Analysis of the von Willebrand coagulation factor

Ph.D. Thesis

Attila Szederjesi

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor: Imre Bodó, M.D., Ph.D.

Official reviewers:
Zsuzsa Bagoly, M.D., Ph.D.
Nikolett Wohner, M.D., Ph.D.

Head of the Examination Committee:
Barna Vásárhelyi, M.D., D.Sc

Members of the Examination Committee:
Zsuzsanna Bereczky, M.D., Ph.D.
András Masszi, M.D., Ph.D.

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1. INTRODUCTION

von Willebrand disease (VWD) is the most common bleeding disorder in the human population. The disease is caused by disruption of the amount and/or function of the von Willebrand factor (VWF) produced by endothelial cells and megakaryocytes. The functions of the giant glycoprotein are protecting the VIII. coagulation factor (FVIII) from degradation in the circulation and binding the platelets to the vessel wall at the site of injury. The most common forms of the bleedings are various mucosal and skin hemorrhages, traumatic or post-operative bleedings. Knowing the family history and bleeding history of the patient, the determination of the levels and function of the VWF and FVIII are required to diagnose and determine the subtype of the disease.

These tests underwent a significant development lately, several new tests appeared on the market, of which the precise interrelationship to each other remains unclear. Finally, we note that analysis of VWF function and structure may be important outside the realm of bleeding diathesis, e.g. in judging prognosis of malignant disorders.

2. OBJECTIVES

The primary objective of my thesis is the better and fully understanding of the von Willebrand factor, and to improve the tools of the laboratory diagnostics, and to use these tools in the widest range. Two important steps in my research are to investigate a high sample number VWF platelet binding activities, statistical evaluation and interpretation of the results, and the use of the multimer analysis of the factor outside the field of von Willebrand disease.

2.1. An international collaborative study to compare different von Willebrand factor glycoprotein Ib binding activity assays: the COMPASS-VWF study

One of the oldest and most important tools of laboratory diagnostics of the disease is the VWF platelet binding test. The aim of our research is to test the commercially available, widespread activities of normal samples and well-known genotyped von Willebrand patients. We tried to select the tests and platforms to represent routine diagnostics, but of course the latest testing tools and techniques are also present. The results obtained were

compared with the values of the "gold standard" von Willebrand ristocetin cofactor activity (VWF: RCo), the statistical parameters were evaluated and the basic parameters typical for the laboratory tests were determined, such as sensitivity, precision and correlation. International laboratories participating in the research have great routine in the large sample VWF studies. The studies and the organization of the samples were supported by the Scientific Standardization Committee of the International Society on Thrombosis and Hemostasis.

2.2. Use of the von Willebrand Factor multimer analysis

The von Willebrand factor multimer analysis plays an important role in the typing of the VWD, but can be used in other areas because of its quantification potential. Platelet count of colon carcinoma patients, von Willebrand factor antigen levels (VWF:Ag), ADAMTS-13 metalloprotease activity, the presence/absence of von Willebrand factor unusual large von Willebrand factor multimer (UL-VWFM), the interactions, prothrombotic efficacy and prognostic significance of these markers were

studied at different stages of the disease. Our laboratory performed VWF multimer analysis of the selected patients, control samples and the densitometric analysis of the resulting pattern, so we focus on this part of the research.

3. METHODS

3.1. The COMPASS-VWF study (Comparison of Assays to Measure VWF Activity)

We investigated 95 samples (52 normal donors and 43 VWD samples). The measurements were carried out in 8 international centers (England, Germany 3, Italy 2, USA, Hungary) with different activity tests on different platforms. The results of the tests were summarized and statistically evaluated. The assays were:

VWF:RCo: BC Von Willebrand Reagent (Siemens), Laboratory 1-6.

VWF:GPIbR: IL HemosIL® Von Willebrand Factor Ristocetin Cofactor Activity (Instrumentation Laboratory, Bedford, USA), Laboratory 1, 2, 3, 5; and IL HemosIL®

AcuStar Von Willebrand Factor Ristocetin Cofactor Activity, Laboratory 1, 2, 3, 5.

VWF:GPIbM tesztek: INNOVANCE® VWF Ac (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany), Laboratory 1-4; and in house ELISA method in Laboratory 6.

VWF:Ab assay: IL, HemosIL® von Willebrand Factor Activity, Laboratory 1, 2 and 5.

Passing - Bablok regression and Bland-Altman method were used for statistical evaluation with the 'r' package software.

3.2. Use of the multimer analysis in CRC patients

In this study, 232 patients with colon carcinoma (CRC) were enrolled at Semmelweis University III. Department of Internal Medicine, 55 of them (divided into high VWF/low ADAMTS-13 and low VWF/high ADAMTS-13 values, due to correlation) were analyzed with the VWF multimer analysis. Blood samples were taken in the beginning of their treatment, and kept frozen until the measurements.

Laboratory tests and statistical analysis

The laboratory performed ADAMTS-13 activity assay using FRETS-VWF73, with standard human plasma calibration curve. The VWF: Ag levels were measured from an EDTA sample by ELISA using multiple antibody labeling calibrated against WHO VWF:Ag. Blood and tumor markers were measured by routine diagnostic technique. Evaluation and demonstration of statistical tests has also been carried out in this case.

Multimer and densitometric analysis were performed according to the protocol used in our laboratory. We run the samples and normal controls in 1.2 w/v% SDS-agarose gel overnight. After Western blotting and double antibody labeling, we made the multimer pattern visible with chemiluminescent reagent, and analyzed it in a gel documentation system (Alpha Innotech). Alpha View software was used to read the densitometric curves and to calculate the presence and amount of the unusually high molecular weight multimers.

4. RESULTS

4.1. COMPASS-VWF study

Of the 5 compared assays, VWF:Ab (IL) and VWF:GPIbM (Innovance® Act.) gave significantly higher and the in-house ELISA methodology gave lower results, i.e. the identity line fell outside of the 95% confidence interval. Although the data show significant differences, it has no clinical consequences. To assess whether the small test differences received were the result of technical problems associated with a particular laboratory, the results of each laboratory were analyzed separately. It was noticed that the VWF:Ab and VWF:GPIbM tests still showed significant differences in all labs, but in this comparison we found the greatest swing in VWF:RCo activity, but these differences were not clinically relevant either.

2 participating laboratories (Laboratories 1-4) performed VWF: RCo and VWF: GPIbM (Innovance Act) tests with double calibration on all samples, with Siemens Standard Human Plasma and SSC Calibrator Plasma. The results were almost the same.

The LLOD values calculated from the measurement's results, and the manufacturer's recommended value, are almost identical in all assay. However, due to some modifications, several laboratories work with lower values than their manufacturer's protocol (10 IU/dL factory value vs. 4 IU/dL modified protocol) in VWF:Ab assay. The best sensitivity was for in-house ELISA and AcuStar VWF:GPIbR (0-0.5 IU/dL).

Most of the new tests tested showed a higher performance than those shown by VWF:RCo (12-16 CV%). As expected, the variability in the low range (i.e., VWD samples) was higher than in the normal samples (13-26 CV%).

Examining individual patterns, we found the following differences:

• All ELISA-based methods had high false results in subtype 2B patients.

• For the p.V1665E mutation, VWF:Ab tests gave higher values.

• Incorrectly low VWF activity in tests using ristocetin for p.P1467S mutations (except AcuStar VWF: GPIbR).

• False values for homozygous p.C2362F, and 1 (from 7) Vicenza patient with VWF:GPIbR (HemosIL) test.

4.2. UL-VWFM analysis in CRC patients

Samples were measured in two groups, Group 1 n=28 samples with highest VWF:Ag (1604 ng/ml) and lowest ADAMTS-13 activity (52%), Group 2 n=27 with lowest VWF:Ag (183 ng/ml) and highest ADAMTS-13 activity (97%). The samples were overexposed to ultra-large multimers, and then all of them (n = 55) were projected onto normal plasma for densitometric analysis. On this basis, in Group 1, the samples showed 67% UL-VWF multimer, while in Group 2 only 32%, which is a statistically significant difference ($p < 0.01$).

5. CONCLUSIONS

The main results of our research are:

• Our laboratory directed an international, large sample (n=95) study, and published the results and conclusions. The unique comparison of this magnitude was supported by the International Society on Thrombosis and Hemostasis.

• We have shown that compared to the results of the "gold standard" VWF:RCo, there are statistically significant but

clinically insignificant differences with new tests (VWF:Ab and VWF:GPIbM), which are the specifics of these methods, therefore the differences are present in all laboratories.

• Examining their sensitivity, we found that the AcuStar VWF:GPIbR test gave the best result (<0.5 IU/dL LLOD), while the original VWF:RCo was the worst (~ 10 IU/dL).

• In precision, the AcuStar VWF: GPIbR test also gave the lowest coefficient of variation (CV%) with a result of 12.1%.

• Using a weighted percent distance screening strategy, we showed that the ELISA methodology incorrectly measures VWD values of subtype 2B.

• AcuStar VWF:GPIbR test is the only test using ristocetin that measured p.P1467S mutation to normal, all other ristocetin-based tests gave false results for this polymorphism

• We have demonstrated that our VWF multimer analysis is not only used for typing VWD, but may be useful in gauging prognosis in malignancy.

• A statistical evaluation of densitometric results of 55 patients with CRC patients demonstrated the presence and significant increase of UL-VWFM in the high-

performance VWF:Ag and low ADAMTS-13 groups, which correlated with patient prognosis.

6. THE BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATION

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