Von Willebrand disease and von Willebrand factor: an old story, a new perspective

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Chapter 1

Introduction

What is von Willebrand factor (VWF)

VWF is a large multimeric protein, found in blood plasma, endothelial cells (within the Weibel-Palade bodies), platelets (within alpha-granules) and in the subendothelium. It is mainly synthesized by endothelial cells (>90%), but also by megakaryocytes. VWF was first purified in the early 1970s and its sequence was reported in 1986.[1-3]
VWF has 2 main functions in haemostasis: interaction with platelets and collagen in the formation of the first platelet plug (primary hemostasis) and stabilization of factor VIII (FVIII).

Characteristic and functions of VWF

VWF is synthesized as a pre-pro-polypeptide of 2813 amino acids and it is characterized by repeating domains. The first 22 amino acids are the signal peptide and are necessary for post-translational changes (glycosylation, sialylation, sulfation, folding) in the endoplasmic reticulum and Golgi apparatus. The signal peptide is cleaved and the VWF monomers can interact to produce VWF dimers and multimers of different sizes. Pro-peptides are released when multimers are secreted.[3] After secretion, ultra-large VWF multimers are cleaved by the metalloprotease ADAMTS-13, that binds to VWF A2 domain.[4] VWF is characterized by a high content of cysteine residues (4 times higher than average human proteins), that were shown to be relevant for proper folding and secretion of the protein.[3]
In case of vascular injury, VWF multimers are secreted from Weibel-Palade bodies of the endothelial cells and exposed to high shear stress, with subsequent unfolding of the A2 domain and formation of long bundles of VWF that expose the A1 domain with binding sites for nearby platelets. The A1 domain interacts with platelet GP1b receptors which initiates platelet aggregation and activation.[2]
VWF also binds to FVIII, protecting it from clearance and therefore prolonging FVIII half-life in plasma.[5]

The main associations between VWF domains and functions are the following:

- the signal peptide initiates post-translational modifications
- the pro-peptide (D1-D2-D'-D3) allows proper alignment during VWF dimerization

- the pro-peptide (D'-D3) binds FVIII to protect it from clearance
- A1 domain binds GP1b on platelets and collagen in the subendothelial matrix (in conditions of high shear stress)
- A2 domain unfolds under high shear stress to expose other vWF binding domains; A2 is also the cleavage site for ADAMTS13
- A3 domain binds extracellular matrix collagens
- C domains interact with GPIIb/IIIa during platelet adhesion
- the cysteine knot domain is important for multimerization and post-translational folding

VWF genetics

The VWF gene was cloned and sequenced in 1989 (Mancuso et al). It is located on chromosome 12 and contains 52 exons. It spans approximately 180 kb and it is highly polymorphic. Exon 28 encodes 1379 base pairs and it codifies almost completely domains A1 and A2.
A partial pseudogene is located on chromosome 22 and it corresponds to exons 22-34 of the VWF gene (that encodes domains A1-A2-A3). The pseudogene presents several missense, nonsense and splice mutations, and cannot generate a functional transcript.[6]

Role of VWF in pathophysiology: preventing bleeding, increasing the risk of thrombosis, and mediating inflammation.

VWF has a key role in the formation of the primary platelet plug. VWF binds to the subendothelial collagen after it is exposed during vascular injuries. Shear stress induces the unwinding of VWF from a loose globular form to an elongated one. This results in exposure of the of binding sites for the platelets' gpIb (in the A1 domain). This finally leads to the recruitment of more platelets to the site of injury and the formation of the platelet plug.[2,7]
The second role of VWF in preventing bleeding is that it is bound to FVIII, protecting it from clearance and localizing it on the sites of vascular injury, where it can amplify the clotting response.[2,5,7]
As can be expected from a protein that stops bleeding, supra-physiological levels of the same protein can have the opposite effect, i.e., increasing the risk of thrombosis.

An increased risk of venous thrombo-embolism (VTE) associated with high levels of VWF, [8] as described by Koster et al in 1995.[9] This was confirmed by Rietveld et al in 2019, in a large case-control study (2377 patients with a first episode of VTE and 2940 controls). In the latter study, high levels of VWF (>99th percentile) were strongly associated with the risk of VTE (compared with low levels, <25th percentile), with an odds ratio of 24.0 (95% CI 15.3-37.3).[10]

High levels of VWF were found to be associated also with arterial thrombosis in several studies and meta-analysis.[11] In particular the ARIC study, which included middle-aged individuals (45–64 years), found an increased risk of ischemic stroke for individuals with VWF plasma levels in the highest quartile compared with those in the lowest quartile, after adjustment for potential confounders.[12] High levels of VWF were also found to be associated to myocardial infarction in the British Regional Heart Study,[13] and in the PRIME prospective study.[14] More recently, high levels of VWF and low levels of ADAMTS-13 were shown to be associated with increased risk of all-cause and cardiovascular mortality in the Rotterdam population–based cohort study.[15]

Besides its hemostatic function, VWF has an important role in inhibition of angiogenesis,[16,17] in enhancing inflammatory responses,[18] and in immuno-thrombosis.[19,20]

In endothelial cells, VWF is essential for the formation of Weibel-Palade bodies that store growth factor Angiopoietin-2. It was shown in vitro that loss of intracellular VWF results in an increased release of Angiopoietin-2 with a consequent increased angiogenesis (through the Angiopoietin-2/ Tie-2/VEGFR-2 pathway). Other pathways involved in the regulation of angiogenesis by VWF are alphaVbeta3 and Gal-3.[16,17]

Acute activation of endothelial cells triggers the secretion of VWF high molecular weight multimers stored within Weibel Palade bodies, together with other inflammation mediators such as P-selectin. Vascular inflammation also results in exaggerated leukocyte recruitment by VWF, either by direct leukocyte binding or by recruiting platelets which in turn will attract leukocytes. Moreover, vascular inflammation is associated with increased vascular permeability, due to weakening of the endothelial junctions.[18] The COVID-19 pandemic was characterized by immuno-thrombosis associated with pulmonary endothelial inflammation and damage. In this setting, markedly increased plasma levels of VWF were observed in patients with severe SARS-CoV-2 infection, suggesting that endothelial activation is the pathogenetic

mechanism in severe COVID-19 infection, which led to the increased mortality associated with thrombotic events.[19,20]

Determinants of VWF levels in plasma.

ABO blood group is the main genetic determinant of VWF plasma levels in individuals without mutations in the VWF gene. Levels of VWF in individuals with O blood group are ~20-30% lower than in individuals with non-O blood groups.[21] Although the effect of ABO blood group is well known, the pathophysiological mechanism is not fully elucidated yet, different levels of glycosylation and sialylation, based on blood group, could lead to low levels of VWF in blood group O, due to increased clearance of VWF.[21,22]

Other genes (i.e., stabilin-2, CLEC4M and LRP1) have also been described as determinants of VWF levels, mainly affecting the clearance of VWF.[22,23]

Levels of several clotting factors increase with age: among these, FVIII and VWF.[24-26] High levels of VWF were described in centenarians, who show a procoagulant phenotype.[27] The increase of VWF with age may reflect the presence of comorbidities (such as inflammation, cancer, hypertension) that increase VWF release or, alternatively, could be due to a change in the production or clearance of VWF.

Von Willebrand disease (VWD) and acquired von Willebrand syndrome (AVWS)

Von Willebrand disease (VWD) was described in 1926 by Erik von Willebrand who observed a severe bleeding disorder affecting consanguineous families from the Åland Islands. Only in the 1950s was it shown that patients affected by VWD could correct their bleeding tendency by transfusion of a partially purified FVIII plasma preparation (i.e., also containing VWF), indicating that VWD is caused by deficiency of a blood protein. Remarkably, these abnormalities also were corrected by transfusion of a similar concentrate prepared from the plasma of patients with severe hemophilia, who are deficient of FVIII.[1]

The symptoms of VWD are typically muco-cutaneous bleeding events, including nose bleeding, skin bruises and hematomas, prolonged bleeding from trivial wounds, oral cavity bleeding, and excessive menstrual bleeding. Gastrointestinal bleeding appears to be rare, but may be severe when it occurs. Severe deficiency of VWF causes a

secondary moderate deficiency of FVIII. These patients may have joint bleeding, muscular hematomas and bleeding of the central nervous system.[28]

Congenital VWD is classified in 3 major types:

- VWD type 3: severe quantitative deficiency, with VWF antigen <1 IU/dL and generally associated to moderate FVIII deficiency
- VWD type 2: qualitative deficiency of VWF, characterized by a reduced activity/antigen ratio; several subtypes are recognized
    - VWD type 2A, with loss of high molecular levels of VWF
    - VWD type 2B, with increased affinity of mutant VWF to gpIb
    - VWD type 2M, with normal VWF multimers
    - VWD type 2N, with low affinity of VWF to FVIII
- VWF type 1: moderate and mild quantitative deficiency of VWF, with normal VWF multimer profile

Acquired VWF deficiency can be associated with several other diseases, and is therefore called acquired von Willebrand syndrome (AVWS). These associated conditions are lung cancer, Wilm's tumor, gastric cancer, monoclonal gammopathies, lymphoproliferative disorders, myeloproliferative neoplasms, systemic lupus erythematosus, hypothyroidism, other autoimmune disorders, drug side effects, and states of high-vascular flow such as aortic stenosis, ventricular assist devices, extracorporeal membrane oxygenation, or metallic cardiac valves. The mechanisms underlying AVWS are heterogenous: decreased synthesis of VWF, increase clearance and increased degradation.[29] Based on the underlying disease causing the AVWS, the therapeutic strategy in case of bleeding is different. In case of hypothyroidism that is associated with decreased VWF production, VWF concentrate can increase levels of VWF. However, in case of monoclonal gammopathies or lymphoproliferative disorders, associated with increased clearance of VWF due to the presence of anti-VWF antibodies, VWF concentrate will increase VWF levels in plasma only for a short period of time. In such cases the use of desmopressin (DDAVP), that releases the endothelial contents of VWF, characterized by ultra-large multimers with strong hemostatic capacity, is a therapeutic alternative.

Bleeding phenotype and GI bleeding in VWD

Except for VWD type 2N, characterized by a defective binding of VWF to FVIII that causes low levels of FVIII, associated with muscle and joint bleeding events, the other VWD types are associated with muco-cutaneous bleeding of different severity. Therefore, easy bruising, epistaxis, gum bleeding, gastro-intestinal bleeding, and heavy menses in women are the most frequently reported symptoms. Depending on the VWD severity, these symptoms can range from mild to life-threatening.[28]

Also, various symptoms are specific for certain ages, although it is not clear why. For example, VWD is characterized by epistaxis in childhood, usually resolved at puberty, while gastro-intestinal bleeding is typical of old age and often associated with angiodysplasia.[28]

The association between gastro-intestinal bleeding and loss of high molecular weight multimers was reported by Fressinaud and Meyer, in the early 1990s.[30] This study reported a higher prevalence of gastro-intestinal bleeding in type 3, type 2A VWD and acquired von Willebrand syndrome (characterized by loss of high molecular weight multimers), than in patients affected by type 2M or type 1 VWD (characterized by a normal multimer profile). A cohort study with 2 years of follow-u, enrolling 107 patients affected by type 2A or 2M VWD showed a higher bleeding risk in patients affected by type 2A than 2M.[31] It should be borne in mind in the interpretation of this study that other unknown factors could play a role, especially in patients belonging to the same family; moreover, the 2-year follow-up was short for GI bleeding, which can fluctuate over time, become chronic and relapse later again as an acute event.

A survey in the US reported that gastro-intestinal bleeding in VWD is more frequently accompanied with angiodysplasia (36.5%) than in patients not affected by VWD (9.5%).[32] Nevertheless, it must be underlined that angiodysplasia reporting is affected by the endoscopic tools that are used, and possibly the bleeding severity and time-frame of endoscopic evaluation in relation to the bleeding events. A French study reported an improvement in angiodysplasia diagnosis when video capsule endoscopy was used on top of conventional endoscopy in VWD.[33]

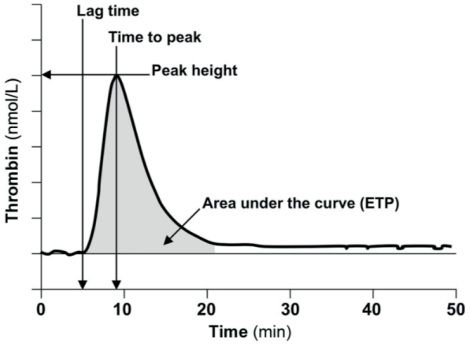
Many patients affected by monoclonal gammopathy and acquired von Willebrand syndrome (AVWS), have low levels of VWD caused by increased clearance of VWF, even in the presence of a normal production. These patients, with presumably normal intracellular VWF, are often characterized by severe GI bleeding. The old median age

of these patients cannot exclude the possible coexistence of intestinal angiodysplasia that become clinically relevant when the AVWS is developed.
Referring specifically to VWD and gastro-intestinal bleeding, the relationship between the two could be explained by the role of VWF in vessel development, that was described by Randi et al.[16-17]

Global assays for the evaluation of thrombotic risk associated with high levels of VWF

Since hemostasis is a complex process that is dependent upon the interaction of cellular and plasma factors, many laboratory tests have been developed to evaluate single components of this system.
Unfortunately, most single tests do not depict the global balance of the hemostatic system, and recent research focused on the development or improvement of global assays, that can give an evaluation of pro-coagulant and anticoagulant factors in the same sample, producing a global hemostatic profile, to better predict the thrombotic or hemorrhagic risk of patients.
In particular, the Thrombin Generation assay, was first described in the 1950s by Macfarlane and Biggs,[34] and largely updated by Hemker et al in the 1980s.[35] This assay measures thrombin generation using a synthetic fluorogenic substrate specific for thrombin and computer software to calculate the parameters stemming from the thrombin generation curve (figure 1). In this assay, the coagulation pathways are activated by tissue factor, phospholipids and calcium and the production of thrombin is initiated. The reaction first accelerates exponentially, and then slows down, until it reaches a plateau. The whole reaction can be measured in time, with as a result the hemostatic capacity. Several parameters are calculated from the curve: lag time (time between application of the trigger and the initiation of thrombin generation), time to thrombin peak (time to maximum generation of thrombin), thrombin peak height (maximum generated quantity of thrombin), endogenous thrombin potential (area under the curve evaluating the total amount of thrombin generation), velocity index (slope of the curve during the amplification phase).[36,37]

Figure 1. Thrombin generation parameters, from Tripodi, Clinical Chemistry 2016).



The type and concentration of reagents in the assay may tailor it to specific clinical conditions. Indeed, a low concentration of tissue factor and phospholipids are suitable to detect hypocoagulability associated with hemophilia A and B and to monitor therapy in these patients, while higher concentrations of tissue factor, with or without thrombomodulin or activated protein C are used to detect hypercoagulability and evaluate the thrombotic risk.[38]

The association between thrombin generation and the risk of both first and recurrent events of VTE was shown by van Hylckama Vlieg et al and Tripodi et al.[39-42]

Another test that can globally evaluate the hemostatic system is D-Dimer, which measures the degradation product of fibrin and can therefore express the activation of the hemostatic system. The automatization of this test on coagulometers has allowed its wide implementation in clinical practice. It has a high negative predictive value to exclude pulmonary embolism in case of low or moderate clinical risk of pulmonary embolism[43] and to predict the risk of recurrent VTE.[44]

Knowledge gap & aim of the thesis

In the past, the measurement of VWF was hampered by cumbersome assays, in particular to evaluate VWF activity. The recent introduction of automatized methods to evaluate VWF antigen and activity has allowed to evaluate VWF levels more frequently in the clinical practice.

The present thesis aimed at evaluating the evolution of VWF levels with age in healthy individuals and in a large population of patients affected by VWD.

The second aim of the thesis was to evaluate the clinical impact of VWD in older people (in particular the occurrence of gastro-intestinal bleeding), and the treatment with desmopressin of AVWS characterized by increased VWF clearance.
Finally, high levels of VWF are a relevant risk factor for first events and recurrent VTE. In this setting, global assays could be used to evaluate the hemostatic balance after a first event of venous thrombosis to help clinicians in the difficult choice of stopping or carrying on the anticoagulant treatment to avoid recurrent VTE.

Study populations

Normal population and patients with VTE

The MEGA study is a multi-center population-based case–control study in the Netherlands on risk factors for VTE, that enrolled 4,956 consecutive patients with a with a first event of VTE and 6,297 controls between 1999 and 2004. Controls were 3297 partners of patients and 3000 individuals recruited via random-digit-dialing method, without VTE, matched on sex, age and geographical area with the patients. Individuals with a history of VTE, severe psychiatric problems or inability to speak Dutch were excluded. Participants were invited to fill in a questionnaire on medical history and current use of drugs and to donate a blood sample (3 months after the discontinuation of anticoagulant therapy). Blood samples, due to logistic reasons, were collected until June 2002 for a total of 2,367 patients.
Patients included in the MEGA case-control study were followed-up for a recurrent event. Between June 2008 and July 2009, patients were sent a questionnaire concerning recurrent VTE. To avoid loss to follow-up, in case of not-returned questionnaires, patients were reached by telephone interview.
Vital status of all follow-up patients was acquired from the central Dutch population register between 2007 and 2009. In case of death, causes of death were obtained from the central register of death certificates of the Central Bureau of Statistics.
Duration of follow-up was defined as the time between the date of discontinuation of anticoagulant treatment and end of follow-up, which was defined as the date of a recurrent event or, in the absence of a recurrence, the date that the follow-up questionnaire was completed. When patients did not fill in the questionnaire, they were censored at the last date known to be free of recurrence. This could be date of death, emigration, last visit to anticoagulation clinic or the last time known to be recurrence free from the information provided from the MEGA case-control study.

Patients affected by VWD

All patients evaluated at the A. Bianchi Bonomi Hemophilia and Thrombosis Center in Milan between 1970 and 2018 with a diagnosis of VWD were included. Clinical chart review yielded data on FVIII and VWF levels for 617 patients, after exclusion of type 3 VWD, severe type 1 VWD, VWD type 2N, carriers of type 3 and 2N and patients with incomplete data (missing date of birth, date of measurement, or incomplete diagnostic workup, n = 23). Clinical chart review also provided information on comorbidities (high blood pressure, diabetes, or cancer), present at the time of FVIII and VWF measurements. Levels of FVIII and VWF were measured during follow-up visits or before surgery. Measurements during pregnancy, puerperium, bleeding episodes, after surgeries, or treatment with desmopressin and VWF concentrate were excluded. The interval between measurements was not fixed.

Thesis outline

In the first section of this thesis (Chapter 1), we presented the multiple functions of VWF and the actual difficulties in the diagnosis and treatment of patients affected by VWD.

In Chapter 2, we describe how levels of VWF increase with age in the normal population.

In Chapter 3, we describe how levels of FVIII and VWF increase with age in a large population of patients affected by VWD type 1 and 2.

In Chapter 4, we describe the treatment of patients affected by VWD with gastro-intestinal bleeding, a rare but potentially life-threatening complication of older age.

In Chapter 5, we describe the biochemical and clinical response to desmopressin in patients affected by AVWS, as a possible therapy, particularly in cases characterized by increased VWF clearance.

In chapter 6, we describe the association between thrombin generation and a first event of VTE. In addition, we describe which thrombin generation condition is the most sensitive to detect hypercoagulability.

In Chapter 7, since high levels of VWF are associated with an increased risk of venous thrombosis, we analyzed the use of 2 global assays (D-Dimer and Thrombin Generation) to evaluate the risk of recurrent VTE in the MEGA study.

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