

STUDIES OF VON WILLEBRAND FACTOR AND GPIIb/IX/V COMPLEX
IN THE DIAGNOSIS OF VON WILLEBRAND'S DISEASE AND
BERNARD-SOULIER SYNDROME

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INTRODUCTION

Haemostasis is a complex and highly regulated process that secures the cessation of bleeding upon vascular injury and degrades the clot once it becomes unnecessary. The first step is the adhesion of platelets to the injured vessel wall followed by their activation and aggregation (primary haemostasis). The coagulation cascade becomes activated and fibrin strengthens the platelet plug (secondary haemostasis). The clot is degraded by the proteolytic system. Under high shear conditions that characterize the arterial circulation, especially the small arteries of the microcirculation, platelet adhesion requires a strong interaction between the platelets and the vessel wall. Once platelets are tethered, they roll slowly along the vessel wall, become activated and attach firmly to the site of the injury. This strong interaction is created by von Willebrand factor (VWF) that bridges its platelet surface receptor, the GPIb/IX/V complex to the subendothelial structures.

VWF is a huge, multimeric protein synthesized by megakaryocytes and endothelial cells. Endothelial cells secrete 95% of the synthesized VWF while 5% is stored in the Weibel-Palade bodies and secreted only upon stimulation (thrombin, adrenergic stress, 1-desamino-8-arginine vasopressin–DDAVP). Megakaryocytes and platelets secrete VWF only through the regulated pathway. Multimers are stored in the α -granules. The molecular weight of the VWF multimers ranges from 500 kD (dimer) to 20,000 kD, and even bigger “ultrahigh molecular weight” multimers can be present in the storage sites. VWF plays two roles in haemostasis: it secures platelet adhesion in high shear environment and it stabilizes coagulation factor VIII and protects it from proteolytic degradation.

Quantitative or functional impairment of VWF leads to a frequent bleeding disorder, von Willebrand’s disease (VWD). The prevalence of VWD is 1%. It is characterized by mucocutaneous haemorrhages. Type 1 means the partial, type 3 the complete deficiency of VWF. Type 2 is qualitative defect: in type 2A the high molecular weight multimers are missing due to a dimerisation/multimerisation defect or due to the increased susceptibility of VWF to the VWF cleaving protease. In type 2B VWD mutations in the VWF A1 domain cause enhanced binding of VWF to GPIb leading to the disappearance of the high molecular weight multimers and thrombocytopenia. Type 2M means impaired function with normal multimeric structure. In type 2N the FVIII binding activity is defective.

The diagnosis of VWD requires an array of special, time consuming tests. There is a need for simple, rapid screening tests that reflect the bleeding tendency of the patients. The traditional *bleeding time* determination is the most widely used screening test of the disorders

of the primary haemostasis. Despite the attempts for the standardisation of the test, its sensitivity is questionable. The result is highly dependent on the skill of the technician. It is not well tolerated by the patients and leaves scars. As a substitute, “in vitro bleeding time” tests modelling primary haemostasis under high shear conditions gained popularity in recent years. The best described such device is the *PFA-100 (Platelet Function Analyzer)*. Citrated blood flows through under constant vacuum a capillary and a 150 μm aperture on a nitrocellulose membrane coated with equine tendon collagen type I. A shear rate of 5,000-6,000 s^{-1} is created. Two types of cartridges are available: in one ADP, in the other epinephrin is added as an aggregating agent. The device reports the closure time, the time needed for the blockage of the aperture by the adhered and aggregated platelets. Another device working under high shear conditions is the O’Brien filter test. Heparinized and citrated whole blood is forced through the capillary sized channels of a glass filter at a pressure of 40 Hgmm. Platelets become activated, aggregate and the aggregates block the filter. Drops passing through the filter are collected in the first 5 seconds (first phase) and between 20-40 seconds (second phase). Platelet counts are determined and platelet retention is calculated in the two phases of the filter test. An other characteristic parameter of the filter test is the blocking drop count. By definition it means the drop count when ≤ 1 drop passes through the filter in 5 seconds. The common feature of the two tests is that anticoagulated whole blood flows through the capillary sized tubes of the instrument. Both tests are dependent on VWF, GPIb and GPIIb/IIIa.

One of the basic specific tests of VWD is the *von Willebrand factor antigen determination (VWF:Ag)* that gives the amount of VWF present. The traditional method is the Laurell-electrophoresis, the best sensitivity is achieved by performing an ELISA test. In recent years, latex immunoassays became widespread due to their easy handling, low cost and rapid results on automated instruments. The other basis of the diagnosis of VWD is the determination of the functional activity of VWF. This can be measured by the ristocetin cofactor activity (VWF:RCo), the collagen binding assay (VWF:CB) and the FVIII coagulant activity (FVIII:C). When the amount and the activity of VWF is decreased to a similar extent, a quantitative defect is diagnosed. When the decrease in the activity is more pronounced than in the antigen level (activity/VWF:Ag $< 0,7$) type 2 VWD is suspected. In Type 2 the subgroups can be differentiated by discriminative tests. These tests are: ristocetin induced platelet aggregation (RIPA), VWF multimeric structure analysis on SDS agarose gels and FVIII binding assay (VWF:FVIII B).

The platelet surface receptor of VWF is the GPIb/IX/V complex. This complex is composed of four transmembrane proteins: GPIb α , GPIb β , GPIX and GPV in a 2:2:2:1 stoichiometry. GPIb α is the receptor of VWF and thrombin, GPIb β and GPIX are needed for the stability of the complex. All proteins contain variable number of leucine rich repeats. GPIb α and GPIb β are connected through a disulphide bridge while the other members of the complex are non-covalently associated.

The reduced amount or the defective function of the GPIb/IX/V complex causes Bernard-Soulier syndrome (BSS), a rare bleeding disorder characterized by mucocutaneous haemorrhages and autosomal recessive inheritance. Its prevalence is 1/1,000,000 on the basis of the reported cases. Patients are thrombocytopenic, and huge platelets are seen in the peripheral smear. Patients are often thought to suffer from immune thrombocytopenic purpura and treated accordingly. The diagnosis is based on the absence of ristocetin induced platelet aggregation that cannot be corrected by the addition of normal plasma. The gene of GPIb α is localized on the short arm of chromosome 17, GPIb β on the long arm of chromosome 22, GPIX and GPV on the long arm of chromosome 3. These genes are small and their structure is simple. All but one (GPIb β) coding sequence is contained within one exon. BSS is caused by mutations in any of the genes of GPIb α , GPIb β and GPIX. No mutations in GPV have been described. The known mutations cause the defective expression or function of the complex by *i*) early termination of protein synthesis and defective insertion of the proteins in the platelet membrane. *ii*) Mutations affecting the structure of the proteins and causing impaired function and reduced expression. *iii*) Mutations affecting the promoter region of the genes and causing diminished transcription.

AIMS OF THE STUDY

1. Evaluation of high shear induced platelet aggregation in the diagnosis and screening of VWD.
 - A, The performance of the O'Brien filter test in VWD.
 - B, The performance of the PFA-100 in VWD.
 - C, Comparison of the two tests
 - D, Comparison of the "in vitro bleeding time" tests with the traditional bleeding time determination.

- 2, To draw attention on the variable effect of rheumatoid factor on the von Willebrand factor latex immunoassays.
- 3, Laboratory examination and genetic testing of a patient with Bernard-Soulier syndrome.
 - A, Determine the possible localisation of the mutation by measuring the amount of the proteins of the GPIb/IX/V complex.
 - B, PCR amplification and sequencing the genes of the GPIb/IX/V complex.
 - C, Determine the homo- or heterozygous state by restriction enzyme analysis.

PATIENTS, MATERIALS AND METHODS

Ad 1. High shear induced platelet aggregation in the diagnosis of VWD.

Patients and control persons: Thirty patients with established VWD were included in the study, twenty women and ten men. Their mean age was 34.5 ± 11 (18-60) years. Twentytwo patients had type 1, two type 2A, three type 2B, three type 3 disease. In type 1 VWD we differentiated between mild and severe form. The bleeding tendency was considered mild if there were no major bleeding complications requiring hospitalization, transfusion and VWF concentrate administration in the history of the patients. The control group consisted of twenty healthy volunteers (4 men and 16 women), their mean age was 32.1 ± 8.5 (23-50) years.

Haemoglobin levels and platelet counts were investigated with a Sysmex K-4500 cell counter (Toa Medical Electronics, Kobe, Japan). *Template bleeding time* was determined by two experienced technicians using Simplate II R disposable device (Organon Teknika, Boxtel, The Netherlands). *APTT* was measured by Platelin LS (Organon Teknika) on Coag-a-Mate MTX (Organon Teknika). *VWF:Ag levels* were determined by a sandwich ELISA technique using two polyclonal antibodies (Dako, Glostrup, Denmark). *VWF:RCO activity* was determined by aggregometry with the commercial Ristocetin Cofactor Assay (Helena Laboratories, Beaumont, Texas) on a Chrono-Log 810-CA aggregometer (Havertown, PA). *FVIII:C activity* test was performed by the one stage clotting assay based on APTT using FVIII-deficient plasma (Organon Teknika). *Ristocetin induced platelet aggregation* was investigated by using ristomycin (Aggristin kit, Reanal, Hungary) in final concentrations of

0.5-0.6-1.0-1.2-1.5 mg/ml, respectively. The multimeric composition of VWF was analysed by SDS gel electrophoresis.

The *O'Brien filter test* has been described in the "Introduction". Briefly: heparinized and citrated whole blood samples are forced through a filter made of fine glass fibers at a pressure of 40 Hgmm. The number of drops passing through the filter is detected by a drop counter and recorded at 5 sec intervals manually. Effluent drops during the first 5 sec (first phase) and between 20-40 sec (second phase) are collected, the pre-filter and post-filter platelet counts in the two phases are determined, and platelet retention is calculated. The method is characterized by the blocking drop count (≤ 1 drop passing through the filter in 5 sec) and the platelet retention expressed in percentage.

The *PFA-100* (Dade International Inc., Miami, FL) has been described in the "Introduction". Citrated whole blood was used and all tests were performed within two hours. The device reported the closure time (CT).

Statistics: The results were given as mean \pm SD. The reference ranges were calculated as mean \pm 2SD. The reference range of the manufacturer was accepted for the bleeding time determination (2.5-9.5 min). The methods were characterized by their sensitivity, specificity, positive and negative predictive values.

Ad 2. The effect of rheumatoid factor on latex immunoassays

A 53 year old male patient has been known to be a bleeder since his childhood. He required repeated blood transfusions because of epistaxis and bleeding after tooth extraction. His children (J.I. and J.L.) are also bleeders. On the basis of thorough laboratory evaluation and genetic analysis type 2M Vicenza was diagnosed. In March 2004 *VWF:Ag levels* were measured in parallel in two independent laboratories of the University of Debrecen. Both laboratories use immunoturbidimetric assays, but in one laboratory the STA-Liatest VWF (Diagnostica Stago, Asnieres, France), in the other the IL Test VWF (Instrumentation Laboratory, Lexington, MA) is available. As a reference, the *VWF:Ag level* was determined by ELISA with polyclonal antibodies against VWF (Dako). The *VWF:RCo* was investigated with the traditional *agglutination method* (Ristocetin Cofactor assay, Helena Laboratories) and with a new *ristocetin cofactor ELISA*, that measures the binding of VWF in the presence of ristocetin to an ELISA plate covered with glyocalicin. Briefly: ELISA plates were incubated with monoclonal antibody 24B3 raised against GPIIb α . After blocking with 3% milk powder, plasma glyocalicin was captured by the incubation of 1/2 diluted platelet rich plasma. Diluted patient plasma samples containing ristocetin (Abp, New Jersey, USA) in a

final concentration of 1 mg/ml were added. VWF bound to glyco-calicin was detected by anti-VWF-HRP and OPD. *VWF:CB* was determined on ELISA plates covered with human type III collagen (Sigma, St.Louis, MO). Rheumatoid factor (RF) was detected by immunoturbidimetric method.

Ad 3. Examination of a patient with Bernard-Soulier syndrome

A 38 years old female patient was investigated. The leading symptom of the disease was meno-metrorrhagia causing iron deficiency anaemia. She had severe bleeding complication after delivery. Her son was also a bleeder, he required blood transfusion after tonsillectomy at the age of 3. The patient has been known to be thrombocytopenic (44-82 G/l, reference range: 160-400 G/l). Giant platelets were observed in the peripheral smear. Bleeding time was over 20 minutes. *Platelet aggregation* was carried out in platelet rich plasma (PRP) with ADP (final concentration: 5 μ M), collagen (2.5 μ g/ml) and ristocetin (1.2 mg/ml). The presence of the GPIb/IX/V complex on the platelet surface was examined by flow cytometry and Western blot analysis. *Flow cytometry* was carried out by Jean-Pierre Delville (Laboratoire D'Hematologie, Hopital Erasme, Université Libre de Bruxelles) on Facscalibur (Beckton Dickinson, San Jose, CA) with indirect immunofluorescence in PRP and with direct fluorescence in whole blood. The quantitative determination of the proteins of the GPIb/IX/V complex was carried out with the Cytoquant CD42 Quantification kit (Biocytex, Marseille, France). For the preparation of platelet lysate washed PRP was resuspended in sample buffer containing 0.34 M Tris-HCl, 39% glycerol, 7.8% SDS. *Polyacrilamide gel electrophoresis* was carried out on 7.5 and 10% gels under non-reducing and reducing conditions. The proteins were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and blocked with 5% milk powder. GPIb α and GPIX were detected with monoclonal, GPIb β with polyclonal antibodies. *Genomic DNA was separated* from whole blood using the QIAamp DNA Blood Midi Kit (Quiagen, Westburg, Hilden, Germany) and the genes of GPIb α , GPIb β and GPIX were amplified by PCR. The PCR-products were cloned into pCR4-TOPO vector using the TOPOTM TA Cloning Kit for Sequencing (Invitrogen, Groningen, The Netherlands). *DNA sequence analysis* was either performed with the T7 Sequencing Kit (Pharmacia Biotech, Roosendaal, The Netherlands) and analysed on a manual sequence apparatus (BioRad, Hercules, USA) or with the ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Warrington, England) and analysed on an ABI PRISM 310 (PE Applied Biosystems). For *restriction enzyme analysis*

control and patient GPIX 3-4 fragments were digested with *Fnu4H1* (New England Biolabs, Hitchin, England) and analysed on agarose gels.

RESULTS

Ad 1. Results with the high shear induced platelet aggregation tests in VWD

Bleeding time: 2/13 (15.4%) patients with mild disease had prolonged bleeding time. In eleven cases (11/13, 84.6%) the bleeding time was normal. Among the nine type 1 patients with severe symptoms, seven had a bleeding time over 9.5 min (77.7%), in two cases it was over 20 min. In type 2A and type 3 all patients had bleeding times over 20 min. In type 2B VWD the bleeding time was prolonged only in the case of a patient with thrombocytopenia (20 G/l). The two other patients with type 2B VWD had normal bleeding times. The sensitivity of the bleeding time determination was 50% if the results of all patients were included.

Results of the O'Brien filter test: Blocking drop count and platelet retention in the two phases were evaluated with two anticoagulants. Although the blocking drop count was higher only in 53.8 % (heparin) and 58.3 % (citrate) in mild type 1 disease, all patients with severe symptoms had higher than normal blocking drop count, and the filter was not blocked in 6/9 cases with heparinized, in 8/8 cases with citrated blood. Patients with mild disease had decreased platelet retention in 11/12 cases (91.6%) with citrate in the second phase of the filter test. All patients with severe symptoms had low platelet retention with citrate in the first phase of the test and 7/8 patients (87.5%) in the second phase. All patients with type 2 and 3 VWD had blocking drop counts above the normal range with citrate and the filter did not block in all but one patient. With heparin the filter did not block in 6/7 cases. Platelet retention was also reduced in all but one patients with both anticoagulants. Platelet retention in the second phase of the filter test using citrate as an anticoagulant showed the highest sensitivity (89.3%). It was also specific (94.7%) and the positive and negative predictive values (96% and 81.8%) were the highest of all in the filter test.

Results with the PFA-100: in mild type 1 VWD the collagen-epinephrin (coll/epi) cartridges showed higher sensitivity (76.9%), the sensitivity of the collagen-ADP (coll/ADP) cartridges proved to be surprisingly low (38.5%). In severe disease and in type 2 the closure time was over the upper range in all but one case. The test was specific with both cartridges (coll/epi: 96.7%, coll/ADP: 100%) and the positive (coll/epi: 96.3%, coll/ADP:100%) and negative (coll/epi: 87.9%, coll/ADP: 78.9%) predictive value was high.

Ad 2. The effect of rheumatoid factor on the VWF:Ag latex immunoassays

Two latex immunoassays from different manufacturers gave different results in case of a patient with VWD (150% vs. 7%). This difference proved to be consistent with repeated testing. VWF:Ag determination with ELISA supported the low antigen level of the IL Test VWF (14%). The patient's children also had low VWF:Ag levels, and we did not get contradictory results by the two immunoturbidimetric tests in their case (J.L.: 12% vs. 10% ELISA: 10%; J.I.: 14% vs. 12%, ELISA:13%). The VWF activity was low with all patients. There was a good correlation between the two ristocetin cofactor tests and the collagen binding activity. We were looking for rheumatoid factor in the patients' samples. In case of the father we found high titer RF (127 U/ml reference range (RR): 0-50 U/ml). There was no detectable RF in the childrens' samples. Since our first observation, we found the same contradictory results in the case of two other severe VWD patients: in one type 1 patient VWF:Ag 53% vs. 10% (VWF:RCo <10%), in a patient with type 3 VWD VWF:Ag: 50% vs. <2% (VWF:RCo <10%). Both patients were RF positive.

Ad 3. Results of the Bernard-Soulier patient

Platelet aggregation was normal with ADP (71%, RR: 64-100%) and collagen (87%, RR: 79-100%). Ristocetin induced platelet aggregation was impaired (6%, RR: 84-100%) and was not corrected by the addition of normal plasma. This finding confirmed the diagnosis of Bernard-Soulier syndrome that was suggested by the patient's clinical symptoms, thrombocytopenia and huge platelets.

Flow cytometric analysis of the GPIb/IX/V complex with monoclonal antibodies showed that GPIb α , GPIb β and GPIX were reduced to a similar extent, the reduction of GPV was less pronounced. Quantitative analysis with the Biocythex kit resulted in a 15%, 9.6% and 21% reduction of GPIb α , GPIX and GPV, respectively. Expression of the GPIIb/IIIa complex and PAR-1 was enhanced due to the bigger size of the platelets.

By *SDS-polyacrilamid electrophoresis and Western blotting* GPIb α was present only in traces, while GPIb β and GPIX were absent. There was no detectable GPIb α -GPIb β complex in the patient's sample.

The genes of GPIb α , GPIb β and GPIX were *PCR amplified and sequenced*. We found only one mutation: an A \rightarrow G change at position 1826 in the gene of GPIX that resulted in an Asn45Ser mutation.

This mutation creates a new restriction site for the *Fnu4H1* enzyme. By *restriction enzyme analysis* we found that the patient was homozygous for the mutation. Surprisingly, the

patient's son was also homozygous for the same mutation. Sample from the patient's husband was not available for genetic analysis.

DISCUSSION

Ad 1. The clinical presentation of von Willebrand's disease is rather heterogeneous: some patients have no symptoms at all, they are identified only through family investigation. Others have severe, life-threatening haemorrhages. But in any case, severe haemorrhagic complications may develop upon tooth extraction, surgery, delivery etc. As these bleeding complications can be prevented by the prophylactic administration of DDAVP or VWF concentrates, it is imperative to diagnose the disease on time.

The exact diagnosis of VWD requires an array of test that measure the amount, the function and the structure of VWF. All these methods are time consuming, require special laboratory equipment. The most widespread screening test, the bleeding time determination is influenced by a number of factors. There is a need for a test, that is easy to carry out, independent of the skill of the operator, well tolerated by the patients and can be carried out in every laboratory.

VWF plays a basic role in platelet adhesion under high shear conditions. Methods examining platelet plug formation in high shear flow are supposed to give information on the function of VWF. We examined the performance of two such devices, the O'Brien filter test and the PFA-100 device in the diagnosis and screening of VWD. In both tests whole blood is forced through capillary sized channels at a high pressure. In the PFA-100 the membrane is coated with collagen, mimicking the situation after endothelial injury. An additional aggregating agent is also added. In the O'Brien filter test platelets adhere to glass fibers and aggregate without the presence of any additional aggregating factors.

Thirty patients were examined, whose diagnosis had been established earlier. The bleeding time was a good indicator of the bleeding tendency in severe type 1 disease or in type 2A and type 3, but in mild cases it was positive only with two patients. It is interesting to notice, that in type 2B VWD, bleeding time was prolonged only in the case of the patient, who was also thrombocytopenic. This patient has been symptomless in the past six years. Another patient with type 2B VWD who had a severe bleeding tendency and a pronounced VWF defect in the O'Brien filter test and in the PFA-100, had normal bleeding time. The O'Brien filter test was more sensitive using citrate as an anticoagulant. Platelet retention in the second phase of the filter test had the highest sensitivity, it was also specific, and showed

the highest positive and negative predictive value. The blocking drop count was less sensitive to the mild cases, but it was always positive in severe type 1, type 2 and type 3 disease. The collagen-epinephrin cartridge of the PFA-100 was more sensitive for mild disease, while there was no difference between the two cartridges in severe disease. The test was more specific with the collagen/ADP cartridge, its positive predictive value was 100%. For screening purposes the collagen/epinephrin cartridges are recommended.

The sensitivity of the two high shear systems was similar for VWD (80-90%). A much lower sensitivity with the collagen/ADP cartridge was found in mild cases. The O'Brien filter test was very useful in these very cases: platelet retention in the second phase was lower than normal in 11/12 cases (91.6%). The specificity, the positive and negative predictive value of the two methods were comparable.

In summary: both high shear tests are suitable for screening for VWD. Their performance is better especially in mild and type 2B VWD. These tests are easy to carry out, their execution does not require special skills and they are well tolerated by the patients. Their use for screening purposes in VWD is highly recommended.

Ad 2. VWF:Ag determination is a basic test of VWD. VWF:Ag latex immunoassays became widespread in recent years. Rheumatoid factor is known to have an effect on immunoturbidimetric tests. We have observed that the effect of rheumatoid factor is dependent on the reagent in use. This effect is of great importance as an incorrectly measured high VWF:Ag level may lead to the false exclusion of VWD, or to the mistyping of the disease. In case of an unexpected result or in autoimmune diseases VWF:Ag should be determined by different tests or rheumatoid factor should be looked for in these cases.

Ad 3. A 38 year old female patient was examined. The diagnosis of Bernard-Soulier syndrome was based on the thrombocytopenia, on the huge platelets seen in the peripheral smear and on the impaired ristocetin induced platelet aggregation with normal VWF antigen level and function. The presence of the proteins of the GPIb/IX/V complex on the platelet surface was examined by flow cytometry and Western blotting. By flow cytometry all proteins were present, with quantitative analysis the reduction of GPIX was the most pronounced, the least reduction was seen in GPV. There were no detectable amounts of GPIX and GPIb β by Western blotting. The amount of GPIb α was reduced. The GPIb α -GPIb β complex could not be detected in the platelet lysate of the patient. Based on the results of the flow cytometric analysis the gene of GPIX was sequenced first. We found a missense point

mutation, an adenine-guanine change at position 1826 that resulted in an Asn45Ser mutation in the polypeptid sequence of GPIX. By restriction enzyme analysis the patient was homozygous for this mutation. No mutations were found in the genes of GPIb α and GPIb β . The Asn45Ser mutation is located in the leucin rich motif (LRM) of GPIX. The exact function of the LRM is not known, but it has a role in forming a non-covalent bond between GPIX and GPIb β . This function shows similarity to other proteins containing LRM sequence, eg. the toll, chaoptin and connectin proteins of the *Drosophila*, that secure cell-cell adhesion by the interaction of the similar receptors on different cell surfaces. In transfection experiments the Asn45Ser mutation inhibited the formation of the GPIb β -GPIX complex. In the absence of this complex the stability of GPIb α is impaired, it is degraded more rapidly, its surface expression is decreased. This results in Bernard-Soulier syndrome. The mutation that was found in our patient was first described in a compound heterozygous English patient, later it was found in a homozygous Austrian, a Swedish and seven Finnish patients. Since the publication of our case it has been described in four German and a compound heterozygous Canadian patient. This patient inherited the Asn45Ser mutation from his mother, who had French-Irish antecedents. It is of note that our patient's son was also homozygous for the mutation, which means that the patient's unrelated husband, who was not a bleeder was an obligate carrier of the mutation. The Asn45Ser mutation was not found in the well studied Japanese and Italian BSS patients. Therefore it seems, that the Asn45Ser mutation is the most widespread mutation causing Bernard-Soulier syndrome in the northwestern population. As most of these patients were diagnosed and treated as suffering from immune thrombocytopenic purpura (ITP), in therapy refracter ITP Bernard-Soulier syndrome or a carrier state of BSS should be considered. Ristocetin induced platelet aggregation is of diagnostic value in these cases.

SUMMARY

Platelet adhesion under high shear flow conditions is mediated by von Willebrand factor. The receptor of VWF on the platelet surface is the GPIb/IX/V complex. If the interaction between VWF and GPIb/IX/V is impaired, a bleeding tendency develops. The aim of our work was to broaden our knowledge on the diagnostic evaluation of the two relevant disease states, vonWillebrand's disease and Bernard-Soulier syndrome.

We examined the utility of two „*in vitro* bleeding time” tests, the Platelet Function Analyzer-100 and the O'Brien filter test in the diagnosis and screening of VWD. The

sensitivity of both tests was superior to the traditional bleeding time test. The sensitivity of the platelet retention in the O'Brien filter test and the closure time with the collagen/epinephrin cartridges in the PFA-100 were similar. The specificity, positive and negative predictive values of the two tests were comparable.

We observed that VWF:Ag immunturbidimetric tests are affected by rheumatoid factor and this effect is dependent on the reagent in use. This may lead to a diagnostic error: either to the false exclusion of VWD or to the mistyping of the disease. In case of an unexpected result or in autoimmune diseases the use of different test or the exclusion of the presence of rheumatoid factor is recommended.

A Belgian patient with Bernard-Soulier syndrome was investigated. A homozygous Asn45Ser mutation in GPIX was found. This mutation destabilizes the GPIIb/IIIa-GPIX complex and causes the rapid degradation of GPIIb/IIIa. The Asn45Ser mutation of GPIX proved to be the most widespread mutation causing BSS in Northern- and Western-Europe. It should be taken into account in the differential diagnosis of therapy refracter immune thrombocytopenic purpura.

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