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Author: Lotta, Luca Andrea

Title: Pathophysiology of thrombotic thrombocytopenic purpura : pathophysiology of thrombotic thrombocytopenic purpura : the "two-hit" paradigm

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SUMMARY

In this thesis, we reported original studies on the pathophysiology of TTP and on severe ADAMTS13 deficiency. The findings of these studies are accompanied by a review of previously published data which collocates the research in the context of available literature. Illustrative clinical cases of the disease are also presented. The work herein reported enables the elaboration of a revised model of the pathophysiology of this rare disease. The manuscript is organized in two Sections. Section I is on congenital TTP, Section II is on acquired TTP. Below we report a summary of the Sections and Chapters of the manuscript.

Section I: congenital thrombotic thrombocytopenic purpura.

Chapter 1: this chapter reports a systematic analysis of *ADAMTS13* gene mutations and polymorphisms. This includes original analyses on the allele frequency and gene localization of variants occurring in the general population and in patients with congenital TTP. This was the first study to report a clustering of age of disease onset in patients with congenital TTP carrying the same disease-causing mutations.

Chapter 2: this chapter reports a study on the residual activity of ADAMTS13 in congenital TTP and its relationships with disease phenotype. The study is the first to (a) describe the presence of some residual activity in the majority of congenital TTP patients; (b) find that the residual activity is associated with the severity of the disease; (c) identify residual ADAMTS13 levels that discriminate patients with unfavorable prognosis; (d) describe a genotype-phenotype correlation in the disease, with mutations in the evolutionary conserved N-terminal domains of

ADAMTS13 being associated with severe disease in an allelic-dose dependent manner.

Chapter 3: a clinical case is presented. Residual ADAMTS13 activity was measured in a patient with congenital TTP during acute disease and compared with levels at remission. Activity was reduced to undetectable levels (i.e. below 0.5%) during the acute phase, whereas it was detectable and above 6% during multiple remission samples. This illustrates how onset of acute disease is characterized by a drop of residual activity, indicating that a “second condition/event” intervened to trigger acute disease in a patient with severe ADAMTS13 deficiency.

Chapter 4: this chapter summarizes emerging evidence on residual ADAMTS13 activity in ADAMTS13-deficient thrombotic thrombocytopenic purpura and discusses its implication for clinical practice and future research.

Section II: acquired thrombotic thrombocytopenic purpura

Chapter 5: the knowledge on the pathophysiology of acquired TTP is reviewed, with particular emphasis on the role of anti-ADAMTS13 autoantibodies. The feasibility of clinical trials in TTP is also discussed.

Chapter 6: this chapter describes the prevalence of clinical symptoms at TTP presentation, the disease-related laboratory measurement and the episode fatality rate of first episodes and recurrences of TTP. A milder clinical course with reduced fatality rate is observed for recurrences of TTP, suggesting that these are diagnosed and managed earlier than first episodes. The study also implies that

there is a time lag between the onset of thrombosis in TTP, the appearance of symptoms and the diagnosis.

Chapter 7: this chapter describes the study of ADAMTS13 and VWF-related laboratory measurement in acute versus remission in TTP. The goal of the study was to discover whether changes in VWF conformation, amount or multimeric pattern were associated with the onset of thrombosis in TTP. The consumption of multimers of ultralarge size of VWF was found to be a marker of acute disease severity. This Chapter shows that changes in the amount and multimeric patterns of VWF may trigger acute thrombosis in ADAMTS13 deficient individuals.

Chapter 8: this chapter describes the study of ADAMTS13- and anti-ADAMTS13 autoantibody-related measurements and their association with acute disease severity and recurrence risk in patients with acquired TTP. The study shows that both the Ig class and subclass of anti-ADAMTS13 autoantibodies are of predictive value for acute episode severity in patients with TTP.

Chapter 9: a clinical case of acquired TTP is presented. Two different thienopyridines, a class of drugs associated with the development of TTP, were safely used in a patient with a history of acquired idiopathic thrombotic thrombocytopenic purpura. This clinical case shows that drug-induced TTP develops with mechanisms that are likely distinct from those of idiopathic acquired TTP, in spite of the presence of anti-ADAMTS13 autoantibodies in both conditions.

Below we discuss the implications of the results of these studies in the understanding of the pathophysiology of TTP.

The emerging concept of residual ADAMTS13 activity and the “two-hit” paradigm for the pathophysiology of thrombotic thrombocytopenic purpura

The discovery that ADAMTS13 plasmatic activity is severely reduced in patients with TTP partially clarified the pathophysiology of the disease. Severe ADAMTS13 activity is associated with the appearance in the circulation of platelet-reactive ultralarge multimers of VWF (ULVWF).¹ These are in turn responsible for platelet aggregation and disseminated microvascular thrombosis that characterize the acute episode of TTP. However, the finding of severe deficiency alone is not sufficient to explain the clinical heterogeneity of TTP.^{2,3} TTP patients with severe deficiency of ADAMTS13 may indeed remain asymptomatic for years, without active thrombosis in spite of the presence of ULVWF in the circulation. Studies on a mouse model of severe ADAMTS13 suggested that a second event is probably required to trigger TTP in the presence of severe ADAMTS13 deficiency. *Adamts13* knock-out mice do not spontaneously develop thrombotic microangiopathy, but they do develop a TTP-like syndrome upon the injection of shiga toxin.⁴ This behavior resembles that of human congenital ADAMTS13 deficiency, in which environmental triggering events such as surgery or pregnancy are often associated with the development of acute episodes.^{3, 5} These conditions might precipitate the onset of acute thrombosis by triggering the release of ULVWF by activated endothelial cells. Consistently, TTP triggering conditions/events are all accompanied by endothelial activation and VWF release.

However, also the postulation of this second event needed to trigger TTP in predisposed patients incompletely explains the clinical heterogeneity of the disease. First, triggering conditions/events are not always apparent. Second, in spite of similarly reduced ADAMTS13 activity (activity below 10%) and of similarly distributed environmental exposures (as one may assume in a sufficiently large population) some patients with congenital or acquired TTP develop frequent recurrences, whereas others may remain thrombosis-free for years.^{2, 3, 5} The discovery of the importance of residual ADAMTS13 activity reconciles this clinical heterogeneity with the “two-hit” paradigm. Below we present a model that incorporates recent evidence.

Environmental exposures (circadian variation, inflammation, infections, traumas, chemicals, etc.) cause ADAMTS13 activity to fluctuate around a genetically determined level. In healthy individuals, these fluctuations never reach the TTP-triggering threshold (Figure 1A). This can be defined as the ADAMTS13 activity level below which ULVWF multimers accumulate to the point that disseminated microvascular thrombosis is eventually triggered. It is unclear which is the actual TTP-triggering threshold, but it is conceivable that this is close to 0% of activity and that it is individually variable. In patients with either congenital or acquired severe deficiency of ADAMTS13, the fluctuations of ADAMTS13 activity occur at a level that is much closer to the TTP-triggering threshold. How close this is depends on the residual activity of ADAMTS13. The type and location of causal *ADAMTS13* mutations are a major determinant of residual plasmatic ADAMTS13 activity in congenital TTP patients, with mutations affecting the evolutionary conserved N-terminal domains of the protein being associated with lower

plasmatic activity. Similarly, in patients with acquired TTP the titre and affinity of anti-ADAMTS13 autoantibodies may determine the residual activity of ADAMTS13. As a result, patients with some degree of conserved activity are more resistant to ADAMTS13 activity fluctuations even under mild challenges of their fragile VWF-ADAMTS13 equilibrium. In these patients, the tiny amount of residual ADAMTS13 may be sufficient to prevent the excessive accumulation of ULVWF, until strong challenges (e.g., pregnancy) overcome the residual activity and trigger acute TTP (Figure 1B). This results in later age of disease onset and less frequent episodes in congenital patients (Figure 1B, 2 and 3). Occasionally, a strong challenge or other disease modifiers may determine early disease onset also in these patients (Figure 2). In contrast, patients with low residual activity are exposed to early disease and frequent episodes, because ADAMTS13 activity fluctuations occur at a level that is very close to the TTP-triggering threshold (Figure 1C). In congenital disease, early-onset disease and frequent recurrences are observed (Figure 2 and 3). A similar scenario occurs in patients with acquired TTP, with the complication that severe ADAMTS13 deficiency is not present since birth and may be corrected by the implementation of plasma exchange (PEX) and immunosuppressive therapy. Another contribution to the greater complexity of acquired TTP comes from the fact that antibody amount fluctuates as well, conceivably resulting in changes in the residual activity of ADAMTS13 of the patients. Nonetheless, a clear association between residual ADAMTS13 activity and risk of recurrence is observed also in acquired TTP, consistent with the proposed model (Figure 4).

The measurement of residual ADAMTS13 activity may help improve the knowledge of TTP-triggering mechanisms, which is necessary to develop adequate preventive strategies. The proposed model also has clinical implications. Monitoring of residual activity may assist in deciding on the implementation of prophylactic therapies aimed at preventing recurrences and may constitute a valuable surrogate endpoint for the preliminary evaluation of preventive strategies in clinical trials. This may improve the predictive value of ADAMTS13 beyond what currently attained by the detection of severe ADAMTS13 deficiency, ameliorating the management of this rare but life-threatening condition.