Accepted Manuscript

Submission Date: 2021-11-25
Accepted Date: 2022-02-18
Publication Date: 2022-02-21

Thrombosis and Haemostasis

The lesson learned from the new c.2547-1G>T mutation combined with p.R854Q:when a type 2N mutation reveals a quantitative von Willebrand factor defect

Alessandra Casonato, Maria Rita Cozzi Cozzi, Silvia Ferrari, Beatrice Rubin, Lisa Gianesello, Luigi De Marco, Viviana Daidone.

Affiliations below.

DOI: 10.1055/a-1777-6881

Please cite this article as: Casonato A, Cozzi M, Ferrari S et al. The lesson learned from the new c.2547-1G>T mutation combined with p.R854Q:when a type 2N mutation reveals a quantitative von Willebrand factor defect. Thromb Haemost 2022. doi: 10.1055/a-1777-6881

Conflict of Interest: The authors declare that they have no conflict of interest.

This study was supported by Grant from the MURST, ex 60% 2020

Abstract:
Type 2N is a rare von Willebrand disease (VWD) variant involving an impairment in the FVIII carrier function of von Willebrand factor (VWF). It has a phenotype that mimics hemophilia A, and FVIII binding to VWF (VWF:FVIIIB) is tested to differentiate between the two disorders. Type 2N VWF defects may also be associated with quantitative VWF mutations (type 2N/type 1), further complicating the identification of cases.
We report on a new quantitative VWF mutation (c.2547-1G>T) revealed by a p.R854Q type 2N mutation acting as homozygous despite being carried as a heterozygous defect. The proband had near-normal VWF levels (initially ruling out a defective VWF synthesis) and slightly reduced FVIII levels, while a VWF:FVIIIB test showed significantly reduced binding. Routine tests on type 2N homozygotes or heterozygotes combined with quantitative VWF defects in our cohort showed reduced FVIII levels in both groups, but it was only in the former that the FVIII/VWF:Ag ratio was always significantly reduced. The two tests are therefore not enough to identify all forms of type 2N VWD. While relatives of type 2N homozygotes usually have normal FVIII levels and FVIII/VWF:Ag ratios, relatives of type 2N/type 1 may have high FVIII/VWF:Ag ratios, but their VWF:FVIIIB and/or VWF:FVIIIB/VWF:Ag ratios are always low.
Measuring FVIII and VWF levels may therefore suggest type 2N VWD in patients carrying type 2N mutations alone, but not in type 2N combined with quantitative VWF defects. The VWF:FVIIIB test should consequently always be included when exploring VWF function, whatever VWD patient's phenotype.

Corresponding Author:
Alessandra Casonato, Universita degli Studi di Padova, Department of Medicine, Padova, Italy, sandra.casonato@unipd.it, vdaidone@gmail.com

Affiliations:
Alessandra Casonato, Universita degli Studi di Padova, Department of Medicine, Padova, Italy
Maria Rita Cozzi Cozzi, Centro di Riferimento Oncologico IRCCS, Centro di Riferimento Oncologico IRCCS, Aviano, Italy
Silvia Ferrari, Universita degli Studi di Padova, Department of Medicine, Padova, Italy
[...]

Viviana Daidone, Universita degli Studi di Padova, Dept of Medicine, Padova, Italy

The Lesson Learned from the New c.2547-1G>T Mutation Combined with p.R854Q: when a type 2N Mutation Reveals a Quantitative von Willebrand Factor Defect

Casonato Alessandra,[1] Cozzi Maria Rita,[2] Ferrari Silvia,[1] Rubin Beatrice,[1] Gianesello Lisa,[1] De Marco Luigi,[3,4] Daidone Viviana[1]

[1]Dept of Medicine, First Chair of Internal Medicine, University of Padua Medical School, Padua, Italy
[2]Immunopathology and Cancer Biomarkers, CRO, Aviano, Italy
[3]Dept of Translational Research, Stem Cell Unit, CRO, Aviano, Italy
[4]Dept of Molecular and Experimental Medicine, SCRIPPS, Research Institute, La Jolla, CA, United States

Address for correspondence: Casonato Alessandra, Dept of Medicine, Via Ospedale Civile 105, 35100 Padova, Italy (e-mail: sandra.casonato@unipd.it).

Abstract

Type 2N is a rare von Willebrand disease (VWD) variant involving an impairment in the FVIII carrier function of von Willebrand factor (VWF). It has a phenotype that mimics hemophilia A, and FVIII binding to VWF (VWF:FVIIIB) is tested to differentiate between the two disorders. Type 2N VWF defects may also be associated with quantitative VWF mutations (type 2N/type 1), further complicating the identification of cases.

We report on a new quantitative VWF mutation (c.2547-1G>T) revealed by a p.R854Q type 2N mutation acting as homozygous despite being carried as a heterozygous defect. The proband had near-normal VWF levels (initially ruling out a defective VWF synthesis) and slightly reduced FVIII levels, while a VWF:FVIIIB test showed significantly reduced binding. Routine tests on type 2N homozygotes or heterozygotes combined with quantitative VWF defects in our cohort showed reduced FVIII levels in both groups, but it was only in the former that the FVIII/VWF:Ag ratio was always significantly reduced. The two tests are therefore not enough to identify all forms of type 2N VWD. While relatives of type 2N homozygotes usually have normal FVIII levels and FVIII/VWF:Ag ratios, relatives of type 2N/type 1 may have high FVIII/VWF:Ag ratios, but their VWF:FVIIIB and/or VWF:FVIIIB/VWF:Ag ratios are always low.

Measuring FVIII and VWF levels may therefore suggest type 2N VWD in patients carrying type 2N mutations alone, but not in type 2N combined with quantitative VWF defects. The

VWF:FVIIIB test should consequently be included when exploring VWF function, whatever VWD patient's phenotype.

Key words

von Willebrand factor

type 2N von Willebrand disease

VWF FVIII carrier function

VWF:FVIIIB

type 2N VWF mutations

INTRODUCTION

Von Willebrand factor (VWF) carries factor VIII (FVIII) in the plasma (**1**), thereby protecting it against proteolysis by activated protein C (**2**), and preventing its clearance from the bloodstream, and the consequent downregulation of its circulating levels (**3**). The FVIII-binding domain of VWF was first identified in the amino-terminal portion of mature VWF, extending between the D'D3 domains, involving residues from 763 to 1036 (**4, 5**), and requiring the cleavage of VWF propeptide (**6**, **7**). It was later demonstrated that the interactive region for FVIII resides mainly in the D' domain of VWF **(8**). Approximately 95-98% of FVIII is in dynamic equilibrium complexed with VWF, with a 1:50 molar ratio of FVIII to VWF (**9**), meaning that most circulating VWF is FVIII-free. Mutations in the D'D3 domain impair the capacity of VWF to carry FVIII, and the associated phenotype features normal VWF levels and lower than normal FVIII levels, with a picture resembling mild or moderate hemophilia A (**10**), but with an autosomal recessive mode of inheritance. The diagnosis of type 2N VWD is suspected when a disproportionate reduction in FVIII levels with respect to VWF levels comes to light, in males or females. This condition was first described in 1987 and called type Normandy (after the area where the first patient was described), and later named type 2N VWD (**11, 12**). The other function of VWF, i.e. primary

hemostasis (platelet adhesion and aggregation), is preserved in type 2N patients. Many type 2N mutations have been reported, most of them located in exons 18-20 of the VWF gene (**13-16**), though exon 17 (**7**), and exons 21-26 have sometimes been involved (**17, 18**). The most common 2N mutation is p.R854Q, which is associated with a relatively mild form of the disease (**13, 14**).

Heterozygotes for type 2N defects generally have no bleeding symptoms (**19, 20**) unless their defect is combined with other hemostatic disorders, such as Glanzmann thromboasthenia or hemophilia A, in which case their bleeding symptoms may even be severe (**21, 22**). Homozygotes or double heterozygotes for type 2N mutations have bleeding symptoms resembling those of hemophilia A, i.e. hemarthrosis or muscle hematomas, and mucocutaneous bleeding (spontaneous or after trauma). This means that establishing a diagnosis of type 2N VWD includes differentiating it from hemophilia A, and there have been numerous reports of patients with type 2N VWD being misdiagnosed as cases of hemophilia A (**23, 24**). Diagnosing type 2N VWD necessitates measuring the exogenous FVIII-binding capacity of a patient's VWF in vitro. The method currently employed is the VWF:FVIIIB test, using exogenous recombinant FVIII (**25**).

In this paper, we report on the complex and elusive phenotype of a heterozygous type 2N defect combined with a new quantitative VWF mutation, which had remained undiagnosed until a VWF:FVIIIB test was performed. The most useful approaches to diagnosing type 2N VWD are also discussed.

MATERIALS AND METHODS

Patients and normal subjects were studied in accordance with the Declaration of Helsinki, after giving their written informed consent, and the study was approved by our institutional review board.

Blood was drawn from patients and normal controls into 3.2% trisodium citrate (1:10 vol/vol) anticoagulant. Basic hemostatic analyses to measure plasma VWF antigen (VWF:Ag), VWF collagen binding (VWF:CB), VWF ristocetin cofactor (VWF:RCo), and FVIII were conducted as described elsewhere (**26**).

The VWF:FVIIIB test was done with a home-made ELISA method (**25**). Briefly, microtitration plates were coated with anti-VWF polyclonal antibody (Dako, Glostrup, Denmark) and left overnight at +4°C. After washing and saturating the plate with 3% fat-free milk, plasma VWF was added and incubated at room temperature for 1 hour. After further washing, bound endogenous FVIII was removed with $CaCl_2$, then recombinant FVIII (rFVIII) (Helixate, Aventis, Marburg, Germany) was added at a concentration of 1 U/mL, and incubated for 1 hour at room temperature. After washing again, the amount of bound FVIII was tested with an anti-FVIII HRP-conjugated polyclonal antibody (Enzyme Research, South Bend, IN, USA). The values were expressed in U/dL, taking as 100 the first dilution of a normal pooled plasma serving as a reference curve. The VWF:FVIIIB values were also normalized to the VWF:Ag values to calculate the VWF:FVIIIB/VWF:Ag ratio (VWF:FVIIIB ratio).

A dose of 0.3 µg/kg of 1-desamino-8-d-arginine vasopressin (DDAVP) (Emosint, Kedrion, Italy) was administered subcutaneously. Blood samples were collected before and then 15, 30, 60, 120, 180, 240, 360, 480 minutes, and 24 hours after administering the DDAVP. The time-course of the post-DDAVP plasma concentrations of VWF:Ag and FVIII was analyzed using a one-compartment model with first-order input and output kinetics (**27**). The model was fitted to each set of concentration-time data using the Prism statistical package (GraphPad, San Diego, CA). The main pharmacokinetic parameters were calculated as explained elsewhere (**28**).

Genetic analysis. Genomic DNA was extracted from peripheral blood leukocytes using the Maxwell® 16 Blood DNA Purification Kit and a Maxwell 16 Instrument (Promega, Wisconsin, USA). PCR reactions for VWF gene sequencing were obtained using the AmpliTaq Gold® DNA Polymerase Kit (Applied Biosystems, AB, California, USA), and primer pairs constructed on the NM_000552 genomic sequence. The sequencing reactions were conducted with the BigDye Terminator Sequencing kit v.1.1 (Thermo Fisher, Massachusetts, USA), and an ABI 3130 XL Genetic Analyzer (AB). RNA was extracted from peripheral blood platelets using the TRIzol™ Reagent (Thermo Fisher) according to the manufacturer's instructions, then retro-transcribed into cDNA using the SuperScript® III First-Strand Synthesis kit (Thermo Fisher).

RESULTS

Patients. Three members of Family 1, coming from northeast Italy, were studied. The proband was a 24-year-old male with a history of bleeding after surgery, dental extractions and minor wounds, and post-trauma hemarthrosis. His bleeding score (BAT) was 7 (0-5 is normal for males). His parents were asymptomatic. Their main hemostatic findings are listed in Table 1.

Six subjects from five families with type 2N VWD combined with a quantitative VWF defect (type 2N/type 1), 4 homozygotes for type 2N VWD (from 3 unrelated families), and 40 type 2N carriers from our cohort were also studied. Their pertinent hemostatic findings are shown in Table 2 and Table 3, respectively.

Hemostatic and genetic findings in the members of Family 1. The proband (Table 1) was characterized by a mildly reduced FVIII (33 U/dL) level and FVIII/VWF:Ag ratio (0.58 vs normal 0.83-1.17), with normal or near-normal VWF:Ag, VWF:CB and VWF:RCo values. His VWF:FVIIIB was significantly reduced (11.3 U/dL vs normal 60-160 U/dL), as was his VWF:FVIIIB ratio (0.2 vs ≥0.74 in normal subjects). This hemostatic pattern was compatible with the presence of homozygous type 2N VWF defects. The father had a reduced

VWF:FVIIIB ratio, while the mother's hemostatic results were all normal, and her FVIII/VWF:Ag ratio was slightly higher than normal (Table 1). Exons 17-22 of the VWF gene were studied to search for the mutation(s) responsible for the defective FVIII binding by VWF. The proband appeared to be heterozygous for the p.R854Q mutation and the novel c.2547-1G>T mutation at the splicing site between intron 19 and exon 20, in a heterozygous state. The father was heterozygous for the p.R854Q mutation, the mother for the new c.2547-1G>T mutation. No other mutations were found.

Since the p.R854Q in a heterozygous state did not explain the patient's VWD phenotype, we suggest that the c.2547-1G>T mutation could induce a quantitative VWF defect. Analyzing the proband's cDNA showed that all genetic polymorphisms seen at heterozygous level in the DNA (including the p.R854Q mutation) were homozygous, and so was the cDNA along its full length (Fig 1). The same pattern emerged in the mother, enabling us to conclude for a degradation of the mRNA carrying the c.2547-1G>T mutation giving rise to a gene null picture. This explains why the proband's heterozygous type 2N defect acts like a homozygous one. The proband was thus classified as having a combination of quantitative and type 2N VWF defects.

Hemostatic and genetic findings in the families with type 2N/type 1 VWD. Patients with combined quantitative and type 2N VWF defects (drawn from our cohort) were analyzed for comparison. They were characterized by a prolonged aPTT, a reduced VWF:Ag and a slightly more severely reduced FVIII, with a normal or slightly reduced FVIII/VWF:Ag ratio (Table 2). VWF:FVIIIB was lower than normal too, and there was a more marked reduction in the VWF:FVIIIB ratio. The patients' mean BAT was 10+/-2.

All the above patients were heterozygous for the type 2N p.R854Q mutation combined with a quantitative VWF mutation giving rise to a gene null pattern (p.P812_fsX3,

p.G2352_2360del, p.T577Sfs*10) or a quantitative VWF defect (p.C524T and p.R760C) (**7**) (Table 2).

The relatives carrying the quantitative VWF defect had lower than normal VWF levels, normal FVIII levels, and a higher FVIII/VWF:Ag ratio (2.1+/-0.93), whereas the results of the above-mentioned routine tests were normal in those carrying the type 2N defect (FVIII/VWF:Ag ratio 0.99+/-0.10). The families with type 2N/type 1 thus included individuals with a normal or increased, or slightly decreased FVIII/VWF:Ag ratio, and with normal or decreased FVIII and VWF levels.

Hemostatic findings in patients carrying type 2N mutations alone. The 4 type 2N homozygotes for the p.R854Q mutation revealed a prolonged aPTT, reduced FVIII levels and a lower FVIII/VWF:Ag ratio, a reduced VWF:FVIIIB and VWF:FVIIIB ratio, but normal VWF:Ag levels (Table 3). While their VWF:FVIIIB ratios were much the same as in the patients with type 2N/type1 VWD, the reduction in FVIII/VWF:Ag ratio was significantly greater (0.31±0.02) in the homozygotes than in the cases of type 2N/type 1 VWD (mean 0.76±0.21). The patients' mean BAT was 7.6+/-2.88.

VWF survival in type 2N. To investigate the consequence of VWF having a FVIII carrier defect, patients' FVIII survival was explored using a DDAVP test lasting 24 hours. The $T_{1/2}$ el of FVIII was calculated with a one-compartment model, adjusted to patients' ABO blood group (Table 4). In the cases homozygous for the p.R854Q mutation, the half-life of FVIII was within normal range, as it was for VWF:Ag (Table 4). A shorter FVIII half-life was found in the 2 subjects with

type 2N/type 1, though the values remained within normal range or at the lower limit of normal range, as seen for VWF:Ag. There was no difference in the half-life of FVIII or VWF between the heterozygote for type 2N VWD and the normal subjects. No correlations

emerged between FVIII survival and the VWF:FVIIIB ratio or the FVIII/VWF:Ag ratio in the subjects investigated (p=0.517 and p=1, respectively).

DISCUSSION

We report a new gene null VWF mutation (c.2547-1G>T) that, combined with the p.R854Q type 2N mutation, gives rise to a type 2N VWD. Without performing the VWF:FVIIIB test, neither the type 2N nor the quantitative VWF defects would have been easy to diagnose, given the patients' normal or near-normal VWF levels and only slightly reduced FVIII/VWF:Ag ratio. The VWF:FVIIIB test enabled us not only to identify the type 2N VWF defect, but also to reveal the presence of a hidden gene null VWF mutation. The patients with this picture were also compared with type 2N homozygotes or type 2N/type 1 VWD patients from our cohort, to clarify the most useful approach to identifying such patients.

Type 2N accounts for 1-2.5% of all cases of VWD (**11**, **29**), so it is a rare variant of a rare disorder, which explains why it is sought less frequently during procedures for diagnosing VWD (**30**). On the other hand, it may be that this variant is rare because it is not usually tested for - as suggested by the finding in various countries that from 1.7% to 5.1% of the general population are heterozygotes for type 2N defects (**11, 31**).

Diagnosing type 2N VWD is made more complicated by an elusive phenotype describing two groups of patients: homozygotes or double heterozygotes for type 2N mutations; and patients carrying a type 2N mutation combined with a quantitative VWF defect. Homozygotes or double heterozygotes for type 2N mutations are characterized by lower FVIII levels and FVIII/VWF:Ag ratios, and a picture indistinguishable from mild forms of hemophilia A. Patients with type 2N/type 1 VWD, on the other hand, have reduced FVIII levels, but normal or only slightly reduced FVIII/VWF:Ag ratios. Their relatives' FVIII/VWF:Ag ratios vary, and may be normal or only slightly lower or higher than normal (**33**), in contrast with subjects whose VWF only reveals a FVIII carrier deficiency. Type 2N/type 1 patients are

characterized by reduced VWF levels, which means that diagnostic procedures tend to focus on their VWF rather than on their FVIII levels or FVIII/VWF:Ag ratio. As a consequence, the type 2N defect may go undiagnosed unless the nature or severity of a patient's bleeding symptoms are inconsistent with their VWF levels, as in the patient described here. It was the presence in our patient of a type 2N VWF defect at heterozygous level that led to us to find the c.2547-1G>T VWF mutation, which does not significantly affect a patient's plasma VWF levels. The novel VWF mutation reported here is paradigmatic inasmuch as it teaches us that algorithms or excessively stringent criteria for diagnosing such a complicated and heterogeneous bleeding disorder as VWD may not always be helpful. According to recent guidelines for the diagnosis of VWD (**32**), it is unnecessary to search for a VWF defect in patients with VWF levels above 50 U/dL (**32**). So, it would have been unnecessary to proceed in the proband or his mother.

Different cases of type 2N VWD may need a different diagnostic work-up: homozygotes or double heterozygotes for type 2N mutations need differentiating from cases of hemophilia A, whereas those with type 2N/type 1 should be distinguished mainly from patients with quantitative VWF defects alone. In both instances, routine tests to ascertain FVIII levels and FVIII/VWF:Ag ratios are not enough for a reliable diagnosis, which can only be achieved by measuring VWF:FVIIIB.

Carrying FVIII is just one of the functions of VWF, which also include binding to collagen or platelet GPIb. The latter functions are measured as part of the diagnosis and characterization of VWD and, by the same logic, VWF binding to FVIII should be as well. Because laboratories do not routinely perform the VWF:FVIIIB test or genetic analyses, it has recently been suggested that one or the other be included in the flowchart for diagnosing type 2N VWD (**32**). Either one may be chosen, where available, but the fact remains that the

VWF:FVIIIB test will always be needed to validate new type 2N genetic variants, so it should be the first choice.

Type 2N VWD is rare, but identifying cases is not just a matter of diagnostic needs or to ensure appropriate genetic counseling for the families involved (though that in itself might suffice), it also affects a patient's treatment. As their reduced plasma FVIII levels are reportedly caused by a shorter FVIII survival (**34, 35**), type 2N patients should only be treated with FVIII concentrates containing VWF. This rule holds, even though DDAVP tests performed in some of our type 2N VWD patients showed a near-normal FVIII half-life (both in patients with type 2N defects alone, and in type 2N carriers). FVIII survival was shorter in those who also had a quantitative VWF defect, partly because their VWF survival was also slightly lower than in normal subjects, and related to ABO blood group (as in healthy subjects).

To sum up, FVIII levels and FVIII/VWF:Ag ratios may not always be useful when a case of type 2N VWD is suspected. These patients can only be identified by measuring VWF:FVIIIB binding and its ratio. Diagnosing type 2N/type 1 can be complicated, and identifying type 2N carriers even more so, as the tests routinely used to diagnose VWD are not informative. That is why we recommend including the VWF:FVIIIB test in the study of VWF function in bleeding patients, whatever the VWD phenotype emerging from routine laboratory tests. We hope that automated VWF:FVIIIB tests can be developed in future, and made available for routine use at all laboratories. This will happen if there is consensus on the need to analyze the FVIII carrier function of VWF for a thorough assessment of VWF function.

What is known about this topic?

- Type 2N is a variant of VWD associated with an impaired FVIII carrier function of VWF.

- FVIII carrier function is rarely explored, unlike other functions of VWF. This is done by VWF:FVIIIB assay.
- Diagnosing type 2N may be challenging when it is combined with quantitative VWF mutations.

What does this paper add?

- As learned from the reported new c.2547-1G>T and p.R854Q mutations combined, measuring FVIII and the FVIII/VWF ratio may not be enough to diagnose type 2N VWD.
- This diagnosis can only be achieved by VWF:FVIIIB assays, which may also be able to reveal hidden VWF mutations.
- We recommend always performing WF:FVIIIB assays when exploring VWF function in bleeding patients.

Authors' Contributions

AC designed the research and wrote the paper; MRC performed the hemostatic tests; SF, BR and LG performed the hemostatic tests and genetic analysis; De Marco L analyzed and discussed the results; VD conducted the genetic analysis, analyzed the data and discussed the results.

Conflict of Interest

None declared.

Acknowledgements

All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest. They have no conflicts of interest to disclose. This work was supported by a grant from the MURST (ex 60% 2020).

References

1) Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem 1998;67:395-424.

2) Fay PJ, Coumans JV, Walker FJ. Von Willebrand factor mediates protection of FVIII from activated protein C-catalyzed inactivation. J Biol Chem 1991;266:2172-2177.

3) Lenting PJ, Vans CJ, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. J Thromb Haemost 2007; 5: 1353–1360.

4) Jorieux S, Magallon T, Mazurier C. Evidence that NH2-terminal but not COOH-terminal moiety of plasma von Willebrand factor binds to factor VIII. Thromb Res 1987; 48:205-210.

5) Foster PA, Fulcher CA, Marti T, et al. A major factor VIII binding domain resides within the amino-terminal 272 amino acid residues of von Willebrand factor. J Biol Chem 1987;262:8443-8446.

6) Wise RJ, Dorner AJ, Krane M, Pittman DD, Kaufman RJ. The role of von Willebrand factor multimers and propeptide cleavage in binding and stabilization of factor VIII. J Biol Chem 1991;266:21948-21955.

7) Casonato A, Sartorello F, Cattini MG, et al. An Arg760Cys mutation in the consensus sequence of the von Willebrand factor propeptide cleavage site is responsible for a new von Willebrand disease variant. Blood 2003;101:151–156.

8) Pietu G, Ribba AS, Meulien P, Meyer D. Localization within the 106 N-terminal amino acids of von Willebrand factor (vWF) of the epitope corresponding to a monoclonal antibody which inhibits vWF binding to FVIII. Biochem Biophys Res Commun 1989;163:618–626.

9) Chiu P-L, Bou-Assaf GM, Chhabra ES, et al. Mapping the interaction between factor VIII and von Willebrand factor by electron microscopy and mass spectrometry. Blood 2015;126:935-938.

10) Pipe SW, Montgomery RR, Pratt KP, Lenting PJ, Lillicrap D. Life in the shadow of a dominant partner: the FVIII-VWF association and its clinical implications for hemophilia A. Blood 2016;128:2007-2016.

11) Casonato A, Galletta E, Sarolo L, Daidone V. Type 2N von Willebrand disease: characterization and diagnostic difficulties. Haemophilia. 2018; 24: 134–140.

12) Goodeve AC. The genetic basis of von Willebrand disease. Blood Rev 2010;124:123-124.

13) Nishino M, Girma J-P, Rothschild C, Fressinaud E, Meyer D. New variant of von Willebrand disease with defective binding to factor VIII. Blood 1989;74:1591–1599.

14) Mazurier C, Jorieux S, Dieval J, et al. A new von Willebrand factor (vWF) deficiency but with normal levels and multimeric pattern of both plasma and platelet vWF. Characterization of abnormal vWF/FVIII interaction. Blood 1990;75:20-26.

15) Gaucher C, Jorieux C, Mercier B, Oufkir D, Mazurier C. The Normandy variant of von Willebrand disease: characterization of point mutation in von Willebrand factor gene. Blood 1991;77:1937-1941

16) Casonato A, Gaucher E, Pontara E, et al. Type 2N von Willebrand disease due to Arg91Gln substitution and a cytosine deletion in exon 18 of the von Willebrand factor gene. Brit. J. Haematol 1998;103:39-41.

17) Jorieux S, Gaucher C, Goudemand J, Mazurier C. A novel mutation in the D3 domain of von Willebrand factor markedly decreases its ability to bind factor VIII and affects its multimerization. Blood 1998; 92:4663-4670.

18) Hilbert L, Jorieux S, Proulle V, et al. Two novel mutations, Q1053H and C1060R, located in the D3 domain of von Willebrand factor, are responsible for decreased FVIII binding capacity. Brit J Haematol 2003;120:627-632.

19) Casonato A, Pontara E, Sartorello F, et al. Identifying carriers of type 2N von Willebrand disease: procedures and significance. Clin Appl Thromb Hemost 2007;13:194-200.

20) Casonato A, Galletta E, Sarolo L, Daidone V. Type 2N von Willebrand disease: characterization and diagnostic difficulties. *Haemophilia* 2018;24:134–140.

21) Casonato A, Pontara E, Sartorello F, et al. Combined hemophilia A and type 2 von Willebrand's disease: defect of both factor VIII level and factor VIII binding capacity of von Willebrand factor. Haematologica 2001;86:1110-1111.

22) Pontara E, Gresele P, Cattini MG, et al. Spontaneous hemarthrosis in combined Glanzmann thrombasthenia and type 2N von Willebrand disease. Blood Coagul Fibrinolysis 2014;25: 401-404.

23) Mazurier C. von Willebrand disease masquerading as Haemophilia A. Thromb Haemost 1992; 67: 391-6.

24) Galletta E, Daidone V, Zanon E, Casonato A. Type 3 von Willebrand disease mistaken for moderate haemophilia A: a lesson still to be learned. Haemophilia 2018;24:e154-e157.

25) Casonato A, Pontara E, Zerbinati P et al. The evaluation of factor VIII binding activity of VWF by means of an ELISA method. Significance and practical applications. Am J Clin Path 1998;109:347-352.

26) Casonato A, De Marco L, Mazzucato M, et al. A new congenital platelet abnormality characterized by spontaneous platelet aggregation, enhanced von Willebrand factor

platelet interaction and the presence of all von Willebrand factor multimers in plasma. Blood 1989;74:2028-2033.

27) Casonato A, Galletta E, Galvanin F, Daidone V. Von Willebrand disease type Vicenza: in search of a classification for the archetype of reduced von Willebrand factor survival. eJHaem 2021;2:340-348.

28) Casonato A, Pontara E, Sartorello F, et al. Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. Blood 2002;99:180-184.

29) Favaloro EJ, Mohammed S, Koutts J. Identification and prevalence of von Willebrand disease type 2N (Normandy) in Australia. Blood Coagul Fibrinolysis 2009;20: 706-714.

30) Seidizadeh O, Peyvandi F, Mannucci PM. Von Willebrand disease type 2N: an update. J Thromb Haemost. 2021;19:909-1916.

31) Bellissimo DB, Christopherson PA, Flood VH, Gill JC, Friedman KD, Haberichter SL, Shapiro AD, Abshire TC, Leissinger C, Hoots WK, Lusher JM, Ragni MV, Montgomery RR. VWF mutations and new sequence variations identified in healthy controls are more frequent in the African-American population. Blood 2012;119:2135-2140.

32) ASH ISTH NHF WFH 2021 Guidelines on the diagnosis of von Willebrand disease. James PD, Connell NT, Ameer B, et al. Blood Adv 2021;12;5:280-300.

33) Eikenboom J, Federici AB, Dirven RJ, et al; MCMDM-1VWD Study Group. VWF propeptide and ratios between VWF, VWF propeptide, and FVIII in the characterization of type 1 von Willebrand disease. Blood. 2013 Mar 21;121(12):2336-9.

34) Tuddenham EG, Lane RS, Rotblat F, et al. Response to infusions of polyelectrolyte fractionated human factor VIII concentrate in human haemophilia A and von Willebrand's disease. Br J Haematol 1982;52:259-267.

35) Mazurier C, Gaucher C, Jorieux S, Goudemand M. Biological effect of desmopressin in eight patients with type 2N ('Normandy') von Willebrand disease. Collaborative Group. Br J Haematol 1994; 88: 849-854.

Fig. 1. DNA and cDNA in the proband and his mother. Panel A, the new c.2547-1G>T mutation identified in the DNA. Panel B, polymorphisms found at heterozygous state in the proband's and his mother's DNA (mainly the c.2561G>A, associated with the p.R854Q type 2N mutation in the proband) were in a homozygous state in the cDNA, as was the whole cDNA in both the proband and his mother, suggesting loss of the allele carrying the new c.2547-1G>mutation.

Table 1. Main hemostatic and genetic findings in Family 1 members

Patients	**Blood gro**	**aPTT** sec	**VWF:Ag** U/dL	**FVIII** U/dL	**FVIII/ VWF:Ag Ratio**	**VWF:FVIIIB** U/dL	***VWF:FVIIIB Ratio**	**VWF:CB** U/dL	**VWF:RCo** U/dL	**VWF mutations**
Proband	O	37.3	56.1	33	0.58	11.3	0.20	69	64	p.R854Q / c.2547-1G>T
Mother	A	29.9	84.9	119	1.4	78.2	0.92	100	92	c.2547-1G>T/ WT
Father	A	30.8	106	104	0.98	62.6	0.59	136	119	p.R854Q /WT
Normal range		25.5-32.5	60-160	60-160	0.83-1.17	60-160	≥0.74	65-150	60-130	

WT= Wild Type
* VWF:FVIIIB/VWF:Ag ratio

Table 2. Main hemostatic and genetic findings in type 2N/type 1 VWD patients.

Family	**Sex/ age**	**Blood gro**	**aPTT** sec	**VWF:Ag** U/dL	**FVIII** U/	**FVIII/ VWF:Ag ratio**	**VWF:FVIIIB** U/dL	***VWF: FVIIIB ratio**	**VWF:CB** U/dL	**VWF:RCo** U/dL	**VWF mutations**
2	F/49	B	40	42	24	0.57	8.53	0.2	44.8	42.8	p.R854Q/ p.P812_fsX31

2	F/54	B	40.5	57.7	34.5	0.59	12.7	0.22	51.4	49.25	p.R854Q/ p.P812_fsX31
3	M/53	A	42.6	19.42	12.2	0.63	4.7	0.24	22.45	24.5	p.R854Q/ p.C524T
4	M/60	O	41.9	41.7	35.9	0.86	14.8	0.35	39.9	41.8	p.R854Q/ p.R760C
5	F/14	A	38.1	46.1	51.1	1.1	14.8	0.36	46.4	50.6	p.R854Q/ p.G2352_2360del
6	F/11	O	41.0	28.8	24	0.83	3.6	0.13	34.2	-	p.R854Q/ p.T577Sfs*10
Normal ran			24-36	60-160	60-160	0.83-1.17	60-160	≥0.74	65-150	60-130	

* VWF:FVIIIB/VWF:Ag ratio

Table 3. Main hemostatic and genetic findings in homozygotes for type 2N VWF mutations, and type 2N carriers

Family	**Sex /age**	**Blood gr**	**aPTT sec**	**VWF:Ag** U/dL	**FVIII** U/dL	**FVIII/ VWF:Ag Ratio**	**VWF: FVIIIB** U/dL	***VWF: FVIIIB ratio**	**VWF:CB** U/dL	**VWF:RCo** U/dL	**VWF mutations**
7	F/28	O	40.4	109	36.7	0.33	5.7	0.23	133.8	120	p.R854Q/ p.R854Q
7	F/24	O	42.6	114.2	34	0.30	28	0.24	118.8	107.2	p.R854Q/ p.R854Q
8	M/7	O	41.9	62.9	18.5	0.29	10.7	0.17	53.3	58.5	p.R854Q/ p.R854Q
9	M/73	O	43.6	95.0	31.1	0.33	23.28	0.25	101.6	91.1	p.R854Q/ p.R854Q
§Heterozygotes			29.9 ±2.8	103.5 ±51.6	85.0 ±34	0.85±0.24	67.0±45.39	0.57±0.09	79.3 ±29.4	97±51	p.R854Q/ WT or p.R760C/
Normal range			24-36	60-160	60-160	0.83-1.17	60-160	≥0.74	65-150	60-130	

§ 39 subjects had the p.R854Q mutation, and 1 had the p.R760C mutation.
* VWF:FVIIIB/VWF:Ag ratio

Table 4. Main post-DDAVP pharmacokinetic parameters in type 2N patients and carriers and healthy subjects

Type 2N subjects	**Blood group**	**VWF:Ag** U/dL	**FVIII/VWF:Ag ratio**	***VWF:FVIIIB ratio**	**T1/2 elimination VWF:Ag** h	**T1/2elimination FVIII** h
Homozygote p.R854Q	A	73.3	0.29 (*21*)	0.24 (*17.5*)	16.8	10.6
Combined p.R854Q/p.R760C	O	38.1	0.94 (*35.9*)	0.23 (*16.8*)	10.2	2.31
Combined p.R854Q/p.G2352_2360del	A	30.5	1.26 (*38.5*)	0.36 (*10.9*)	8.89	3.43
Heterozygote p.R854Q	A	99.4	1.4 (*139.4*)	0.65 (*65.2*)	21.3	9.85

Heterozygote p.R854Q	O	57.0	1.31 (*75.1*)	0.6 (*34.2*)	7.98	5.0
Normal subjects		**O 84.4±35.1 Non-O 96±26.5**	**O 0.9±0.2 Non-O 0.97± 0.15**	**≥0.74**	**O 12.5±5.9 Non-O 16.6±6.5**	**O 4.5±2.1 Non-O 6.5±3.8**

In the brackets are included FVIII and VWF:FVIIIB values

* VWF:FVIIIB/VWF:Ag ratio