Pathophysiology of von Willebrand factor in bleeding and thrombosis

Pagliari, M.T.

CHAPTER 9

GENERAL DISCUSSION AND PERSPECTIVES

This thesis discusses the role of mutated *VWF* as the cause of VWD, a common bleeding disorder caused by mutations within *VWF* and it also explores VWF's role in the pathogenesis of deep vein thrombosis (DVT) with a particular interest on the ADAMTS13-VWF equilibrium.

PART I: GENETICS OF VON WILLEBRAND DISEASE

VWF is located on chromosome 12 and spans about 178 Kilobases. It is composed of 52 exons encoding for a 2813 amino acids (aa) protein that includes a signal peptide (22 aa), a propeptide (741 aa) and the VWF (2050 aa). Each VWF monomer (2050 aa) is made up of conserved domains arranged in a specific order (D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C6-CK) [1]. The mature VWF molecules are obtained through a complex mechanism that includes monomer dimerization, dimer multimerization and several post-translational modifications (O- and N- glycosylation, sialylation, sulfatation and propeptide cleavage [2, 3]). Then, the VWF can be stored in the Weibel Palade bodies (WPBs) within endothelial cells, in platelets' α-granules or it can be released in the circulation [2, 3].

VWF mutations are responsible for VWD, an inherited bleeding disorder. The classification of VWD distinguishes between VWF quantitative defects that can be partial or complete (type 1 and type 3 VWD, respectively) and qualitative defects that may affect different VWF function (type 2 VWD) [1].

Type 1 *VWF* variants are mostly missense, although other genetic defects have been identified such as small insertions and deletions, splice variants and nonsense mutations. These variants act following three main mechanisms: reduced VWF synthesis, increased intracellular VWF retention and increased VWF clearance or a combination thereof [1]. Type 3 VWD is mainly due to null variants leading to a complete lack of VWF, whereas missense variants are less common.

Differently from type 1 and type 3 variants, that may be found across the whole gene without a specific localization, the qualitative type 2 variants show a target distribution in specific VWF domains. Type 2A can be considered the more complex subtype as mutations can be localized in different functional domains, although they all result in an altered multimeric pattern [4]. These variants are mostly found within the A2 domain, but they can be also found within D1-D3 and CK domains [5, 6]. Type 2B variants are responsible for an increased affinity for the GPIbα receptor and are localized in the A1 domain [7]. Type 2M variants cause a decreased VWF affinity for GPIbα and are localized in the A1 domain, however in some cases the variants cause a collagen binding defect and are located in the A3 domain [8, 9].

Contrary to the other type 2 variants, those causing type 2N are inherited recessively. These variants impair the capacity of VWF to bind FVIII and can be identified in a small portion of *VWF* corresponding to the D' and part of D3 domains [5].

Performing *VWF* genetic analysis can be a valid support to corroborate the VWD diagnosis. The diffusion of new genotype techniques such as next-generation sequencing (NGS) represents a huge advantage, especially for type 1 and 3 defects. On the other

hand, these techniques also led to the identification of a large number of *novel* variants with an unconfirmed causal role [10].

In silico tools can be helpful to predict the possible pathogenic role of these variants. To date, several tools based on different algorithms have been freely released and they can be applied to either single nucleotide variants (SNVs) or they permit testing if a variant may impact the mRNA causing alternative splicing. Since these tools suffer from specific weaknesses that may affect the prediction results, it is recommendable to apply different tools and combine the obtained results [10]. However, in spite of their utility in filtering potentially damaging variants, they are not affirmative for pathogenicity and other approaches are required to proof variants' effect.

From heterologous systems to endothelial colony forming cells (ECFCs)

The use of heterologous systems represents the most common approach to evaluate the pathogenic effect of a *novel* variant. Briefly, the expression vector containing the wild-type (WT) or mutant VWF (obtained by site direct mutagenesis) cDNA are transiently transfected in non-endothelial cell lines. Then, the respective recombinant (r) proteins released in cell media or present in cell lysates are collected and characterized [11]. So far, different cell lines characterized by different properties, have been used to study VWF. Among all, Human Embryonic Kidney (HEK) 293 cells are those preferred due to their capacity to form pseudo-WPBs, allowing for evaluation of VWF structure, its storage within cells, and its secretion [12, 13].

The extensive application of this method raised some limitations that result in discrepancies between patients' phenotypes and *in vitro* results such as: (i) the overexpression of recombinant (r)VWF due to vectors' strong promoter; (ii) the impossibility of completely reproduce the synthesis and secretion pathways and (iii) the difficulty to reproduce patients' heterozygous state by co-transfecting the same amount of WT and mutant expression vectors. Despite all, heterologous systems still contribute to increase the knowledge about the mechanisms responsible for VWF synthesis, storage and secretion as reported in Chapters 3 and 4 of this thesis.

A valid alternative approach is the use of ECFCs, previously referred as blood outgrowth endothelial cells (BOECs) [14]. These cells can be isolated from the peripheral blood mononuclear cells by performing a density gradient [15, 16]; they show the classical cobblestone morphology typical of endothelial cells, express endothelial markers (CD31, CD34, CD51/61, CD144, CD146, CD309) and form WPBs [17]. ECFCs can be isolated from VWD patients allowing to study naturally mutant VWF [18] overcoming the limitation of the heterologous systems mentioned above, and they can be used to evaluate other VWF functions such as its role as an angiogenesis regulator [19].

Even in this case, some limitations have been identified, starting from the variable isolation rate that has been recently resized from 80% [16] to 50% [20]. Moreover, the studies so far published showed variability of ECFCs parameters including ECFCs

morphology, VWF expression and angiogenic properties, [19-21] highlighting the necessity to reach a consensus either on both ECFCs specific characteristics as well as the methods used to characterize these cells [19, 20].

Nevertheless, ECFCs represents a promising research tool in the field of personalized treatments such as the selective inhibition of the mutant allele using small-interfering (si) RNA [22] or to perform VWF CRISPR editing [23].

PART II: TYPE 3 VON WILLEBRAND DISEASE: RESULTS FROM THE 3WINTERS-IPS

Type 3 VWD is a recessively inherited bleeding disorder due to the complete absence of VWF [1, 24, 25]. Despite the rarity of this disorder, with a reported prevalence between 0.1 and 5.3 per million [26], the severe clinical manifestations along with the requirements of replacement therapy and the related complications such as inhibitor development and anaphylactic reactions make it extremely relevant from the clinical point of view.

The 3WINTERS-IPS is the largest cohort of type 3 patients so far collected that includes 265 patients with a previous diagnosis of type 3 VWD who were enrolled in Europe and Iran. At enrolment, patients underwent blood sampling and a bleeding questionnaire was administered. Blood samples were used to confirm phenotype and genotype diagnosis, and by the bleeding questionnaire their bleeding history was evaluated. The evaluation of patients' bleeding phenotype was compared to that of type 1 VWD patients enrolled in the frame of another study, the Molecular and Clinical Markers for the Diagnosis and Management of type 1 von Willebrand Disease (MCMDM-1VWD) [27].

As expected, type 3 VWD patients showed a more severe bleeding manifestation than type 1 VWD patients, with a bleeding score that increased with age. In addition, some bleeding manifestations such as central nervous system bleeding seem to be typical of type 3 patients, whereas cavity bleeding, hemarthroses, and deep hematomas were found to be from 7 to 10-fold most frequently reported than in type 1 VWD patients.

The authors also evaluated if some bleeding symptoms may cluster together resulting in non-random bleeding patterns. The results showed two patterns, one including hemarthroses, gastrointestinal bleeding and epistaxis, whereas oral cavity bleeding was reported together with post-surgical and post-extraction bleeding [27]

The 87% of type 3 patients enrolled in the 3WINTERS-IPS have been genotyped using NGS in combination with Sanger sequencing and multiplex ligation-dependent amplification techniques. The overall analyses showed that European and Iranian type 3 patients have different genotypes and a different variant distribution throughout the gene. Indeed, most of the variants found in Iranian patients were located at the NH2 terminal of the pro-VWF, whereas the variants found in European patients span across the pro-VWF [28, 29]. These data were in line with previous studies on Indian, French and the one recently published on American populations indicating that geographical localization and consanguinity played a role [29-31]. In most of the cases, null alleles

or putative null alleles have been found (79%), whereas the remaining 21% consist of missense variants mainly found among European patients. Of them, about one-half of these variants cause the loss of cysteines suggesting a deleterious effect. A small portion of patients had incomplete or inconclusive genotyping, probably due to technical limitations that do not allow for the identification of variants localized in deep intronic regions or gene conversion [28].

The 3WINTERS-IPS cohort of patients has been described in two chapters of this thesis. At first, we focused on the evaluation of VWFpp over VWF:Ag and FVIII:C over VWF:Ag ratios to explain the pathophysiological mechanism underlying type 3 VWD (Chapter 5). This approach was based on previous studies performed on type 1 VWD patients reporting that the VWFpp/VWF:Ag ratio can be used to attribute the reduction of VWF levels to a reduced secretion and/or increased clearance from circulation. Similarly, the FVIII:C/VWF:Ag was indicative of a reduced VWF synthesis [32].

We found out that the VWFpp/VWF:Ag ratio was significantly higher in type 3 patients carrying missense variants than in those carriers for nulls alleles, indicating that the remaining circulating VWF in patients with missense variants has an increased clearance and suggesting that the reduced VWF level was at least partly due to an increased protein clearance. Therefore, VWFpp/VWF:Ag ratio may also be used to discriminate patients carrying missense variants from those with null defects. Differently, FVIII:C/VWF:Ag ratio was similar among all patients, independently of the type of genetic defect identified, thus in type 3 VWD this FVIII:C/VWF:Ag ratio cannot be used to identify a defective VWF synthesis [33].

Type 3 VWD treatment requires the use of plasma derived concentrates containing VWF and different amounts of FVIII or recombinant VWF. The use of these products may be responsible for serious side effects such as the development of VWF inhibitors, which make the treatment ineffective, and the occurrence of anaphylactic reactions in a minor percentage of patients [34,35]. Up to now, the available data were limited and mainly related to small groups or single type 3 patients. Moreover, the identification of VWF inhibitors is complicated by the limited accessibility of the laboratory tests used to detect them and because of the lack of a standardized method [36]. Based on this background, we assessed the prevalence of alloantibodies in the cohort of 3WINTERS-IPS as reported in Chapter 6. The type 3 patients from 3WINTERS-IPS cohort were tested for the presence of VWF alloantibodies. Of them, 8.4% were found to be positive for alloantibodies using an indirect ELISA able to detect all alloantibodies against VWF. The detection of alloantibodies with inhibitory effect was performed with a modified version of the Bethesda assay using VWF:CB ELISA assay and resulted in a prevalence of 6% inhibitors. Subsets of samples have been evaluated with two other Bethesda-based methods. Samples positive for non-neutralizing anti-VWF antibodies were tested with a Bethesda-based method using the semi-automated gain-of-function glycoprotein-Ib binding (VWF:GPIbM) assay. Patients positive for VWF:GPIbM inhibitors also had VWF:CB inhibitors, but one only had

VWF:GPIbM inhibitors. These results point out that the estimation of inhibitor prevalence is related to the type of assay performed (i.e., by the different epitopes recognized by alloantibodies). The Bethesda assay using VWF:Ag ELISA was limited to samples positive for VWF:CB inhibitors, and was able to detect VWF:Ag inhibitors in half of the patients highlighting its poor sensitivity.

The inhibitor data were also interpreted based on the genotype characterization confirming that they are mainly found in patients carrying null alleles [37-39]. Nevertheless, not all carriers of the same variants developed VWF inhibitors thus confirming a variable penetrance of inhibitor development [40].

Altogether, the data obtained from the 3WINTERS-IPS allowed us to highlight new information about the pathogenesis of type 3 VWD and corroborate the previously reported data obtained on smaller populations. The information that will stem from the ongoing perspective phase of this project, will lead to a better knowledge of type 3 VWD starting from the diagnostic process to the management of patients' treatment.

PART III: ROLE OF VON WILLEBRAND FACTOR IN DEEP VEIN THROMBOSIS

Deep vein thrombosis is a common life-threatening disorder that can be caused by environmental, behavioral and genetic risk factors [41]. However, part of its etiology remains unclear.

In this part of the thesis, we evaluated the equilibrium existing between the metalloprotease ADAMTS13 and VWF as a possible mechanism involved in DVT onset. This hypothesis is plausible considering that in physiological conditions, ADAMTS13 cleaves VWF multimers at the cleavage site in the A2 domain reducing its size and as consequence its procoagulant activity [42]. The alteration of ADAMTS13 levels is the cause of thrombotic thrombocytopenic purpura (TTP), a thrombotic microangiopathy characterized by VWF-mediated platelet microthrombi [43]. Moreover, the synergistic effect of low ADAMTS13 activity and high VWF levels were reported to be associated with myocardial infarction and coronary artery disease in a meta-analysis [44].

Initially, high VWF levels were found to be associated with arterial thrombosis [44-46], whereas their role in venous thrombosis started to be considered later. Moreover, most of these studies refer to venous thromboembolism which includes both DVT and pulmonary embolisms.

In 1995, Koster et al., were the first to describe that increased VWF levels were associated with an increased risk of VTE [47]. Over the years, these results have been replicated in different case-control studies. Recently, Rietvield et al. [48], showed that the association of VWF and FVIII with VTE was stronger than that found considering thrombin, FVII, FIX, FX and FXI, in a large case-control study. The same authors also reported that the increased VWF and FVIII levels are stable over time, contributing to sustains a direct causal effect [48]. Indeed, most of the studies evaluating VWF and FVIII

association with DVT, as well as ours, are case-control studies in which sample collection occurred after the thrombotic event. Therefore, the causality of the association cannot be proven.

Further confirmations also derive from prospective studies showing that the increased levels of VWF and FVIII are also associated with an increased dose-dependent risk of recurrent VTE [49]. This association was stronger for those patients with unprovoked VTE [50].

Even in our study, we found an association between increased VWF and FVIII levels with DVT (Chapter 7). We also showed that a moderate decrease in ADAMTS13 activity was sufficient to increase the risk for DVT in comparison to controls. However, the most interesting result was obtained by analysing the combined effect of low ADAMTS13 activity levels and high VWF levels that lead to a dramatic 15-fold increase in DVT risk. This latter finding suggested that ADAMTS13 and VWF act synergistically and even a small alteration of this equilibrium is enough to contribute to DVT risk [51].

To date, the role of ADAMTS13 levels has been evaluated in a few studies, involving smaller populations of VTE patients [52-54]. Of them, two were in line with our results, although they measure ADAMTS13 antigen levels instead of activity [53-54]. Differently, another study refers to high ADAMTS13 and VWF levels in presence of high inflammatory markers for a long time after VTE events [52]. This information was not available in our study population and therefore a proper comparison cannot be made.

The genetic component of DVT

DVT and more in general VTE have a strong genetic component that accounts for 50-60% of the hereditability, although part of it remains unexplained [55,56]. Most well-known genetic risk factors are factor V Leiden (FVL), prothrombin G20210A mutation, fibrinogen gamma chain C10034T mutation and the deficiencies of natural anticoagulants proteins (antithrombin, protein C and protein S) [57].

Large genome-wide association studies (GWAS) and meta-analyses contributed to identify *novel* common variants in hemostasis-related genes, even if they were performed in populations of VTE patients rather than DVT subjects alone [58,59]. Among them, different common variants on *VWF* and other genes involved in the modification of its plasma levels have been reported. Sabater-Lleal et al., performed a meta-analysis of GWAS results from 46354 individuals of European, African, East Asian, and Hispanic ancestry and identified 13 *novel* loci associated with FVIII and VWF levels. The same authors further evaluated the association between VWF level and VTE performing two-sample mendelian randomization in the population of 7507 VTE cases and 52632 controls enrolled in the frame of the INVENT consortium, confirming a causal role of VWF levels [60].

GWAS are not able to identify rare variants and therefore a different approach is required. Desch et al. [61], performed a whole-exome study of 393 individuals with unprovoked VTE and 6114 controls. Results showed an excess of rare variants in *PROS1*, *STAB2*, *PROC*, and *SERPINC1* in VTE patients than in controls. Then, the authors focused

on *STAB-2* because of its role in the clearance of VWF and *in vitro* characterized 7 missense variants, proving that they are responsible for a reduced expression of STAB-2 protein on the surface of endothelial cells. Patients carrying one of these variants had higher VWF levels than non-carriers. Taken together, these data support that the impaired VWF clearance may contribute to increased VWF levels [61].

In our study, reported in Chapter 8, we used the NGS to sequence the coding region and intron/exon boundaries of *ADAMTS13, VWF* and *F8* in a population of DVT cases and controls. The evaluation of common/low frequency variants showed inconsistent results. Only 9 common/low frequency variants were found to be associated with *DVT*, 7 in *ADAMTS13* and 2 in *VWF*. Nevertheless, the adjustment for multiple testing resulted in high false discovery rate. Instead, we confirmed the association between rare *ADAMTS13* variants and DVT [62], as previously reported by Lotta et al., [63]. Moreover, patients carrying at least one rare *ADAMTS13* variant showed lower ADAMTS13 activity than non-carriers. Conversely, there was no association between rare *VWF* and *F8* variants and DVT [62]. These results together with those already discussed above may further suggest that the defective VWF clearance is an adjunctive mechanism underlying the increased VWF levels.

Targeting VWF as a therapeutical approach in thrombosis

VWF represents an attractive target in the treatment and prevention of both arterial and venous thrombosis. This may be achieved through the regulation of VWF levels including its synthesis or modulating VWF interaction with specific targets such as GPIb, collagen and ADAMTS13.

Conventional drugs routinely used for other scopes may be taken into account as they unselectively target VWF. Heparin, usually administrated as an anticoagulant, also exerts an anti-platelet effect blocking the interaction between the A1 domain and GPIb [64].

A meta-analysis showed that statins, lipid-lowering drugs, may reduce VWF levels, although their use as anti-thrombotic agents is still debated [65]. N-acetylcysteine used to reduce mucins multimer size for the treatment of chronic obstructive pulmonary disease can also reduce UL-VWF multimers by reduction of the intrachain disulfide bonds. In addition, experiments performed in ADAMTS13 deficient mice demonstrated that N-acetylcysteine can rapidly reduce thrombus size, suggesting a potential use for TTP treatment [66].

Among the proposed specific target, there is the regulation of WPBs size. Indeed, smaller WPBs correspond to shorter VWF strings characterized by a reduced capacity to recruit and bind platelets on the endothelial cell surface. Recently, Ferraro et al., evaluated the capacity of 37 compounds to reduce WPBs size performing *in vitro* studies on HUVEC cells. These authors showed that both the pharmacological and physiological (e.g., hyperglycemia) status should be monitored and considered in a way to control VWF pro-coagulant activity [67].

Another class of potential drugs consists of specific VWF antagonists, as they may have the potential to reduce therapy side effects. Different monoclonal antibodies targeting VWF-GPIb have been tested in animal models. Results showed a good antithrombotic action, with limited side effects in terms of bleeding and thrombocytopenic [64].

Aptamers are single-stranded DNA/RNA oligonucleotides characterized by a specific and stable three-dimensional structure that allows for the recognition of a target with high specificity. Aptamers targeting VWF-GPIb interaction or VWF–collagen binding have been proposed to treat other thrombotic disorders [68,70]. Among them, the aptamer BT200 which inhibits VWF platelet dependent function has been shown to prevent arterial occlusion in non-human primates [71].

Nanobodies are small functional fragments of single-chain antibodies with a potential application in several fields. Of them, Caplacizumab, approved in 2018 for the treatment of TTP, specifically binds the VWF-A1 domain and thereby inhibits VWF-GPIb interaction [72]. However, there are no data about application of nanobodies in VTE.

The administration of rADAMTS13 may also be considered. Positive results have been reported in preclinical studies in TTP patients [73], whereas mice models of ischemic stroke highlighted a protective effect from inflammation and ischemic damages [74]. However, at the moment there is no information regarding their application to treatment of venous or arterial thrombosis.

CONCLUSIONS AND REMARKS

The increasing use of next-generation sequencing approaches simplified the genotyping of *VWF* in the frame of the VWD diagnostic process and led to the identification of several *novel* VWF variants with an unconfirmed causal role. In this thesis, we showed that *in silico* tools and conventional heterologous systems still contribute to evaluating the effect of variants, thus explaining patients' phenotype. More in general, we showed that the combination of phenotype and genotype tests continues to be necessary to properly classify VWD patients.

In the second part of this thesis, we focused on the characterization of type 3 VWD using conventional phenotyping tests to evaluate the mechanisms at the basis of the most severe form and we evaluated the prevalence of potential side effects due to replacement therapy. These data along with those generated in the frame of the 3WINTERS-IPS study add information on the characterization and management of type 3 VWD.

In the last part of this thesis, VWF has been evaluated as a risk factor for DVT. We found that a modest variation of the equilibrium between ADAMTS13 activity and VWF antigen levels increases the DVT risk. While the modest reduction of ADAMTS13 activity may be explained by the excess of rare *ADAMTS13* variants found in DVT patients, rare *VWF* and *F8* variants do not seem to play a role. The results reported by other authors suggested that the increased VWF levels may be partially due to variants localized in genes

involved in VWF clearance. Nevertheless, the comprehension of underlying mechanisms responsible for DVT and more in general in venous thrombosis requires further effort to be elucidated.

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