# Laboratory Diagnosis of von Willebrand Disease

Muriel Meiring,[1] Philip N Badenhorst[2] and Mareli Kelderman[3]

1. Associate Professor and Specialist Scientist; 2. Professor and Chairman; 3. Medical Technologist,
Department of Haematology and Cell Biology, University of the Free State

## Abstract

von Willebrand disease (VWD) is a bleeding disorder caused by either quantitative (type 1 and 3) or qualitative (type 2) defects of von Willebrand factor (VWF). No single available test provides appropriate information about the various functions of VWF, and the laboratory diagnosis of VWD is based on a panel of tests, including the measurement of factor VIII coagulant activity (FVIIIC), VWF antigen levels (VWF:Ag), VWF activity as measured by the ristocetin co-factor activity (VWF:RCo), the collagen-binding activity of VWF (VWF:CB), VWF multimer analysis, ristocetin-induced platelet agglutination (RIPA), the factor-VIII-binding assay of plasma VWF and VWF propeptide levels. Due to the heterogeneity of VWF defects and the variables that interfere with VWF levels, a correct diagnosis of types and subtypes may sometimes be difficult, but is very important for therapy. Furthermore, the RCo assay and the RIPA test are based on platelet agglutination in reaction with the non-physiological antibiotic ristocetin. These tests also have low sensitivity and are difficult to standardise. Therefore, several analyses (tests) are required to diagnose VWD and it is important to be aware of the pitfalls to which these tests are subjected in terms of the diagnosis. In this article, the laboratory diagnosis of patients with type 1, 2A, 2B, 2M, 2N and 3 VWD will be explained by using a modified algorithm that was first proposed by the guidelines for diagnosis and treatment of VWD in Italy.

## Keywords

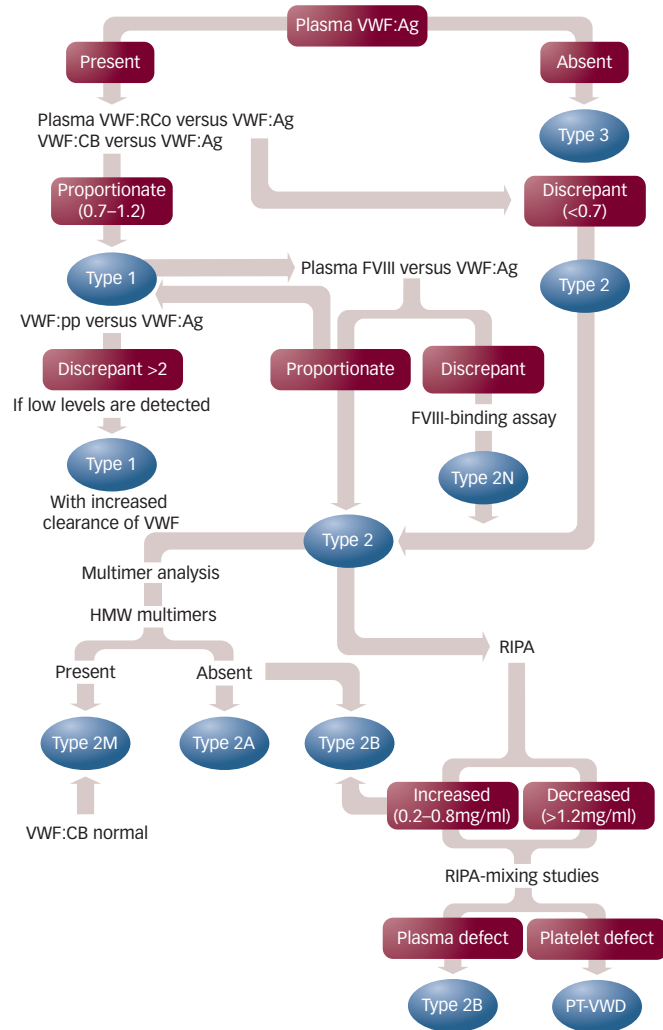von Willebrand factor, von Willebrand disease, classification, subtypes

von Willebrand disease (VWD) is a bleeding disorder caused by quantitative or qualitative defects of von Willebrand factor (VWF). VWF is a high-molecular-weight (HMW) glycoprotein that plays an essential part in the early phases of haemostasis by promoting platelet adhesion to the subendothelium and platelet aggregation under high shear stress conditions.[1] VWF is also the carrier of factor VIII (FVIII) in plasma, and a deficiency or abnormality of VWF also results in an impairment of blood coagulation. By the non-covalent interaction between VWF and FVIII, FVIII is protected against binding to membrane surfaces and to proteolytic attack by a variety of serine proteases, including activated protein C.[2] In the majority of cases, VWD is a congenital disease that is inherited in an autosomal-dominant fashion. Patients with VWD may have had a mild, moderate or severe bleeding tendency since childhood, usually proportional to the degree of the VWF defect. Inherited VWD has been classified into three types that reflect its pathophysiology. Type 1 VWD is characterised by partial quantitative deficiency of VWF. It is the most common type, with a prevalence of about 80%. The mechanisms involved include reduced synthesis and secretion of VWF or increased clearance of VWF from plasma.[3] The treatment is simple, as 1-deamino-8-D-arginine vasopressin (DDAVP) causes the release of structurally normal VWF from endothelial stores. However, in patients with increased clearance of VWF, DDAVP treatment would not be effective as VWF in plasma is cleared very quickly from the circulation. Therefore, it is important to diagnose patients with an increased clearance rate of VWF.

Type 3 VWD is the least common subtype and reflects a virtually complete absence of VWF. Again, DDAVP treatment is not effective in type 3 VWD. The current treatment of choice for type 3 VWD, as well as most type 2 VWD, is transfusional therapy with plasma-derived FVIII/VWF concentrates.[3]

Type 2 VWD is a quantitative defect that is subdivided into four subtypes (2A, 2B, 2M and 2N VWD). Type 2A refers to variants with decreased platelet-dependent function and is associated with the absence of HMW multimers; type 2B refers to variants with increased affinity for platelet glycoprotein 1b$\alpha$; type 2M refers to variants with decreased platelet-dependent function not caused by the absence of HMW multimers; and type 2N refers to variants with markedly decreased affinity for FVIII. DDAVP treatment is not recommended in patients with type 2 VWD, as it would only increase the dysfunctional VWF.

Platelet-type VWD (PT-VWD) is a rare autosomal-dominant bleeding disorder. The genetic defect is in platelets rather than VWF, and the disease is characterised by abnormally high binding affinity of the platelets to the VWF, similar to type 2B VWD.[4] Therefore, most people with PT-VWD are misdiagnosed as type 2B VWD. However, these may require different therapeutic management, so discrimination is clinically important. The spectrum and severity of VWD is wide, ranging from a few doubtful haemorrhagic symptoms to severe life-threatening bleeding episodes. This is due not only to the heterogeneous VWF gene,

# Coagulation Disorders

**Figure 1: Algorithm for a Laboratory Diagnosis of von Willebrand Disease**



VWF = von Willebrand factor; RCo = ristocetin co-factor; Ag = antigen; CB = collagen-binding; pp = propeptide; FVIII = factor VIII; HMW = high-molecular-weight; RIPA = ristocetin-induced platelet agglutination; PT = platelet type.

which may impair its haemostatic function, but also to the influence exerted by other genes (e.g. those for ABO blood groups).[6] In addition, many acquired conditions – either physiological (stress, pregnancy) or pathological (inflammation) – can induce fluctuations in VWF levels).[6] This highly variable clinical picture and the presence of many different defects in the VWF molecule complicate the diagnosis of VWD.[6] The guidelines for diagnosis and treatment of VWD in Italy[7] propose the use of an algorithm (see *Figure 1*). We adopted and modified these guidelines in our VWD testing facility.

## Laboratory Diagnosis of von Willebrand Disease
### Sample Collection
Blood samples must be collected into tubes containing 0.105M sodium citrate in a ratio of 1:9 with blood. Platelet-poor plasma (PPP) is prepared by centrifugation of whole blood at 2,000g for 20 minutes at room temperature. Samples must be stored immediately after centrifugation in polypropylene tubes at -70ºC until analysed. It is important to note that a cryoprecipitate may form if plasma samples are stored at temperatures over -70ºC. Cryoprecipitate contains large quantities of VWF, especially HMW multimers.[8] All tests must be performed on original aliquots that were not previously thawed, and

plasma samples should be thawed to 37ºC before performing the diagnostic tests. Special care should be taken to ensure that no cryoprecipitate is present in the samples; therefore, it must be dissolved before the tests are performed as it will influence the results.[8]

## Screening Tests
Screening tests for bleeding disorders include a platelet count, a bleeding time, prothrombin time (PT) and activated partial thromboplastin time (APTT), plasma FVIII levels and the blood group of a patient. These tests are usually carried out by a routine coagulation laboratory.

## Laboratory Tests
The first line of tests includes the VWF concentration in plasma (VWF:Ag), the ristocetin co-factor assay (VWF:RCo) and the collagen binding assay (VWF:CB). VWF:Ag is measured with an enzyme-linked immunoabsorbent assay (ELISA). An ELISA plate is coated with a specific rabbit antihuman VWF antibody that captures the VWF to be measured. The plasma to be measured is added in 1:50 and 1:100 dilutions in blocking buffer. Afterwards, a rabbit antihuman VWF antibody conjugated to peroxidase is added. This antibody binds to the remaining free antigenic determinants of VWF, subsequently forming a 'sandwich'. The bound enzyme peroxidase is revealed by its activity in a pre-determined time on the substrate ortho-phenylenediamine (OPD) in the presence of hydrogen peroxide. After stopping the reaction with a strong acid, the intensity of the colour produced bears a direct relationship to the VWF concentration initially present in the plasma sample. A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured.

The RCo assay is performed with formalin-fixed washed platelets in an aggregometer with software suited for the test. Formalin-fixed washed platelets can be obtained commercially or can be self-prepared from normal platelet-rich plasma (PRP). Washed platelets do not agglutinate in the presence of the antibiotic ristocetin unless normal plasma is added as a source of VWF. The agglutination follows a dose–response curve that is dependent on the amount of plasma VWF added. Usually, plasma concentrations of 1:2 and 1:4 in Tris-

*A functional assay that more laboratories have been starting to use over the last seven years is the collagen-binding assay of von Willebrand factor.*

buffered saline (TBS) are used. A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured. Another functional assay that more laboratories have been starting to use over the last seven years is the collagen-binding assay (CBA) of VWF.[9] The CBA is based on the ability of the HMW multimers of VWF that preferentially bind to collagen. This is an ELISA-based assay where dilutions of the patient's plasma (comparable to the VWF:Ag 1:50 and 1:100 dilutions) are added to a collagen-coated ELISA plate. The type of collagen seems to be important, but discordance still exists about which type of collagen to use (type 1,

type 3 or a combination thereof).[10] The amount of bound VWF is determined by using an anti-horseradish peroxidase (HRP)-conjugated VWF antibody. The values are expressed in U/dl or per cent. A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured. This assay has been shown to be sensitive in the discrimination of type 1, 2A and 2B VWD.[11] Casonato et al.[12] demonstrated that the CBA was consistently more sensitive to large- and intermediate-VWF multimer representation than the RCo assay. The decreased values of the CBA in type 2A and 2B patients are more consistent than that of the RCo assay. However, the CBA is insensitive to type 2M VWD patients.[13]

The ristocetin-induced platelet agglutination (RIPA) and the VWF multimer patterns are performed to diagnose the type 2 subtypes. RIPA is measured by mixing different concentrations of ristocetin ranging from 0.2 to 2mg/ml in increments of 0.1mg/ml with the patient's PRP in an aggregometer. The results are expressed as the concentration of ristocetin (mg/ml) able to induce 30% agglutination. RIPA-mixing studies are performed to distinguish between type 2B VWD and PT-VWD. In short, the patient's PRP and PRP from a control person are centrifuged and gently resuspended to 200x10^{6} platelets per millilitre in the following manner: patient platelets/patient plasma; normal platelets/normal plasma; control platelets/patient plasma; and patient platelets/normal plasma. Different ristocetin concentrations are added to each of the platelet suspensions and the agglutination is measured in an aggregometer, similar to the RIPA assay.[5] PT-VWD is diagnosed when the RIPA-mixing studies confirmed a platelet origin. The multimeric structure of VWF in plasma is determined by a highly sensitive and rapid method originally described by Krizek and Rick in 2000.[14] This method utilises submerged horizontal agarose gel electrophoresis followed by transfer of the VWF onto a polyvinylidine fluoride membrane and immunolocalisation and luminographic visualisation of the VWF multimer pattern. This method distinguishes type 1 from type 2A and 2B VWD. The density of the high-, intermediate- and low-molecular-weight multimers of each multimer pattern is determined using a gel-documentation system.[15] VWF multimer patterns from normal plasma and type 1, 2A, 2B and 2M VWD is shown in *Figure 2* and the density graphs in *Figure 3*.

The capacity of plasma VWF to bind exogenous FVIII is measured with an ELISA. A microplate (Maxisorp, Nunc, Denmark) is coated by incubation for two days at 4°C with a rabbit polyclonal antihuman VWF. After washing with a TBS buffer of 0.1% bovine serum albumin (BSA) and 0.05% Tween, the wells are saturated with TBS containing 3% BSA. Next, 100µl of serial dilutions of plasma from patients and normal pooled plasma are added and incubated overnight at 4°C. Each patient sample is tested in six serial dilutions, the first adjusted to 5% VWF-antigen level. After removal of endogenous FVIII using 350mmol/l $CaCl_2$ (twice for 10 minutes), 70mU of recombinant FVIII is added to each well. After incubation for two hours at 37°C and washing, bound FVIII is quantified using 1µg/ml of peroxidase-conjugated sheep polyclonal antihuman FVIII. After washing, immobilised VWF is measured using a peroxidase-conjugated rabbit polyclonal antihuman VWF. The colour is developed by addition of OPD and the optical density read at 490nm. Two reference curves are established in parallel. One is for the quantification of immobilised VWF and the other one for the quantification of bound rFVIII. For each plasma dilution, the values of bound rFVIII are plotted against the amount of immobilised VWF. The slopes of the obtained regression lines reflect the binding capacity of VWF to FVIII.[16]

**Figure 2: The Multimeric Structure of von Willebrand Factor in Normal Plasma and von Willebrand Disease Types 1, 2A, 2B and 2M**
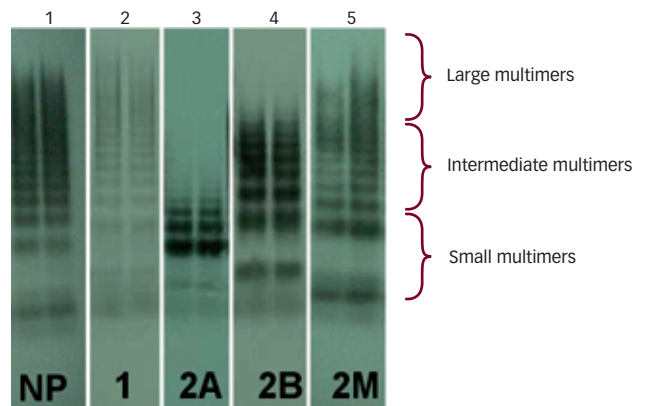


*Source: Meiring et al., 2005.[15]*

**Figure 3: Densitometric Tracing of Lane Sets 2–5**
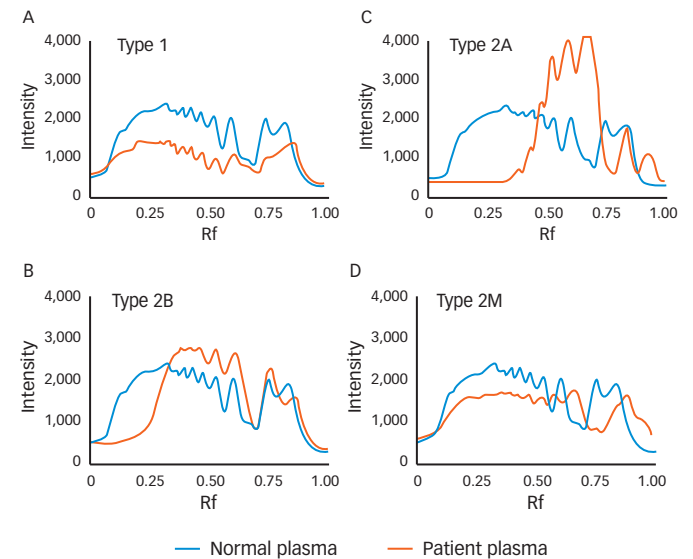


*The density of the different bands is plotted against the relative front (Rf value) of the different lanes.*

The VWF propeptide (VWF:pp) level in the patient's plasma is also measured with an ELISA. Microtitre wells are coated with CLB-Pro 35 antibody and incubated with the plasma sample. Subsequently, the wells are washed and the bound propeptide-containing protein is detected with CLB-Pro 14.3 coupled to peroxidase. Calibrators for the VWF:propeptide assay are used to set up a standard curve against which the patient's plasma is measured.[17]

## Diagnosis of von Willebrand Disease

The algorithm used for the laboratory diagnosis of VWD is outlined in *Figure 1*. A proportional reduction of both VWF:Ag and VWF:RCo with a RCo/Ag ratio >0.7 as well as a proportional reduction of both VWF:Ag and VWF:CB with a CB/Ag ratio >0.7 suggest type 1 VWD. If type 1 VWD is diagnosed, it is important to determine the clearance rate of VWF; consequently, the VWF:pp is performed. If the ratio between the VWF:pp and the VWF:Ag is more than two, an increased clearance rate of VWF is suspected in this patient.

If the RCo/Ag ratio and/or the CB/Ag ratio is <0.7, type 2 VWD is diagnosed. Type 2B VWD can be identified with an enhanced RIPA (<0.8mg/ml). Type 2B VWD is distinguished from a PT-VWD (pseudo-

VWD) by performing the RIPA-mixing studies. Type 2A and 2M may have a low RIPA (>1.2mg/ml). Multimeric analysis in plasma is necessary to distinguish between type 2A VWD (lack of largest and intermediate multimers) and type 2M VWD (all the multimers are present). The VWF:CB is usually normal in type 2M VWD due to the presence of the HMW multimers, except where a collagen-binding defect is diagnosed in patients with type 2M VWD. In type 1 VWD, the ratio between FVIII and VWF:Ag is always concordant. When this ratio is discrepant with a FVIII level of <20%, type 2N VWD is suspected, and this type of VWD can be confirmed by performing a FVIII-binding assay.

## Conclusion

We have outlined a systematic way to diagnose VWD. This algorithm is also recommended by the International Society for Thrombosis and Haemostasis (ISTH). It is important to note that the RCo/Ag ratio and the CB/Ag ratio are necessary to distinguish between type 1 and type 2 VWD.

The CBA is extremely sensitive in the discrimination of type 1 and types 2A and 2B VWD.[11] In contrast, however, the RCo test has a poor sensitivity of 50%,[18] which is difficult to standardise,[19] and lacks a physiological analogue; however, it remains the standard method for measuring VWF activity approved by the Standardisation Committee of the ISTH. Casonato et al.[12] demonstrated that the VWF:CB was consistently more sensitive to large and intermediate VWF-multimer representation than the VWF:RCo. None of the type 1 VWD patients studied showed a greater decrease in CBA than in Ag levels, which was not the case with the RCo assay. The decreased values of the CBA in type 2A and 2B patients were more consistent than those of the RCo assay. The VWF:CB is normal in type 2M VWD patients due to the presence of the HMW multimers. This assists in the diagnosis of type 2M VWD.

The mechanisms involved in type 1 VWD include reduced synthesis and secretion of VWF or increased clearance of VWF from plasma.[20] In patients with increased clearance of VWF, the DDAVP treatment would not be effective, as VWF in plasma is cleared from the circulation very quickly. The ratio between the propeptide and the antigen levels is used as an indication of VWF clearance in these patients.[3]

The RIPA test is necessary to distinguish between type 2B and type 2A VWD. It is also important to carry out the RIPA test with ristocetin concentrations ranging from 0.2 to 2mg/ml in increments of 0.1mg/ml. This will ensure a range of concentrations to determine the maximum agglutination value of 30%. Currently, the RIPA test that is being used by most laboratories in developing countries is performed with only three ristocetin concentrations (0.5, 1 and 1.25mg/ml). It is sometimes difficult to determine whether the RIPA is enhanced or reduced on only three values. Laffan et al.[21] also found RIPA to be normal in patients with a VWF:RCo value of less than 30%. They found RIPA decreased only in severe forms of VWD. When RIPA is increased and type 2B is suspected, it is important to perform the RIPA-mixing studies to distinguish PT-VWD from type 2B VWF.

VWF multimeric analysis in plasma is necessary to distinguish between subtypes of type 2 VWD. Our method includes rapid processing, simplicity of gel preparation, high sensitivity to low concentrations of VWF and elimination of radioactivity.[15] Type 2N VWD can be suspected in case of discrepant values of FVIII. In typical type 2N cases, the FVIII level is usually less than 20%.[22] The diagnosis of type 2N VWD should be confirmed by the FVIII-binding assay. As VWD is such a complex disease to diagnose, this systematic approach makes the diagnosis of VWD more accurate, which is of vital importance for the treatment of the disease. However, there are pitfalls in this diagnostic process that are due to the limitations in sensitivity, reproducibility and inter-laboratory variability of the agglutination-based RCo and RIPA tests.[23]

In conclusion, it is crucial to use a systematic method to diagnose VWD. Each laboratory test only forms one piece of the diagnostic puzzle and therefore it is necessary to put all of the puzzle pieces together for the whole diagnostic picture to emerge. ■

Muriel Meiring is an Associate Professor and Specialist Scientist in the Department of Haematology and Cell Biology at the University of the Free State at Bloemfontein in South Africa. She established the Specialised Haemostasis Laboratory at the University of the Free State as a reference centre for von Willebrand disease (VWD). Professor Meiring's list of publications and congress presentations has won various awards at local and national congresses.

Philip N Badenhorst is Chairman of the Department and a Professor of Haematology and Cell Biology at the University of the Free State and the National Health Laboratory Service at Bloemfontein in South Africa. Professor Badenhorst is a former President of the South African Society of Haematology (SASH). He has received the University of the Free State Centenary Medal in recognition for his strong role and national profile in establishing and developing haematology as a discipline at the university.

Mareli Kelderman is a Medical Technologist (specialising in haematology) in the Department of Haematology and Cell Biology at the University of the Free State in Bloemfontein in South Africa, and is a member of the Society of Medical Laboratory Technologists of South Africa (SMLTSA). She developed, streamlined and implemented several tests for diagnosing von Willebrand disease (VWD), especially the von Willebrand factor (VWF) multimer test and the factor VIII-binding assay.

1. Tuddenham EG, *Blood Rev*, 1989;3: 251–62.
2. Vlot AJ, Koppelman SJ, Bouman BN, Sixma JJ, *Thromb Haemost*, 1998;79: 456–65.
3. Sadler JE, *Thromb Haemost*, 2005;3:1702–9
4. Kessler C, *Eur J Haematol*, 2007;2(1):16–17.
5. Favaloro EJ, Patterson D, Denholm A, et al., *Br J Haematol*, 2007;139:621–8.
6. Laffan M, Manning R, *Dacie and Lewis Practical Haematology*, Philadelphia: Elsevier, 1995;379–440.
7. Federici AB, Castman G, Mannucci PM, *Haemophilia*, 2002;8:607–21.
8. Favaloro EJ, Mehrabani PA, *Haemophilia*, 1996;2:218–23.
9. Favaloro EJ, Bonar R, Kershaw J, et al., *Haemophilia*, 2004;10:232–42.
10. Favaloro E, *Thromb Haemost*, 2000;83(1):127–35.
11. Favaloro E, Koutts J, *Patology*, 1997;29:385–90.
12. Casonato A, Pontara E, Bertomoro A, et al., *Br J Haematol*, 2001;112:578–83.
13. Meiring SM, Badenhorst PN, Kelderman M, *Clin Chem Lab Med*, 2007;45(8):1068–72.
14. Krizek DR, Rick ME, *Thromb Res*, 2000;97:457–62.
15. Meiring SM, Badenhorst PN, Kelderman M, *Medical Technology SA*, 2005;19(2):15–20.
16. Casonato A, Pontara E, Zerbinati P, et al., *Am J Clin Pathol*, 1998;109(3):347–52.
17. Borchiellini A, Fijnvandraat K, Ten Cate JW, et al., *Blood*, 1996;88:2951–8.
18. Scott JP, Montgomery RR, Retzinger GS, *J Biol Chem*, 1991;266:8149–1.
19. Casonato A, Pontara E, Bertomoro A, et al., *Thromb Haemost*, 1999:81:994–5.
20. Haberichter S, Castaman G, Budde U, et al., *Blood*, 2008;111:4979–85.
21. Laffan M, Brown SA, Collins PW, et al., *Haemophilia*, 2004;10:199–217.
22. Schneppenheim R, Budd U, Krey S, et al. *Thromb Haemost*, 1996;76:598–602.
23. Favaloro E, Smith J, Petinos P, et al., *Thromb Haemost*, 1999;82:1276–82.