Epidemiological Investigation of the Prevalence of von Willebrand's Disease

By Francesco Rodeghiero, Giancarlo Castaman, and Enrico Dini

To evaluate the prevalence of von Willebrand's disease (vWd) we carried out an epidemiological investigation among school children of the Veneto region in northern Italy. A total of 1,218 of 1,281 possible children participated in the study. They were 11 to 14 years of age, and all attended secondary schools in two distinct small areas, 70 km apart, between which there is no social contact. A blood sample was taken from each subject for determination of the blood group and von Willebrand factor (vWF) level (measured as ristocetin cofactor and expressed in IU/dL after calibration of the internal pool against an international standard), and the parents were given a questionnaire concerning hemorrhagic symptoms in the members of the family in the last three generations. Separate normal ranges were calculated for blood group O and non-O subjects (1,168 children and 289 adults) with a nonparametric method because the distribution curves of the reference values did not fit the gaussian distribution.

VON WILLEBRAND'S DISEASE (vWd) is an autosomally inherited disorder resulting from a quantitative or a qualitative defect of von Willebrand factor (vWF). This protein circulates in plasma complexed to factor VIII (VIII/vWF complex) and is required for the normal adhesion of platelets to subendothelium and for in vitro ristocetin-induced platelet aggregation. Clinical expressions of the disease are variable. In addition to severe cases requiring replacement therapy to stop or prevent hemorrhages, there are mildly affected patients. Furthermore, some subjects carrying the mutant gene have no hemorrhagic symptoms.

The mutant gene has multiple phenotypic effects consisting essentially of a prolonged bleeding time and reduced factor VIII/vWF complex-related activities (factor VIII coagulant activity, vWF antigen, ristocetin cofactor activity of vWF). The bleeding time and VIII/vWF-related activities can be easily measured by laboratory tests, but the diagnosis of this disorder may be difficult because the laboratory data may overlap with the normal range and fluctuate with time in a given patient. Recently, the identification by multimeric sizing techniques of qualitative variants of vWd has added to the complexity of the diagnosis.

So far, the prevalence of vWd has been estimated only from the cases registered at specialized centers and indicates a prevalence similar to or even lower than that of hemophilia. This paper reports the results of an epidemiological investigation of 1,218 children attending secondary school in two distinct areas of North Italy.

MATERIALS AND METHODS

Subjects. All 1,281 children aged 11 to 14 attending the three grades of secondary school in two distinct areas of the Veneto region (Province of Vicenza) were asked to participate in the investigation. The first area consists of two small neighboring towns 30 km north of Vicenza (Santorso and Piovene Rocchette, 11,676 inhabitants), and the second consists of two small neighboring towns 40 km south of Vicenza (Poiana Maggiore and Noventa Vicentina, 11,638 inhabitants). The possibility of investigating all resident children, the adequate size of the sample, and the administrative facilities caused us to choose these particular areas. Moreover, less than 10% of the two populations had a common ancestor, as judged by an analysis of family names, so that the two areas can be considered to be independent samples. Parents and physicians in charge of the children were given detailed information about the purpose and methodology of the investigation. All children for whom parents had provided written consent were admitted to the study.

Hemorrhagic symptoms were investigated through a questionnaire consisting of four sections that concerned the child under investigation, or her siblings, the father and his sibship and parents, and the mother and her sibship and parents. Specific symptoms investigated included epistaxis, hemorrhage after tooth extraction, hemorrhage after surgery, prolonged bleeding after superficial cuts, menorrhagia, postpartum hemorrhage, and easy bruising. Families for which a hemorrhagic history could be suspected were interviewed to get more detailed descriptions of reported bleeding episodes and additional information about relatives not specifically investigated in the questionnaire. Each symptom was recorded only when sufficiently fully reported and of not trivial importance. Easy bruising was considered only for male adults and menorrhagia and postpartum hemorrhage only when specific causes could be reasonably ruled out. Subjects complaining of epistaxis, whenever possible, were examined to exclude Rendu-Osler disease. A subject was recorded as definitely positive if at least two hemorrhagic symptoms were present. A family was recorded as positive if at least two members of the genetic line were positive. Laboratory investigation was offered to all available members of these families. The questionnaire...
answers and interviews of family members were examined by one of us before the laboratory results were known.

Laboratory investigations. Blood was taken by venipuncture with a 19-gauge needle in the inframary of each subject between 8:30 AM and 10:00 AM after the subjects had been resting for at least 30 minutes. The samples were placed in precooled tubes and transported in refrigerated boxes to our laboratory within two hours. Repeat blood samples were taken 2 to 4 months later from all children of hemorrhagic families, and samples were also obtained from as many family members as possible.

Blood cell counts were made on EDTA-anticoagulated blood using an electronic counter (Coulter Counter IV Plus, Coulter Electronics, Hialeah, FL). ABO blood groups and Rh typing were determined using standard slide methods; secretors were identified by a saliva test. Blood for coagulation tests, collected in 3.8% sodium citrate (1:10), was centrifuged at +4°C (15 minutes at 1,500 g) immediately after arrival at our laboratory, and platelet-poor plasma was deep-frozen and stored at −80°C.

The ristocetin cofactor activity of vWF was measured essentially according to the method of Macfarlane et al. The rate of agglutination of formalin-fixed washed platelets to ristocetin (final concentration, 1.5 mg/mL) was measured by calculating the slope of the steepest part of the agglutination curve (Chrono-Log Corp aggregometer, Havertown, PA). Four dilutions of local standard plasma in buffered saline (1:1 to 1:8) were assayed in reverse order, at the beginning and at the end of each set of measurements, and the reference curve was calculated on the average of the rate of agglutination of each dilution. No significant temporal drift occurred if the assay was completed within two hours. Each sample was assayed at three different dilutions and its potency in terms of the local standard plasma calculated by fitting a straight line parallel to the reference line. When major departures from parallelism were observed, the sample was retested. Two samples, one with normal and the other with low ristocetin cofactor activity, were included in each set of measurements; the interassay variability of both samples was less than 10%. The coagulant activity of factor VIII (VIII:C) was measured using a one-stage method based on the activated partial thromboplastin time (Actin reagent, Dade, Aguada, Puerto Rico) and a dilution of the test plasma in hemophilic plasma from patients with an unmeasurable VIII:C level.

vWF antigen was measured by the Laurell electroimmunoassay technique using a polyclonal antisem anti-vWF antigen from Behringwerke (Marburg, West Germany). The ratios of the potency of the samples to the local standard plasma were calculated as in the ristocetin cofactor assay.

The local standard plasma, plasma from 98 healthy hospital staff members, was collected on a single morning and pooled. The same local standard, stored at −80°C, was used throughout the study. It was calibrated for factor VIII:C, vWF:Ag, and ristocetin cofactor activity against the First International Reference Preparation for Factor VIII Related Activities in Plasma (IRP) (kindly supplied by Dr T.W. Barrowcliffe, National Institute for Biological Standards and Control, London). Calibrations, three at the beginning and three at the end of the study, were made as suggested by Kirkwood and Snape. The local plasma standard and the lyophilized IRP were assayed in plasma severely deficient in vWF for the purpose of calibrating ristocetin cofactor activity. The potency estimations were combined by taking a weighted geometric mean, the weights being inversely proportional to the variances of the log potency estimates. The ristocetin cofactor activity of vWF in plasma is simply referred to as “vWF” in the text. The multimeric composition of factor VIII/vWF was analyzed in the laboratory of Professor Mannucci (Milan, Italy), as previously published.

Definition of the normal range of vWF. Values of vWF obtained from children without a family history of hemorrhage were used for the definition of the normal range of vWF. An analysis of the variance and the Newman-Keuls test showed significantly lower level of vWF in blood group O subjects. Separate normal ranges were calculated for O and non-O subjects. The values were not normally distributed and showed significant positive kurtosis and skewness. Several transformations including log, square root, and inverse hyperbolic sine did not correct all kurtosis and skewness, and the Kolmogorov-Smirnov test showed significant deviation from normality. Normal ranges were defined by calculating 2.5 and 97.5 percentiles by a nonparametric method. A 90% confidence interval for each end point thus obtained was calculated on the basis of a binomial probability distribution. All reference values were included because no outliers were found. The normal range of vWF in adults was defined in 289 subjects of both sexes aged 19 to 65 without a personal or family history of hemorrhage and who were not taking contraceptive drugs. One hundred twenty-four of them were regular blood donors, 98 were healthy hospital staff members, and 67 were teachers or employees of the schools.

Criteria for the diagnosis of vWD. The diagnosis was considered only for subjects belonging to a family recorded as positive for bleeding history. Subjects satisfying this criterion and also having vWF levels below the normal range were classified as cases with “probable vWD.” A definite diagnosis of vWD was considered when at least one other member, on the hemorrhagic side of the subject’s family, had a low vWF level.

RESULTS

The potency ratios of the local standard relative to the international standard measured at the beginning and at the end of the study 18 months later showed similar potencies. Potency estimations were combined and assigned to the local standard plasma (Table 1).

A total of 1,218 children participated in all stages of the research, with similar rates of participation in the areas north (594/620) and south (624/661) of Vicenza; the male-to-female ratio was 1.01. Among the questionnaires, 202 were selected because one or more of the reported episodes could be regarded as hemorrhagic. Parents of children of these families were interviewed. In this way, 49 families (21 north and 28 south of Vicenza) could be classified as positive (107 members with two or more hemorrhagic symptoms). Epistaxis accounted for 39% of the 268 identified symptoms, followed by bleeding after tooth extraction (20%), menorrhagia (18%), postpartum hemorrhage (8%), prolonged bleeding after superficial cuts (4%), after tonsillectomy or adenoidectomy (4.5%), after surgery (3.5%), easy bruising (2%), and other manifestations (1%). Values of vWF in the

| Table 1. Potency Ratios of Local Standard Plasma Relative to the International Standard for Factor VIII/vWF-Related Activities |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | At the Beginning | At the End       | Combined Potency |
|                                 | of the Study     | of the Study     | Potency          |
|                                 | (3 Calibrations) | (3 Calibrations) | Ratios           |
|                                 | Mean 95% CL      | Mean 95% CL      | Mean 95% CL      |
| vWF                             | 1.07 0.97-1.16   | 1.08 0.96-1.19   | 1.07 1.01-1.13   |
| VIII:C                          | 0.93 0.78-1.07   | 0.86 0.76-0.94   | 0.89 0.83-0.94   |
| vWF:Ag                          | 1.11 0.94-1.31   | 1.03 0.89-1.19   | 1.06 0.97-1.15   |

Student’s t test did not show statistically significant differences between potency ratio values found at the beginning and at the end of the study for each activity.

Abbreviation: CL, confidence limits.

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children of these families (52 subjects, 24 male and 28 female) were treated separately and normal ranges calculated from the remaining 1,166 children.

Subjects of group O had a significantly lower mean vWF value than subjects of group A, B, or AB (P < .001; Newman-Keuls multicomparison test) (Table 2). The age distribution of vWF levels was independent of sex, Rh phenotype, secretory status, or area of residence. In 289 adults, vWF was also similarly lower in group O subjects, whereas no significant differences were observed in relation to sex or Rh phenotype. Only in subjects of group O was there a significant correlation with age (r = .33; P < .001). Table 2 shows the mean values and normal ranges of vWF for children and adults of O and non-O groups. A correction for age in group O adults was not done.

Figure 1 shows the distribution of vWF levels in the 52 children belonging to families with hemorrhagic histories.

Six subjects of group O and four of group non-O fell below the 2.5 percentile. Fourteen children had vWF levels below the upper limit of the 90% confidence limits (nine of group O, five of group non-O), and seven were below the lower limit (three of group O, four of group non-O). Subjects of group O in the normal range seem to be clustered near its lower end.

Table 3 lists the laboratory findings and hemorrhagic symptoms in the 14 children with low vWF levels and their symptomatic family members.

Six children (four to nine according to the 90% confidence interval of the 2.5 percentile) could be definitely classified as having vWD because other family members had vWF levels below the normal range, whereas for another four (three to five) the diagnosis of vWD was "probable." Similar prevalence of the disease was found in the two areas. vWD was more frequent in females (eight of 14) and in O group children (nine of 14), (P values, .091 and .105, not significant). vWF was below the normal range in nine of 24 relatives in whom it was measured (38%).

The strict correlation between vWF and vWF:Ag and between vWF and VIII:C (in both cases r = .83, P < .001) suggests that these subjects may have type I vWD. This was confirmed by multimeric analysis of all affected children. vWF levels in these subjects are distributed around half of the normal value, as would be expected in heterozygotes of an autosomal dominant trait. A clear hemorrhagic story could be traced in all families, and symptoms, present in all the children, appeared to be transmitted in an autosomal dominant fashion.

<table>
<thead>
<tr>
<th>Table 2. Distribution of the vWF Level in Normal Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF Level (IU/dL)</td>
</tr>
<tr>
<td>Subjects</td>
</tr>
<tr>
<td>Children</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Adults</td>
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</tr>
</tbody>
</table>

Abbreviation: CI, confidence interval.

Repeat blood samples were obtained from 44 of the 52 children of hemorrhagic families 2 to 4 months after the first investigation. The mean values of vWF were the same as before (87.9 v 88.2), with a mean difference between first value and second values for each subject of 11.8 ± 9.5. The vWF values of the second samples confirmed the diagnoses for 12 of the 14 children, and four additional cases of vWD were detected.

DISCUSSION

The criteria for the diagnosis of vWD used in this investigation were conservative and aimed at reducing to a minimum the rate of inclusion of false-positive subjects. Subjects belonging to hemorrhagic families that had vWF below the normal range were recorded as cases of "probable vWD." Definite diagnoses were made only when, in addition, at least one other member with low vWF could be found among relatives on the hemorrhagic side of the family.

Diagnostic criteria (laboratory and clinical). The lower normal limit of the vWF level was chosen as the value below which lie 2.5% of normal subjects and was defined on the basis of the values found in more than 1,000 normal subjects. Because the vWF distribution showed a positive kurtosis and skewness that was not completely removed by several transformations, we used a nonparametric method for the definition of the 2.5 and 97.5 percentiles and the 90% confidence intervals around these limits. Nonparametric methods make no assumption about the type of distribution of the reference values and are thus more precise and recommended in situations that differ from the ideal model of normality.24-26 Homogeneity between the reference and investigated groups was assured because both were drawn from the same population of children. Moreover, because vWF was found to be lower in group O subjects, separate normal ranges were calculated for O and non-O subjects. Uniformity of measurement of vWF during the study was guaranteed by using a single reference standard that showed no loss of potency relative to the international standard (Table 1) and by performing the vWF assay shortly after sample collection. No changes in vWF levels were found in relation to the time of blood sampling.
Table 3. Laboratory Findings and Hemorrhagic Symptoms in the Children Classified as Affected by vWd and Their Relatives

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Area</th>
<th>Blood Group</th>
<th>vWF (IU/dL)</th>
<th>vWF:Ag (IU/dL)</th>
<th>VIII:C</th>
<th>Diagnosis</th>
<th>Family Members</th>
<th>Bleeding Symptoms‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>O</td>
<td>8*†</td>
<td>5</td>
<td>7</td>
<td>D</td>
<td>afg</td>
<td>Mother</td>
<td>21 abcddeg</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>S</td>
<td>50*†</td>
<td>38</td>
<td>50</td>
<td>D</td>
<td>ab</td>
<td>Sister</td>
<td>19 afg</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>N</td>
<td>51*†</td>
<td>51</td>
<td>53</td>
<td>P</td>
<td>ag</td>
<td>Uncle</td>
<td>64 ab</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>N</td>
<td>56†</td>
<td>53</td>
<td>37</td>
<td>D</td>
<td>a</td>
<td>Mother</td>
<td>59 a</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>S</td>
<td>56†</td>
<td>63</td>
<td>40</td>
<td>P</td>
<td>abfg</td>
<td>Sister</td>
<td>48 ac</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>N</td>
<td>58†</td>
<td>53</td>
<td>54</td>
<td>D</td>
<td>ac</td>
<td>Cousin</td>
<td>53 ac</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>N</td>
<td>64</td>
<td>50</td>
<td>46</td>
<td>D</td>
<td>a</td>
<td>Sister (case 7)</td>
<td>62 ac</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>N</td>
<td>64</td>
<td>53</td>
<td>57</td>
<td>D</td>
<td>ag</td>
<td>Father</td>
<td>49 a</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>N</td>
<td>64</td>
<td>36</td>
<td>36</td>
<td>D</td>
<td>a</td>
<td>Aunt</td>
<td>48 ac</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>S</td>
<td>59*†</td>
<td>54</td>
<td>57</td>
<td>D</td>
<td>a</td>
<td>Aunt (case 2)</td>
<td>53 ad</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>S</td>
<td>62†</td>
<td>68</td>
<td>56</td>
<td>P</td>
<td>acg</td>
<td>Cousin (case 2)</td>
<td>53 ac</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>S</td>
<td>67†</td>
<td>54</td>
<td>60</td>
<td>P</td>
<td>ab</td>
<td>Aunt (mother, case 2)</td>
<td>53 ac</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>S</td>
<td>67*†</td>
<td>50</td>
<td>64</td>
<td>D</td>
<td>c</td>
<td>Mother</td>
<td>56 ac</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>B</td>
<td>78</td>
<td>72</td>
<td>61</td>
<td>P</td>
<td>a</td>
<td>Mother</td>
<td>56 ac</td>
</tr>
</tbody>
</table>

VIII:C and vWF:Ag determined in 60 normal children ranged 56 to 182 and 53 to 188 respectively.

Abbreviations: ND, not done; NV, normal value.
*Below the lower limit of the 90% confidence interval of 2.5 percentile.
†Below the 2.5 percentile.
‡a, epistaxis; b, bleeding after tooth extraction; c, menorrhagia; d, bleeding after surgical procedures; e, postpartum hemorrhage; f, prolonged bleeding after superficial cuts; g, easy bruising (considered for the purpose of defining hemorrhagic diathesis only in male adults).

Conservative criteria were used for the definition of hemorrhagic diathesis, and two or more positive members were required to consider a family as positive. The frequency of “bleeders” in the general population is unknown, but using criteria less strict than the ones adopted in this study, Miller et al.27 were able to classify as symptomatic as many as 23% of normal subjects, a figure that would undermine the discriminant power of this parameter. In contrast to their data, only 26 children (2%) and only 107 relatives among the approximate 14,000 for whom we had direct or indirect information through questionnaires and interviews were classified as positive. In this way, a total of 52 subjects (from 49 families) out of 1,218 investigated were recorded as belonging to hemorrhagic families.

The a priori probability of a child being a member of a hemorrhagic family is thus .042 (52/1218), which, when multiplied by .025 (the probability of false-positive classification on the basis of vWF measurement), yields a probability of about .001 of a “normal child” being falsely classified as a case of “probable vWd.” The probability of misclassification is, of course, greatly reduced in the presence of other relatives with low vWF levels, and in this case it seems appropriate to consider the diagnoses as definite.

Epidemiology of vWd. Among the 1,218 children investigated, ten were classified as affected (four cases as “probable vWd,” six cases as definitely proven). More precisely, taking into consideration the 90% confidence interval of the lower end point of the normal range (in fact, all the values in

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this interval have identical significance), there would be from seven (three “probable cases,” four definite cases) to 14 (five "probable cases," nine definite cases). The calculated prevalence of vWD in our population is thus estimated to be between 0.57% and 1.15% (Table 3). All these subjects appear to be heterozygous and have inherited the disorder in an autosomal dominant fashion. The phenotypic and laboratory characteristics and multimeric composition were those of classic vWD (type I).

A theoretical prevalence of 25 of 1,000,000 homozygotes is anticipated according to the Hardy-Weinberg equation on the basis of 1% heterozygotes in the population, but a possible genotypic heterogeneity of classic vWD, unrecognized by our methodology, would make the Hardy-Weinberg equation inapplicable. Nevertheless, homozygous patients for the classic type I dominant vWD are likely to be more frequent than homozygotes for type III recessive vWD, who are estimated to be between 0.11 and 0.55 out of 1,000,000 in Europe29 and 3.1 and 3.2 out of 1,000,000 in Sweden and Israel.14,29,30 Thus, in diagnosing type III vWD, one has to be very careful that the propositus is not the offspring of two nonmanifesting type I patients because rare instances are known in which severely affected cases were born of asymptomatic type I vWD parents.27,31

Although repeated measurements of vWF in patients with vWD may yield variable results,7 the classification of cases in this investigation showed little dependence on repeating the vWF assay. In fact, 12 of the 14 subjects had their diagnoses confirmed by the second value. Moreover, four additional children would have been detected by considering the lowest value found in the two determinations.

An assay for vWF (ristocetin cofactor activity) was found to be the single most useful test for identification of transmitters and symptomatic members in kindreds with the disease.32,33 In this study a vWF assay was preferred to other tests such as a vWF antigen (vWF:Ag) on the basis of this consideration and because variant forms of vWD are known in which vWF:Ag is normal or near normal. Nevertheless, Miller et al35 could identify only 42% of the transmitters by using separate normal ranges for O and non-O subjects; we were able to identify about 50% of the transmitters (data not published), and in reasonable accord with these figures, in this study we were able to identify as transmitters about 40% of the hemorrhagic relatives in families of children with low vWF.

Considering the limitations of the vWF assay for diagnosis and the conservative criteria for the definition of hemorrhagic diathesis, probably 50% or more of vWD cases could go unrecognized with a methodology like the one used in this study (false-negative subjects). Nevertheless, the prevalence of vWD found in this investigation is much higher than the prevalence previously estimated from cases registered at specialized centers, which appeared to be similar to or even lower1-3,16 than that of hemophilia.14 This method of calculating the prevalence of a disease with highly variable clinical expression is unreliable. Moreover, the significantly lower level of ristocetin cofactor activity (this study) and vWF antigen in subjects of group O35,36 leads to an overestimation of vWD in O subjects and to an underestimation in non-O subjects unless strictly defined normal ranges are used.

Although the two geographic areas investigated here can be regarded as independent samples, almost identical prevalences of vWD were found. Hence, because these areas do not present particular features, we think that the prevalence of vWD estimated in this study can apply to the Caucasian population in general. However, in extrapolating the prevalence found in children aged 11 to 14 to subjects of all ages we must consider a possible bias in the upward direction. In fact, children from larger families have a greater probability of being included, and thus there is a potential “founder effect.” Actually, only one family among those who contributed with positive cases had additional children (either positive or negative) included in the study. Thus, although subjects 4 and 7 in Table 3, who are siblings, should be considered as a single case, this does not substantially change our prevalence estimation.

Practical implications. Assuming a 2% prevalence of subjects carrying the mutant gene for vWD in the population and a 50% sensitivity of the vWF assay in identifying these carriers, the vWF assay would have a predictive value of 29% for positive results (true positives/total positives) and 98.9% for negative results (true negatives/total negatives). The efficiency of the test (percentage of true-positive and true-negative results) would be 96.5%. Furthermore, limiting its use for subjects with evidence of personal or family histories of hemorrhage would virtually abolish the misclassification of normal subjects (about one in 1,000 false-positives in this study). It has to be emphasized that, to obtain these results, normal ranges have to be defined separately for O and non-O subjects.

Considering the frequency of vWD suggested by this study, we think that a vWF assay (ristocetin cofactor activity) should be included among the first level screening tests for evaluation of subjects with mild bleeding diathesis even in presence of normal routine screening tests.

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