# Evaluation of an automated method for the quantification of von Willebrand factor antigen. Its application in the study of vascular dysfunction

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*Background and Objectives.* Abnormally high levels of von Willebrand factor (VWF) have been described as a response to physiologic and chronic alterations. It is therefore of great interest to have sensitive, accurate, fast and easy to use assays to quantify VWF. The aim of this study was to evaluate the performance characteristics of the immunoturbidimetric assay IL Test ™ VWF:Ag and the use of this determination in the study of vascular dysfunction.

*Design and Methods.* The reproducibility, accuracy and linearity of the method were determined. A method comparison study was performed using an ELISA as the reference test. The assay reference range and its age-dependence were established. To evaluate the utility of the assay in vascular dysfunction, a cohort of 30 type 1 diabetes mellitus (DM) patients were analyzed, 11 of whom showed microvascular complications.

*Results.* The linearity range found was 10-150 IU/dL. Reported values above 150 IU/dL should be diluted and reanalyzed, expanding the range up to 600 IU/dL. The coefficients of variation were below 5% and inaccuracies below ±15% within the linearity range. The comparison with the reference ELISA showed a good correlation coefficient (r = 0.985, VWF values from 5-680 IU/dl). The reference range was found to be 40.8 – 158.1 IU/dL. Significant differences between the DM type 1 patients with microvascular complications and a sex-age matched control group were observed. This difference was not observed for the DM patients not showing vascular alterations.

*Interpretation and Conclusions.* The method is reproducible, accurate and linear. The assay correlates well with the reference ELISA. There is a relationship between patients showing microvascular diabetic complications and high levels of VWF:Ag. The assay is easy to perform, fully automated, and suitable for analyzing a small number of samples.
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Key words: von Willebrand Factor, automated assay, microvascular dysfunction, diabetes mellitus.

The von Willebrand factor (VWF) is a large multimeric plasma glycoprotein formed by 2 to 100 monomers of approximately 250 Kd each.[1] VWF is synthesized in megakaryocytes and endothelial cells first as a large precursor form that initially dimerizes, and then undergoes subsequent multimerization and proteolytic processing to produce the mature VWF subunit.[2] In plasma, VWF serves two major functions. At sites of vascular injury it provides a major adhesive link between platelets and the vessel wall via binding to specific receptors on the platelet surface, as well as to one or more ligands in the subendothelium.[3] In addition, VWF serves as the carrier for factor VIII (FVIII) in plasma, stabilizing and localizing FVIII to sites of platelet plug and fibrin clot formation.[4,5]

von Willebrand's disease (VWD) is the most common of the inherited hemorrhagic diatheses, and is characterized by the decrease of VWF or partial or total loss of its functionality.[6] The measurement of VWF:Ag, together with that of von Willebrand factor ristocetin cofactor activity (VWF:RCo) and FVII, is an essential laboratory assay in the diagnosis algorithms for VWD. Further characterization of VWD types and subtypes may require more complex assays, for example ristocetin-induced platelet agglutination (RIPA) analysis, VWF binding to normal FVIII test or VWF

**Table 1. Reproducibility.**

| | | | |
|---|---|---|---|
| *Within run precision* | | | |
| Mean (IU/dL) | 119.1 | 59.1 | 29.9 |
| SD | 0.86 | 1.52 | 0.86 |
| CV (%) | 0.7 | 2.6 | 2.9 |
| SEM (%) | 0.1 | - 0.7 | 0.6 |
| *Inter-day precision* | | | |
| Mean (IU/dL) | 120.0 | 60.1 | 31.2 |
| SD | 3.08 | 1.96 | 1.22 |
| CV (%) | 2.6 | 3.3 | 3.9 |
| SEM (%) | 0.9 | 1.0 | 5.0 |

*Mean reported VWF:Ag, standard deviation (SD), coefficient of variation (CV) and standard error mean (SEM) calculated as the difference in percentage between the expected and the mean reported values.*

**Table 2. Linearity.**

| Dilution | Expected value | Reported value | SEM(%) | SD | CV (%) |
|---|---|---|---|---|---|
| Undiluted | 613.5 | 227.8 | -62.8 | 10.7 | 4.7 |
| 1/2 | 306.8 | 233.1 | -24.0 | 4.1 | 1.8 |
| 1/4 | 153.4 | 142.5 | -7.1 | 14.0 | 9.8 |
| 1/8 | 76.7 | 84.1 | 9.6 | 12.0 | 14.2 |
| 1/16 | 38.3 | 43.8 | 14.2 | 4.4 | 10.1 |
| 1/32 | 19.2 | 21.5 | 12.0 | 0.8 | 3.7 |
| 1/64 | 9.6 | 9.6 | 0.0 | 0.7 | 7.7 |
| 1/128 | 4.8 | 6.2 | 29.9 | 1.0 | 16.1 |

*VWF:Ag expected and mean reported values expressed in IU/dL.*

multimeric analysis.[7,8] On the other hand, elevated concentrations of VWF have been described in a variety of physiologic situations[9] and several chronic conditions including hyperthyroidism,[10] renal failure,[11,12] liver disease,[13] atherosclerosis[14] and diabetes.[15,16] Its measurement is also helpful in the follow-up of VWD patients treated with the vasopressin analog 11-deamino-(8-D-arginine)-vaso-pressin (DDAVP) and desmopressin.[17] Furthermore it is useful to follow-up some treatments causing a decrease of VWF.[18]

Most laboratories determine VWF:Ag by an enzyme-linked immunosorbent assay (ELISA) which, although accurate and sensitive, has the drawback of being time-consuming and not adequate for analyzing a single or small number of samples or in an emergency. Recently, new assays overcoming these inconveniences have been reported in the literature.[19-21]

The scope of this study was to evaluate the performance of an immunoturbidimetric assay to quantify VWF:Ag as a marker of endothelial dys-

function. For this purpose, this method was used to analyze a group of diabetes mellitus (DM) type 1 patients. Some of them showed microvascular complications. A comparison was made between this cohort and a group containing an equal number of healthy individuals matched by age and sex.

## Design and Methods

### *Blood sampling and VWF:Ag determination*

Blood samples were obtained by puncture of an antecubital vein. The blood was collected into vacuum tubes containing 0.127 M trisodium citrate, centrifuged at 2,000×g for 15 minutes to obtain platelet-poor plasma, and stored below –70°C until tested.

IL Test™ VWF:Ag kit consists of a latex reagent (suspension of polystyrene latex particles coated with a polyclonal antibody directed against VWF) and a reaction buffer. When plasma containing VWF:Ag is mixed with the latex reagent and the reaction buffer in the reaction cell of a coagulation analyzer, the coated particles agglutinate proportionally to the VWF:Ag present in the sample. The degree of agglutination is measured as a decrease of the transmitted light at 405 nm. The assay was performed on an ACL 7000 analyzer (Instrumentation Laboratory, Milan, Italy). The IL Calibrator Plasma, assigned for VWF:Ag against the 4th International Standard FVIII/VWF (NIBSC 97/586), was used as the calibrator. For the method comparison study, VWF:Ag was also determined by ELISA. This assay was performed as described previously.[23]

### *Plasma samples from patients and controls*

To determine the reference range, plasma from 146 healthy individuals (age range: 2-93 years) were used. For the method comparison study we used the two VWF:Ag assays to analyze 89 samples distributed as follows: 36 from healthy individuals, 23 from type 1 VWD untreated patients, 12 from VWD patients treated with DDAVP, and 18 from patients admitted to the Emergency Unit, selected because of their high fibrinogen values, indicative of an acute phase reaction.

### *Diabetes mellitus patients*

Thirty patients diagnosed according to National Diabetes Group criteria[24] as having diabetes mellitus (DM) type 1, and not showing other pathologies, were consecutively selected. Patients with high fibrinogen values were excluded to eliminate other possible causes of VWF:Ag elevation. Eleven out of these 30 patients with DM had microvascular complications (6 retinopathy plus nephropathy and 5 retinopathy only).
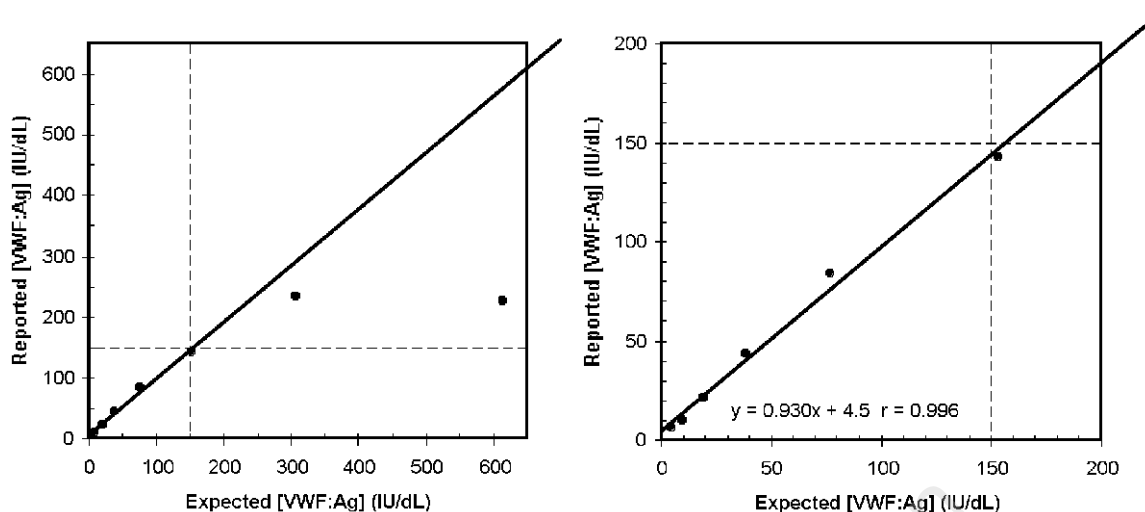
**Figure 1. Linearity of the IL Test™ VWF:Ag. Solid line: least squares fit of values below 150 IU/dL. Dotted lines: Upper limit of the test range according to the manufacturer. Right plot: magnification of the lower end of the left plot.**

### Other assays

Fibrinogen was determined on an ACL 7000 (Instrumentation Laboratory, Milan, Italy) using the method derived from the prothrombin time. Glucose, total cholesterol (C), HDL-C, LDL-C and triglycerides were determined by an automated enzymatic method and colorimetric method (DAX-72 Bayer, Tarrytown, New York, USA). HbA1c was measured by high performance liquid chromatography (Model L-9100, Hitachi, Tokyo, Japan).

### Results

*Reproducibility.* A lyophilized pool of plasma assigned for VWF:Ag against the NIBSC 97/586 standard was analyzed with the IL Test™ VWF:Ag undiluted (119 IU/dL), diluted 1+1 and diluted 1+3 with physiologic saline. Intra-assay (n=5) and inter-day (n=7 days) precision results (summarized in Table 1) show CVs below 4% at the three levels assayed.

*Linearity.* One plasma sample assigned by the ELISA method against the NIBSC 97/586 standard was analyzed in quadruplicate with the IL Test™ VWF:Ag at dilutions up to 1:128 in physiologic saline. Results are summarized in Table 2 and plotted in Figure 1. The assay showed errors smaller than ±15% in the range 10-150 IU/dL of VWF:Ag. Samples below 10 IU/dL were reported with poor accuracy and precision. Samples above 150 IU/dL reported inaccurate results, and needed to be diluted 1+3 with physiologic saline and retested and the result multiplied by four.

*Method comparison.* The method comparison statistics are listed in Table 3. The curve was fitted following the methodology described by Passing and Bablok.[25] The slope 1.002 very close to one, the intercept 3.40 close to the origin and the Pearson correlation coefficient 0.985 suggest that the IL Test™ VWF:Ag and the reference ELISA are equivalent. Figure 2 shows the comparison plots.

*Reference Range.* The reference range for the IL Test™ VWF:Ag was determined by analyzing 146 apparently healthy individuals distributed in four age groups. Calculations were performed following the guidelines of the International Federation of Clinical Chemistry (IFCC).[26] The statistics are summarized in Table 4. The mean value and the reference range increase with age.

*Diabetes mellitus Type 1 patients.* Significant differences were not found between patients with

**Table 3. Method comparison statistics.**

| Slope (95% CI) | Intercept (95% CI) | CC (95% CI) | Mean X Mean Y | Max X,Y Min X,Y | p |
|---|---|---|---|---|---|
| 1.002 (0.96 – 1.04) | 3.397 (0.74-6.11) | 0.985 (0.98 – 0. 99) | 134.9 135.6 | 640, 596 5.0, 6.2 | < 0.0001 |

*CC: correlation coefficinet. Slope & intercept calculated by the Passing and Bablok method. Correlation coefficient according to Pearson's method.*

**Table 4. Reference interval; dependence on age.**

| Age | 1-15 | 16-40 | 41-60 | > 60 | All |
|---|---|---|---|---|---|
| 95% R.I. | 51.7 | 37.5 | 39.5 | 72.8 | 40.8 |
| | 125.8 | 132.2 | 164.9 | 183.3 | 158.1 |
| Mean | 88.7 | 84.9 | 102.2 | 128.0 | 99.2 |
| SD | 18.9 | 24.2 | 32.0 | 28.2 | 30.6 |
| N | 37 | 44 | 33 | 32 | 146 |
| Max | 133.4 | 141.6 | 169.0 | 182.9 | 182.9 |
| Min | 56.5 | 39.8 | 40.7 | 78.2 | 39.8 |

*Lower and upper limits of the 95% reference interval (R I) for the different age ranges (in years). The last column shows the reference interval for all samples studied. VWF:Ag expressed in IU/dL.*

**Table 5. VWF:Ag for the group with diabetes mellitus type 1 and the age- and sex-matched control group.**

| | Diabetes Mellitus Type 1 | | | *Controls | | |
|---|---|---|---|---|---|---|
| | Total | With Complications | Without | Total | With Complications | Without |
| Mean | 133.9 | 176.1 | 109.5 | 102.8 | 115.5 | 95.5 |
| SEM | 12.27 | 26.62 | 8.04 | 6.81 | 14.48 | 6.52 |
| N | 30 | 11 | 19 | 30 | 11 | 19 |
| Max | 428.4 | 428.4 | 194.4 | 225.9 | 225.9 | 156.8 |
| Min | 60.2 | 123.2 | 60.2 | 51.6 | 53.5 | 51.6 |
| p | 0.0136[a] | 0.0002[a] | 0.4236[a] | | 0.0105[b] | 0.2371[b] |
| | | 0.0007[c] | | | | |

*VWF:Ag expressed in IU/dL. *Controls matched for age and gender to the patients with and without complications. p[a]: with respect to the total control group. p[b]: with respect to its own control group. p[c]: with respect to the two patient groups.*

or without microvascular complications when controlled for glycemia and lipemia: $HbA_{1c}$ 8.86±0.31 vs 8.55±0.43%; total cholesterol 208.8±13.3 vs 283.7±95.91 mg/dL; HDL-C 65.6±4.91 vs 60.18±4.91 mg/dL; LDL-C 124.2±14.36 vs 112.8±5.18 mg/dL and triglycerides 96.0±12.44 vs 77.95±10.25 mg/dL.

The patients´ age was similar to that of the control group: 34.00±2.64 vs 34.83±2.82 years, although after dividing the patients into two groups (with or without complications), patients showing complications were older than those without them (44.9±4.5 vs 27.4±2.3, $p$=0.0018). This could contribute to an increase in the VWF:Ag concentration as shown in Table 4, and to a greater possibility of suffering microvascular alterations.

The VWF:Ag concentration in the 30 DM patients (Table 5) was significantly different from its corresponding value in the gender- and age-matched control group ($p$=0.0136). However, when the patients without microvascular complications were compared with the normal population or with the control group made up of by the same number of individuals of the same gender and age, no statistical differences were found. The patients presenting complications (6 retinopathy plus nephropathy and 5 retinopathy only) had a higher VWF:Ag concentration than the normal control group ($p$=0.0002) or the gender/age matched group ($p$=0.0105), and even the group of DM type 1 patients not showing complications ($p$=0.0007).
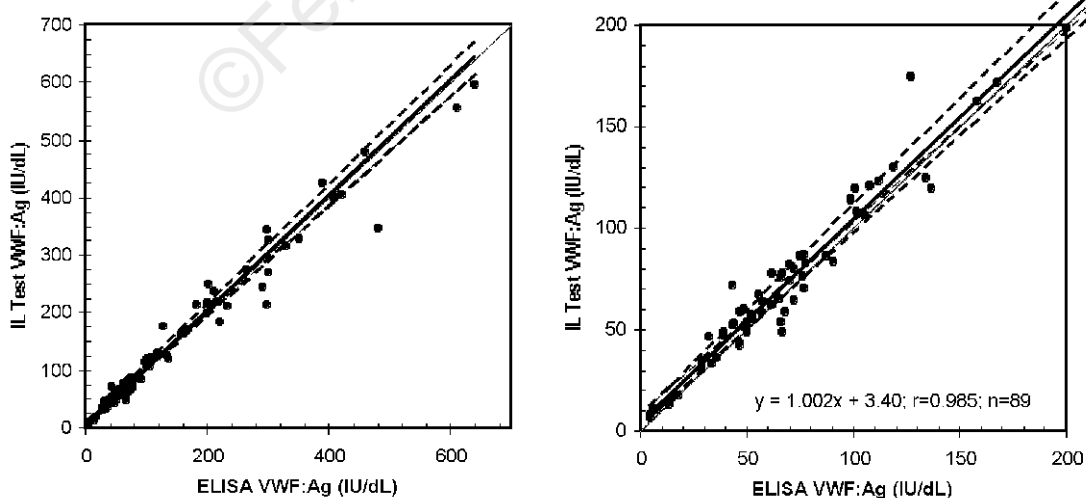


**Figure 2. Method comparison plot. Passing & Bablok fit and 95% confidence interval: solid and dotted lines. The diagonal line is the identity. The right plot is a magnification of the lower end of the left one.**

y = 1.002x + 3.40; r=0.985; n=89

These data suggest a relationship between the rise in VWF:Ag concentration and microvascular dysfunction.

## Discussion

The new method was compared to our reference ELISA, and an excellent agreement within the range 5-600 IU/dL of VWF:Ag was found. Samples with VWF:Ag below 5 IU/dL cannot be accurately quantified with IL Test™ VWF:Ag and should be reported as less than 5 IU/dL. In contrast, such samples can be re-assayed with the ELISA at a lower sample dilution with good accuracy. Although this was not the case in the population analyzed in our study (lowest reported value 5 IU/dL), accuracy down to 1 IU/dL can be important to distinguish patients with severe type 1 VWD from those with type 3.

The determination of VWF:Ag can be affected by rheumatoid factor (RF) when the immunoassay uses global IgG. The RF can bridge the capture antibody and the conjugate in an ELISA or can link IgG coated to different particles causing agglutination in a latex immunoassay. Therefore, the presence of RF in samples may result in overestimation of the VWF:Ag content. Immunoassays can avoid this problem using fragments of antibodies, e.g. $F(ab´)_2$.[21,22] The ELISA and the latex immunoassay used in this study are prepared with whole IgG and are, therefore, susceptible to interference.

The reference interval described in the literature is quite broad due to a variety of physiologic conditions. On average it ranges from 40 to 240 IU/dL including the influence of blood group and age.[27] Covering a wide age span, we determined the reference interval as being 40.8 to 158.1 IU/dL. When the individuals were divided in four age intervals, a small but clear rise of VWF:Ag concentration was observed with age.

VWF is released when endothelial cells are damaged, making it a possible marker of endothelial dysfunction.[28,29] In this sense and in order to prove the validity of the VWF:Ag assay evaluated in this study, a group of patients with type 1 DM (some with microvascular complications) was selected and compared with a control group consisting of an identical number of apparently healthy individuals of the same age and sex. The VWF:Ag assay reported higher concentrations for the insulin-dependent DM patients with microvascular complications when compared with the control group or even with the DM patients not showing complications, suggesting the possibility of using this method to analyze VWF:Ag as a risk marker of

atherogenic-dependent microvascular complications in diabetes and to follow-up their progress.

This method is reproducible, accurate and linear within the range 10–150 IU/dL. Values above that range may be diluted and reanalyzed. The assay correlates well with the reference ELISA (r = 0.985, VWF values from 5–680 IU/dL). The reference range is 40.8–158.1 IU/dL, with a clear tendency to increase with age. There is a relationship between patients showing microvascular diabetic complications and high levels of VWF:Ag.

The IL Test™ VWF:Ag is a fully automated assay. It has the advantages over the ELISA of being easier to perform, faster and suitable for analyzing a small number of samples.

### Disclosures

*Conflict of interest: none.*

*Redundant publications: no substantial overlapping with previous papers.*

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### Potential implications for clinical practice

The new methodology was used to determine von Willebrand factor not only as a diagnostic aid for von Willebrand's disease but also as a marker of microvascular alterations in other pathologic conditions.

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