Pathophysiology of von Willebrand factor in bleeding and thrombosis

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CHAPTER 10

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von Willebrand factor (VWF) is a multimeric glycoprotein mainly known to be involved in primary hemostasis recruiting platelets at the site of damaged vessels and acting as factor VIII (FVIII) carrier.

Quantitative or qualitative alteration of VWF protein is responsible for von Willebrand disease (VWD). Conversely, increased VWF levels have been found to be associated to different thrombotic disorders such as arterial thrombosis, ischemic stroke and venous thromboembolisms. The dual role of VWF in both bleeding and thrombosis, with a focus on VWD and deep vein thrombosis (DVT), are discussed in this thesis.

In the first part, we described the approach used to perform VWD diagnosis, including the use of *in silico* tools and heterologous cell systems to confirm the disease-causing role of VWF variants.

In **Chapter 2**, we described the approach used to perform a differential diagnosis between type 2A and 2B VWD in a pediatric patient carrying a *de novo* and novel variant. The biochemical characterization allowed us to exclude type 2M due to a loss of high molecular weight multimers and reduced VWF:CB. The finding of a *novel* deletion, despite its localization in the A2 domain, did not allow for discrimination between type 2A and 2B VWD. The conventional approach would require to perform the ristocetin-induced platelet aggregation (RIPA) to discriminate type 2B VWD from the other type 2. This assay needs a relatively large amount of fresh blood, which is especially undesirable in the case of a pediatric patient. Therefore, we opted for an alternative approach using a platelet-dependent VWF activity (VWF:GPIbM) ELISA. This assay is able to discriminate type 2B VWD patients from the other type 2 VWD patients and it can be done using a small amount of frozen plasma sample. This led us to confirm that our patient was affected by type 2A VWD.

In **Chapters 3** and **4**, we combined the use of predictive *in silico* tools and heterologous cell systems to prove the pathogenic role of the VWF variants identified in our VWD patients. In both chapters, *in vitro* expression studies have been performed by transient transfection of wild type and mutant expression vector into HEK293 cells. In **Chapter 3**, we evaluated the effect of the previously reported type 1 variant p.Arg1379Cys, identified in five unrelated patients. Of them, one was diagnosed as affected with type 1 VWD, whereas the other four had a type 2M diagnosis. These latter patients also carried a polymorphism p.Ala1377Val, which role has been initially underestimated. The *in silico* evaluation showed that both variants destabilize the A1 domain, leading us to hypothesize a synergistic effect resulting in a decreased capacity of VWF to bind GPIb. Then, we tested the capacity of the wild-type, mutant, and hybrid recombinant (r) VWF to bind recombinant glycoprotein Ibα (rGpIbα) in presence of an increasing concentration of ristocetin. In this way, we were able to confirm that the synergistic effect exerted by these two variants was responsible for patients' type 2M phenotype.

Chapter 4 described the characterization of two unrelated Italian patients diagnosed as affected by type 1 VWD. Both were found to be heterozygous carriers for the same *novel* variant p.Thr274Pro localized in the VWF propeptide (VWFpp). This variant showed a dominant negative effect in contrast with other previously reported variants found nearby it, which have been described to be responsible for type 3 or type 2A/IIC VWD.

Therefore, we decided to perform an *in vitro* study including immunofluorescence with the aim to elucidate the disease-causing mechanism. Our results showed that the p.Thr274Pro was responsible for patients' phenotypes through a combined mechanism including defective synthesis, secretion and an impaired multimerization process.

The second part of this thesis focused on the characterization of type 3 VWD, the rarest and most severe manifestation of this bleeding disorder. In **Chapter 5** we evaluated if the VWFpp over VWF antigen (VWF:Ag) ratio and FVIII coagulant activity (FVIII:C) over VWF:Ag ratio can be used to determinate the pathophysiological mechanism of this disease. We showed that the VWFpp/VWF:Ag ratio was able to identify homozygous/compound heterozygous carriers for a missense variant as already described for type 1 VWD patients. Moreover, it also indicated that the extremely reduced VWF levels measured in these patients were at least partly due to a faster VWF clearance from the circulation. On the contrary, FVIII/VWF:Ag ratio failed to discriminate carriers for null defects from those carrying missense variants.

Treatment for type 3 VWD patients includes the administration of concentrates containing VWF or rVWF. The development of VWF alloantibodies and/or anaphylactic reactions are rare but important side effects of replacement therapy administrated to type 3 VWD patients.

In **Chapter 6**, the prevalence of alloantibodies against VWF in the 3WINTERS-IPS cohort has been assessed. Because of the lack of a gold-standard, we chose to carry out an indirect ELISA assay able to detect all anti-VWF antibodies and a Bethesda-based method using VWF:CB. Anti-VWF alloantibodies were found in 8.4% of the study population. We confirmed that the development of neutralizing antibodies represents a rare event with a prevalence of 6% and it is mainly found in type 3 patients homozygous for null defects. Two other Bethesda-based methods using either VWF:GPIbM or VWF:Ag ELISA were performed in subgroups of patients. Both methods were able to identify VWF inhibitors in a lower number of patients. However, the Bethesda-based method using VWF:GPIbM identified a further patient who tested negative for VWF:CB inhibitors. Taken together, these data confirmed that prevalence estimation is strongly affected by the epitope recognized by the alloantibodies and therefore by type of assay used.

Last part of the thesis aimed to evaluate whether the reduction of ADAMTS13 activity or the alteration of ADAMTS13-VWF equilibrium may play a role in DVT pathogenesis.

In **Chapter 7**, we performed a case-control study to evaluate the association between ADAMTS13, VWF, and FVIII plasma levels and DVT. We showed that a slight decrease in ADAMTS13 activity levels was associated with a moderately increased risk for DVT,

whereas we confirmed the strong association between increased VWF:Ag and FVIII:C levels and DVT.

More interestingly, we showed that the combination of slightly reduced plasma ADAMTS13 activity levels and increased VWF levels was responsible for a markedly increased DVT risk. These latter findings were further confirmed performing a sensitivity analysis after the exclusion of patients whose samples were collected less than three months from DVT event and/or during anticoagulant therapy.

Subsequently, we decided to investigate if variants localized in *ADAMTS13*, *VWF*, and *F8* genes may contribute to explain the altered levels of the respective, encoded proteins, as described in **Chapter 8**. For this purpose, a larger population of DVT cases and healthy volunteers has been sequenced using NGS. We confirmed that rare *ADAMTS13* variants alone are associated with DVT risk. Moreover, DVT patients carrying a rare *ADAMTS13* variant had a lower ADAMTS13 activity than non-carriers. In contrast, neither rare *VWF* nor *F8* variants were associated with DVT, thus indicating that other mechanisms are responsible for increased VWF and FVIII plasma levels.

Finally, **Chapter 9** includes a general discussion and future perspectives about the topics described in this thesis.