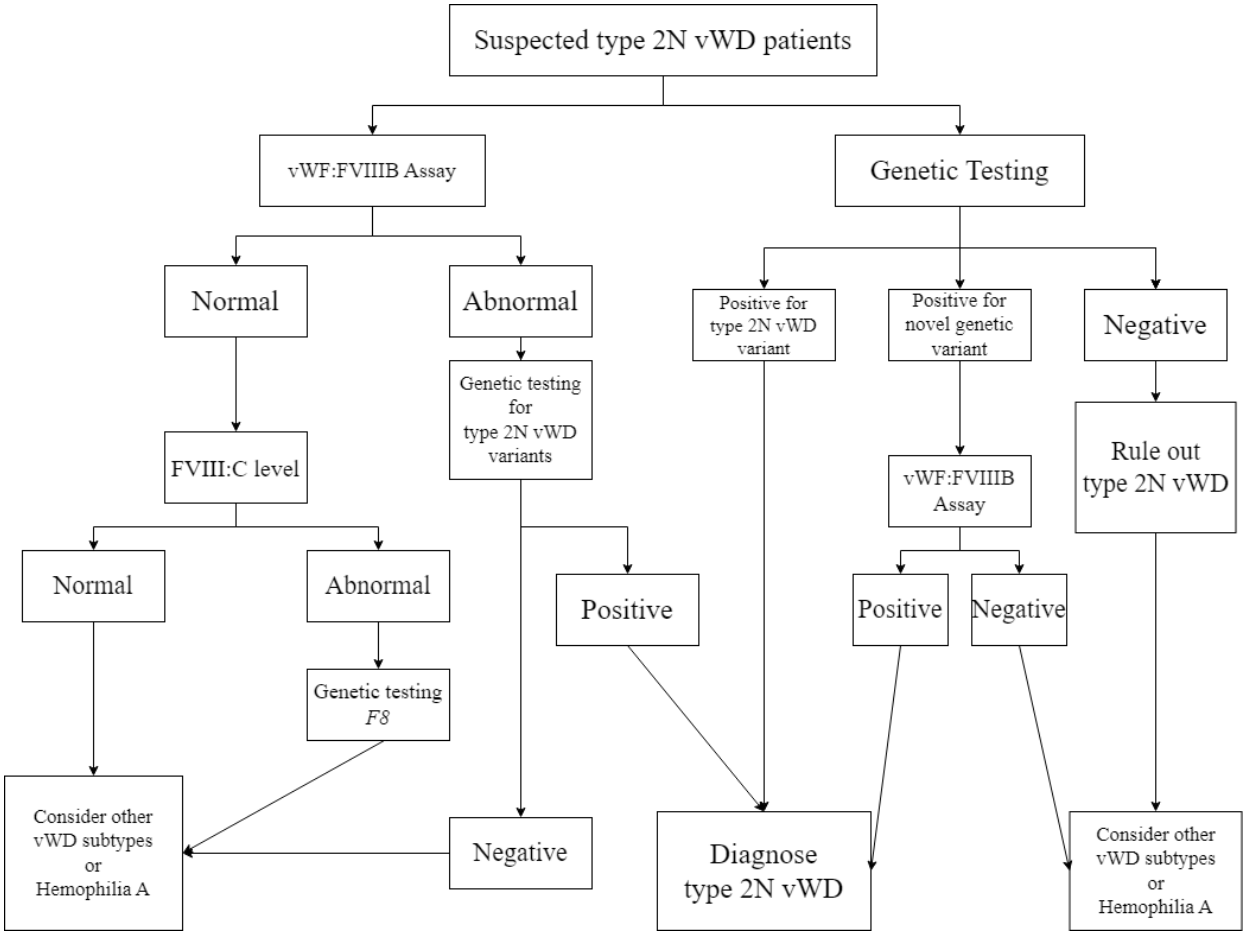

Figure 2. ISTH's suggested algorithm for the diagnosis of type 2N vWD.

vWF:RCo is a highly specific assay that measures the binding capacity of GPIbα; therefore, it can reflect the functional status of vWF. Despite its specificity, high coefficient of variation limits the usage of vWF:RCo [50, 55]. Moreover, reports indicate variations in the A1 domain of vWF can result in reduced levels of vWF:RCo without altering the vWF function [56]. Nowadays, vWF:GPIbR and vWF:GPIbM assays are used as alternatives to the vWF:RCo for measuring activity levels of vWF [57]. vWF:GPIbR assay uses ristocetin to measure the binding capacity of vWF to plasma-derived or recombinant GPIb . At the same time, vWF:GPIbM is a ristocetin-independent assay that utilizes a modified version of a GPIb to evaluate vWF's activity [57, 58]. Compared to vWF:RCo, both assays have higher precision and sensitivity with reduced intra- and inter-laboratory variability [57-60]. In contrast to other types of von Willebrand disease that show decreased antigen and activity levels of vWF, patients with type 2N vWD usually have normal values for both assays [5]. Moreover, in type 2N vWD, vWF:RCo/vWF:Ag ratio values are above 0.6, which can differentiate it from other type 2 vWD subtypes [61].

Due to the critical role of vWF as a plasma carrier for FVIII, activity levels of this coagulation factor are usually proportional to antigen levels of vWF [29, 48]. Thus, it is recommended to include the measurement of FVIII:C in the workup of vWD. [47]. As the clearance rate of FVIII increases in type 2N vWD, affected patients exhibit decreased FVIII:C levels. Several studies report that the FVIII activity levels can vary from 1 to 40 U/dL in type 2N vWD, which can be a typical laboratory finding I mild to moderate hemophilia A cases [9, 36]. Moreover, FVIII:C/vWF:Ag ratio values below 0.6 may indicate type 2N vWD [62].

In 2018, Casonato et al. conducted a cohort study to evaluate the prevalence of type 2N vWD in Northeast Italy. In this study, primary laboratory findings of homozygous or compound heterozygous type 2N vWD patients indicated

[DOI: 10.61186/ijbc.16.2.60]

Table 1. Main hemostatic findings of type 2N vWD patients among multiple studies.

Authors	Year	Country	Number of Patients	vWF:Ag	vWF:RCo	FVIII:C
Lapic et al.	2022	Croatia	1	53.6	57.9	66.0
Seidizadeh et al.	2020	Iran	5	62.2	62.4	22.0
Casonato et al.	2017	Italy	9	69.5	69.6	32.2
Liang et al.	2017	China	5	48.8	49.9	6.5
Yadegari et al.	2012	Germany	4	70.5	71.75	20.5
Casonato et al.	2007	Italy	4	57.3	-	28.5

a mean of 39.4±3.5 seconds for aPTT, which is prolonged compared to the normal range (24.4-36.5s). Moreover, vWF:Ag and vWF:RCo values revealed a mean of 69.4±39.7 U/dL and 69.6±36.8 U/dL, respectively. Based on the average values for these assays, both values were slightly above the lower cut-off limit (60 U/dL). In addition, FVIII:C levels showed a mean of 32.2±14.7 U/dL [63]. According to the results obtained, primary laboratory data is not a reliable diagnostic tool for type 2N vWD, as it cannot distinguish between individuals affected by the disease and those with mild or moderate Hemophilia A. Hence, vWF:FVIIIB assay or molecular genetic studies must be ordered in addition to primary laboratory test [10].

3.2. vWF:FVIII Binding Assay

vWF:FVIII binding assay was first introduced by Nishino *et al.* in 1989 [12]. It is an ELISA-based method that measures the binding capacity of a patient's vWF to exogenous recombinant FVIII [45, 46]. It is a crucial and discriminant assay to identify and differentiate type 2N vWD from mild to moderate hemophilia A because type 2N vWD patients show markedly reduced values due to their compromised binding capacity, while the latter group has normal results [6, 8].

Currently, there are two standard methods to perform vWF:FVIIIB assay [12, 40]. The first method involves quantifying both bound FVIII and immobilized vWF using a single well, which requires special sample preparation or complex standardization. However, with thorough standardization, the measurement of immobilized vWF can also serve as an indicator of the vWF:Ag level [46].

The second method utilizes parallel experiments to determine two analytes and reports the ratio of bound recombinant FVIII to immobilized vWF [64]. Compared to the simultaneous measurement of bound FVIII and vWF, it is a more convenient method that can be easily automated and used in the clinical laboratory [43].

In 2015, Boylan *et al.* evaluated the vWD phenotypes and genotypes in affected individuals with and without presence of an identified mutation in *F8* gene. In this study, the binding capacity of vWF was measured using a commercially available ELISA with recombinant FVIII. Results of this study showed that out of 37 participants with no mutations in their *F8* gene, four cases had below 80% vWF:FVIIIB rates. After conducting additional evaluations, it was discovered that two of the cases under investigation were found to have a heterozygous p.R854Q mutation, which is known to be a causative mutation for type 2N vWD [65].

In a more recent study, Seidizadeh *et al.* conducted a 10-month screening program that included 71 mild to moderate factor VIII deficient cases with their FVIII:C rates ranging from 1% to 40%. In this study, the evaluation of the FVIII:C/vWF:Ag ratio indicated a ratio of 0.6 or lower in 26 cases. Additionally, the vWF:FVIIIB assay was performed, and five index patients with no shared family relations were found to have abnormal results when using 80% as the cutoff value for healthy subjects. Among these five cases, none were under clinical requests to investigate the possibility of type 2N vWD [10].

Studies indicate that some patients with homozygous or compound heterozygous mutations of type 2N vWD may exhibit normal vWF:FVIIIB results [31, 36]. Moreover, the complex setup of this crucial assay has led to limited use in a small percentage of specialized laboratories. Thus, Type 2N vWD may be underdiagnosed or misdiagnosed as mild to moderate hemophilia A [10, 66].

[DOI: 10.61186/ijbc.16.2.60]

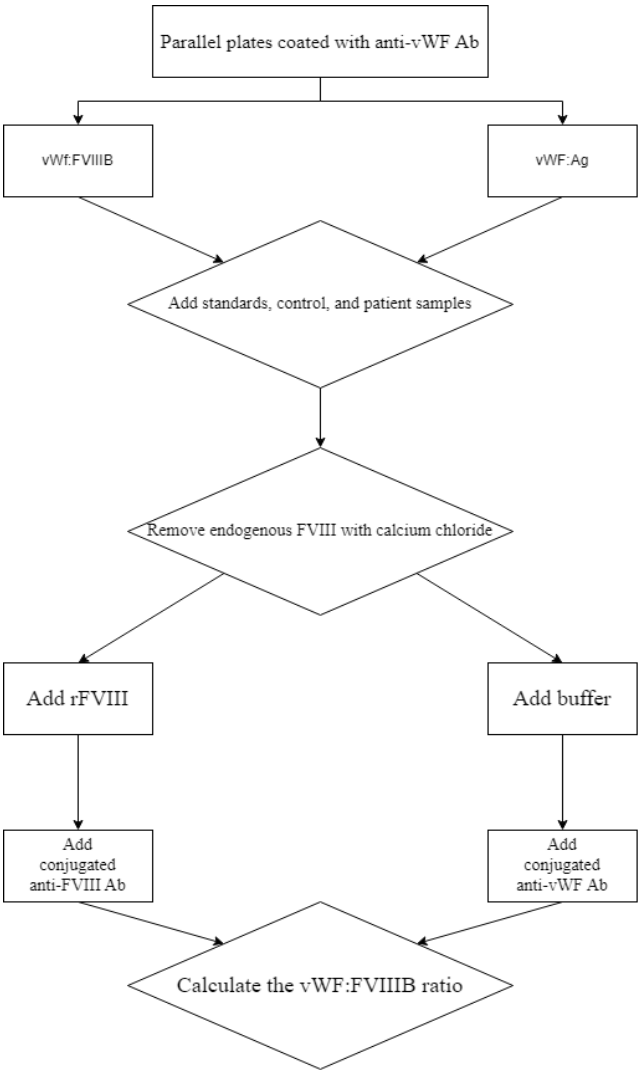

Figure 3. **Scheme of vWF:FVIIIB assay.**

3.3. Molecular Genetic Study

While considered a valuable diagnostic tool for vWD, vWF:FVIIIB assay has limited availability. As a result, targeted molecular analysis has emerged as an alternative means of diagnosing type 2N vWD patients [3]. This approach involves identifying and analyzing specific genetic mutations associated with vWD type 2N, which can provide valuable data to discriminate type 2N vWD from hemophilia A [67]. In contrast with other types of vWD, type 2N is an autosomal recessive disease; thus, affected patients can exhibit a single homozygous 2N mutation or compound heterozygous mutations. Additionally, co-inheritance of a null VWF mutation with a heterozygous 2N mutation can result in symptomatic type 2N vWD patients [6, 68-72]. In a single heterozygous state, patients are considered carriers [67]. Due to their normal or subnormal primary laboratory findings, type 2N vWD carriers are often asymptomatic and identified by chance during studies on families with a history of vWD [36]. In 2018, Casonato *et al.* designed a study that divided heterozygous individuals into two groups: asymptomatic carriers and patients with bleeding symptoms suffering from epistaxis, hematomas, or surgery-related bleeding. In this study, symptomatic carriers showed higher bleeding assessment tool (BAT) scores with significantly lower vWF:Ag levels; however, both groups had similar vWF:FVIIIB/vWF:Ag ratios. According to the study results, type 2N vWD carriers tend to experience symptoms when their vWF antigen levels drop below the cut-off limit. This suggests a correlation between vWF:Ag levels and the manifestation of symptoms in type 2N vWD carriers. Individuals with this condition need to monitor their vWF:Ag levels regularly, as this can help them identify and manage symptoms more effectively [63].

The D'D3 domain of vWF, encoded by exons 18 through 28 of the VWF gene, is responsible for binding with FVIII. As a result, mutations in these regions can impair the interaction between FVIII and VWF, which can lead to type 2N vWD [27]. Studies indicate that the majority of these missense mutations are found in the D' domain, which extends from exon 18 to exon 20; therefore, it is a recommended approach to analyze these exons as primary targets, but when no abnormalities are found, exons 17 and 21-28 must be checked for any other causative mutations [72-75].

At the time of writing this article, more than 50 mutations have been reported in the European Association for Hemophilia and Allied Disorders (EAHAD) vWF variant database. Based on this database, more than 90 percent of these mutations are missense; however, some studies reported non-sense mutations in rare cases [76, 77]. Among these mutations, p.Arg854Gln, p.Arg816Trp, and p.Thr791Met account for the most reported cases. Moreover, studies indicate that affected patients with p.Glu787Lys, p.Thr791Met, and p.Arg816Trp mutations can exhibit the most severe forms of type 2N vWD [28]. While numerous pathogenic variants of VWF have been identified, there is a possibility of discovering new mutations that have not been reported. In these cases, phenotypic evidence, such as decreased binding of FVIII to vWF, which can be measured by vWF:FVIIIB assay, is

phenotypic evidence, such as decreased binding of FVIII to vWF, which can be measured by vWF:FVIIIB assay, is required to determine the pathogenic state of that novel variant [2].

[DOI: 10.61186/ijbc.16.2.60]

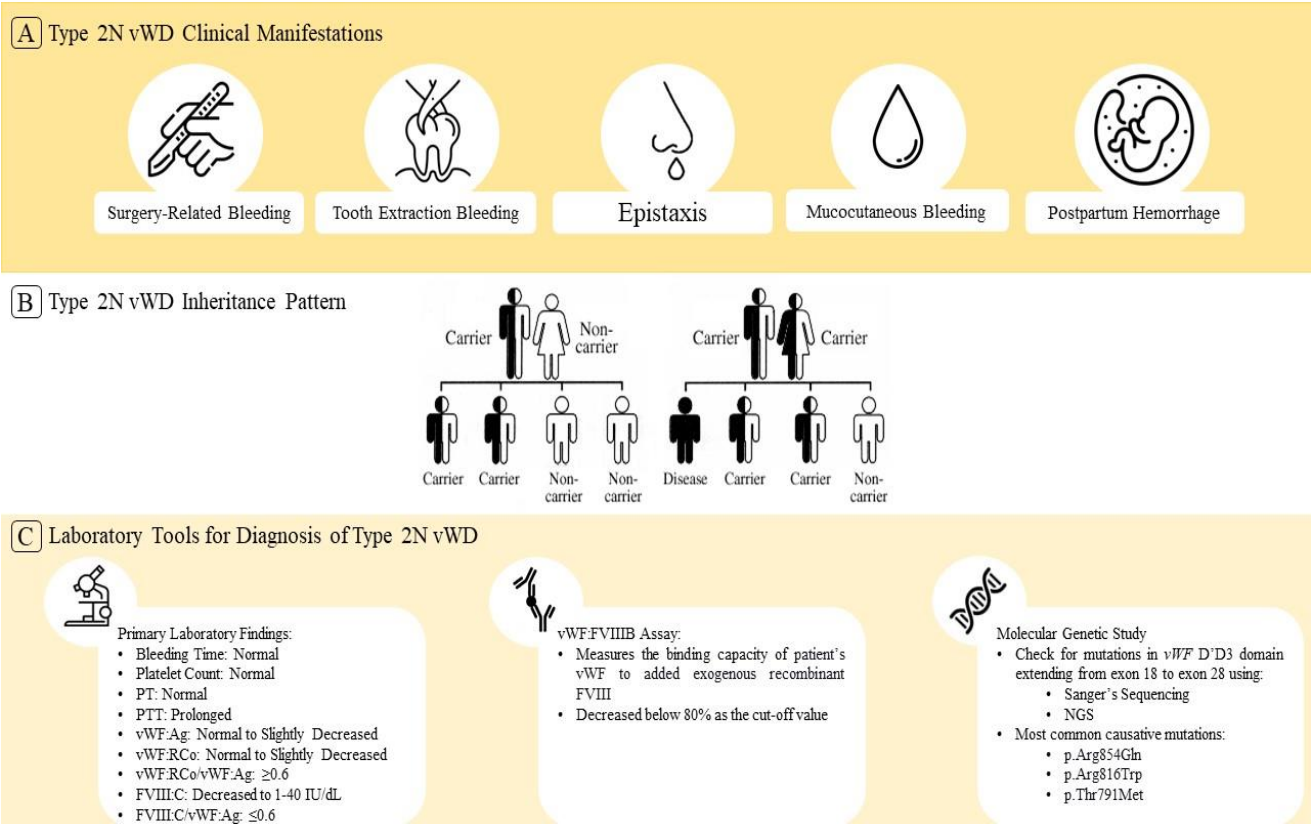

Figure 4. **Overview of type 2N vWD. A)** Most common clinical manifestations of type 2N vWD include mucocutaneous bleeding or bleeding following trauma or surgery**. B)** Type 2N vWD is inherited in an autosomal recessive manner, which differs from hemophilia A as it is an X-linked disease. **C)** Tools for diagnosing type 2N vWD include primary laboratory findings, vWF:FVIIIB assay, and molecular genetic studies that can provide helpful data in distinguishing this disease from mild to moderate hemophilia A.

Nowadays, the emergence of new advanced and state-of-the-art sequencing techniques such as next-generation sequencing (NGS), alongside other methods like Sanger's sequencing, made molecular studies simpler with shorter turn-around times and more reliable results [3].

In 2021, a research study was conducted in Spain by Perez-Rodriguez *et al.*, which aimed to investigate the prevalence of type 2N vWD in the local population. The study included 63 participants who were either previously diagnosed with type 2N vWD or showed FVIII:C/vWF:Ag ratios below 1. Using NGS, the results of this study revealed that 28 individuals had type 2N vWD or carried a heterozygous type 2N mutation. Based on their findings, only three different causative mutations were detected. These mutations were p.Arg854Gln, p.Arg816Trp, and p.Arg763Ser. Among these mutations, the most frequently occurring one was p.Arg854Gln [67].

In a study of 200 Chinese individuals from 90 families with vWD, Liang et al. aimed to correlate genotype and phenotype using the same diagnostic approach. Results of this study indicated that only five patients were type 2N vWD cases based on their genetic findings. Among them, one patient was homozygous for p.Arg816Trp, while others were found to be heterozygous for p.Cys799Tyr, p.Cys858Trp, p.Thr791Met, and p.Arg816Trp mutations [78].

Overall, studies demonstrate the value of molecular studies as an accurate differential diagnostic method with a high detection rate for type 2N vWD mutations, which is essential for appropriate treatment strategies and genetic counseling. However, genetic testing is only available at some laboratories and specialized centers. Furthermore, molecular studies are more expensive than the vWF:FVIIIB assay with variable insurance coverages. Surveys indicate that some patients are concerned about genetic studies, but most are willing to undergo either test [2].

[DOI: 10.61186/ijbc.16.2.60]

4. CONCLUSION

According to published articles, Type 2N vWD is a relatively rare autosomal recessive disease characterized by a qualitative defect in von Willebrand factor, impairing its binding capacity to factor VIII. The disease resembles mild to moderate hemophilia A. Type 2N vWD is usually associated with low levels of factor VIII coagulant activity but normal levels of von Willebrand factor antigen.

Accurate diagnosis of type 2N vWD requires laboratory testing to distinguish it from hemophilia A. The vWF:FVIIIB assay is a definitive method of diagnosis, as it measures the binding of factor VIII to von Willebrand factor. Genetic testing is also necessary to confirm the diagnosis, as it can identify the specific genetic mutations responsible for the disease.

Unfortunately, not all laboratories have access to these diagnostic methods. As a result, misdiagnosis of type 2N vWD can occur. To prevent this, it is essential to establish reference coagulation centers with the expertise and resources to diagnose type 2N vWD accurately. These centers can provide guidance and support to other laboratories to ensure that patients receive an accurate diagnosis and appropriate treatment.

Acknowledgment

The authors would like to express their gratitude to Iranian Blood Transfusion Organization (IBTO).

Conflict of interest

The authors declare no conflicts of interest.

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[DOI: 10.61186/ijbc.16.2.60]

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[DOI: 10.61186/ijbc.16.2.60]

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[DOI: 10.61186/ijbc.16.2.60]