Advantages and limits of ADAMTS13 testing in thrombotic thrombocytopenic purpura

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare disease (5-10 cases per million persons per year) characterised by the massive formation of platelet rich-thrombi in the microcirculation of multiple organs[1,2]. It affects both sexes, although the incidence is two to three times higher among females[3]. TTP was first described by Moschowitz in 1924 in a 16-year old girl who presented with fever, anaemia, thrombocytopenia and focal changes in the central nervous system and kidneys[4]. Several reports of TTP followed this initial description leading to a definition based upon the following pentad of symptoms: fever, mechanical haemolytic anaemia, thrombocytopenia, central nervous system abnormalities and renal impairment[5]. However, fever, neurological abnormalities and renal impairment are not constant symptoms, especially during the early stage of the disease, thus leading to the currently accepted definition of TTP consisting of the association of mechanical haemolytic anaemia with fragmented erythrocytes and thrombocytopenia (platelet count $<100 \times 10^9$/L) without alternative causes[6].

In parallel to the evolution of clinical criteria, the pathophysiological mechanisms of TTP were elucidated when Joel Moake and his colleagues observed that the plasma of a patient with recurrent TTP contained very high molecular weight [so-called ultralarge (UL)] multimers of von Willebrand factor (VWF), a multimeric adhesion glycoprotein contained in endothelial cells, platelets and plasma[7]. Once released from the abnormally stimulated endothelial cells, these ultralarge forms of VWF, present in the endothelium but not in plasma in physiological conditions, promote intravascular aggregation of platelets and the consequent microvascular thrombosis and haemolytic anaemia caused by mechanical damage, particularly in blood flow conditions characterised by high fluid shear stress such as in the microcirculation[3].

Moake himself hypothesised the deficiency of a cleaving protease as being responsible for the presence of UL VWF[7], but it was Furlan *et al.* and Tsai and Lian in 1996 who managed to isolate from human plasma a metal-dependent protease able to cleave the peptide bond between the tyrosine at position 1605 and the methionine at position 1606 in the central A2 domain of VWF. The same investigators subsequently and independently found that the VWF-cleaving protease was deficient in a retrospective cohort of patients clinically diagnosed as having TTP[8,9]. The protease, which is responsible for regulating the multimeric structure of VWF, was identified in 2001 by Zheng *et al.*[10] as a new (the thirteenth) member of the ADAMTS (A Disintegrin And Metalloprotease with ThromboSpondin 1 repeats) family of metalloproteases and was called ADAMTS13[2]. While the discovery of ADAMTS13 has renewed interest in TTP, as documented by the exponential increase in the number of publications on this topic in the last few years, it has raised the issue of the diagnostic and prognostic value of ADAMTS13 testing in this condition.

Classification of TTP

There are two different forms of TTP: congenital and acquired[2]. Congenital TTP, caused by mutations in the *ADAMTS13* gene (which is located on chromosome 9q34 and codes for the metalloprotease), is an extremely rare (incidence 1:1,000,000) autosomal recessive condition which manifests often, but not exclusively, at birth or during childhood[11-14].

The acquired forms can basically be distinguished into two types: immune-mediated forms, due to autoantibodies against ADAMTS13[15-17], and those probably secondary to massive endothelial stimulation with consequent release of UL VWF multimers in amounts exceeding the system's ability to degrade them, despite the presence of normal or only mildly reduced levels of ADAMTS13[18]. The most common physiological or pathological conditions present in the immune-mediated forms, which are often associated with severe ADAMTS13 deficiency (levels less than 10% of the normal), are pregnancy, infections, autoimmune diseases and the use of drugs such as ticlopidine and clopidogrel. The most frequent concomitant conditions associated with TTP forms presenting with normal or mildly reduced levels of ADAMTS13 (greater than 10%) are metastatic tumours, organ transplantation (particularly allogeneic bone marrow transplantation and solid organ transplants) and the use of drugs such as cyclosporine, mitomycin and a-interferon[19]. In most cases TTP occurs as a single, sporadic acute episode, but there are chronic recurrent forms (20% - 30% of the cases), which have a genetic basis or are associated with the formation and persistence of autoantibodies.

ADAMTS13 assays

The possibility of using plasma ADAMTS13 values to manage TTP patients stems from the current availability of ADAMTS13 assays to measure ADAMTS13 activity, ADAMTS13 antigen and neutralising or non-neutralising anti-ADAMTS13 autoantibodies.

Several assays of ADAMTS-13 activity have been developed[20-22]. They are based on the cleavage of plasma-derived or recombinant VWF multimers by test plasma and the direct or indirect detection of cleaved VWF by ADAMTS13. Direct assays focus on the detection of VWF cleavage products by using agarose or polyacrylamide gel electrophoresis (PAGE), western blotting, and fluorescence resonance energy transfer (FRET) techniques[22]. The last assay, which uses a truncated synthetic 73-amino-acid VWF peptide as a substrate for the determination of ADAMTS13 activity (FRETS-VWF73 assay)[23], is a rapid technique[24]. Indirect assays depend on measuring the residual substrate (i.e., VWF) or its disappearance. They include the collagen-binding assay, ristocetin-induced aggregation and enzyme-linked immunosorbent assays[25]. The general principle of ADAMTS13 activity assays is illustrated in figure 1. A multicentre study comparing several of these assays on 30 plasma samples with varying levels of ADAMTS13 activity showed a generally good agreement for the identification of severe ADAMTS13 deficiency, although one false negative and some false positive results were reported by laboratories using the collagen binding assay[26]. The interlaboratory agreement on samples with mildly reduced or normal activity values was less good.

A number of variables may interfere with the results of these assays. Firstly, all the aforementioned assays measure ADAMTS13 activity upon cleaving VWF in static conditions and thus do not reflect the *in vivo* physiological blood flow conditions. A flow-based test system, capable of observing *in vitro* under flow conditions the capacity of plasma ADAMTS13 to cleave UL VWF multimers secreted from stimulated endothelial cells, has been recently proposed but is not quantitative nor clinically validated[27]. Another important variable is that denaturing agents (e.g., guanidine or urea) are required to make VWF susceptible to cleavage by ADAMTS13[25]. The use of shorter peptides, such as the FRET-VWF73, instead of full-length VWF in enzyme immunoassay-based methods helps to deal with intra- and inter-laboratory variability only to a limited degree[21]. Finally, while the stability of ADAMTS13 from normal patients at –70°C has been documented, protease activity in various pathological conditions is not stable *in vitro* and may be reduced during storage or incubation[19].

ELISA to monitor plasma antigen levels of ADAMTS13 have recently become available[25,28]. Feys *et al.*[28] compared ADAMTS13 antigen and activity in a large set of plasma samples collected from subjects in various physiological states (neonatal period, pregnancy, oral contraceptive intake) or with pathological conditions (liver cirrhosis, inflammatory bowel disease, cardiac surgery) and found that the antigen assay showed less variability than the collagen binding-based activity assay. Thus, the authors concluded that the parallel measurement of both ADAMTS13 activity and antigen levels should be preferred to the measurement of only one parameter.

With regards to the detection of anti-ADAMTS13 autoantibodies, most of them are inhibitory and they can, therefore, be titrated *in vitro* using classical mixing studies with mixtures of patient's heat-

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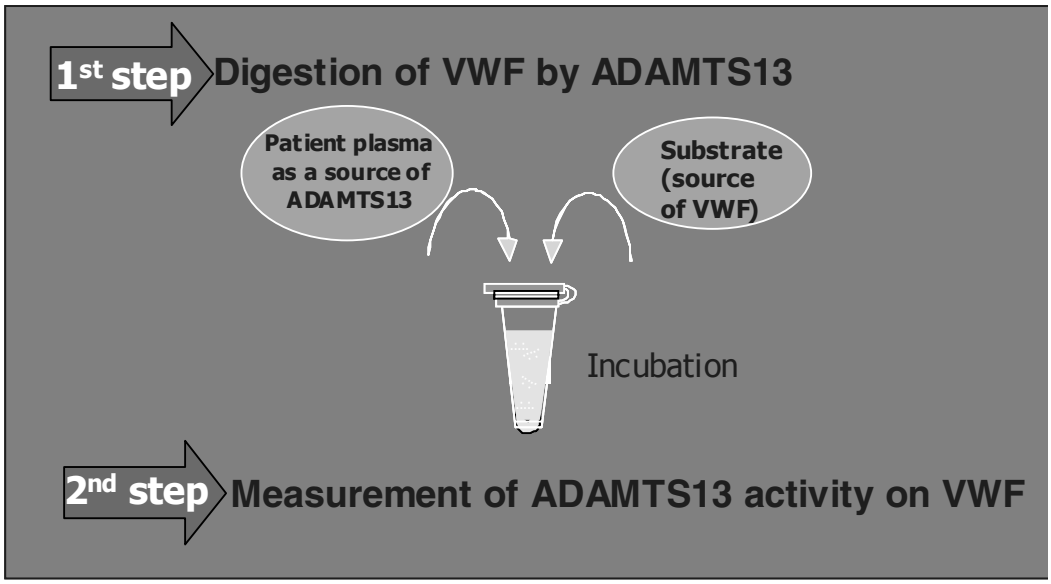

Figure 1 - General principle of ADAMTS13 activity assays

inactivated plasma and normal plasma[8,9]. Less frequently, autoantibodies promote the clearance of ADAMTS13 from blood without inhibiting its activity. These non-neutralising antibodies can be detected with more sophisticated methods using recombinant ADAMTS13[16].

ADAMTS13 activity testing

Diagnostic value of ADAMTS13 testing in acute TTP

A number of studies have assessed the diagnostic value of ADAMTS13 testing in acute TTP. In the context of two pioneering studies, Furlan *et al*.[8] measured VWF cleaving protease levels in 30 patients with clinically diagnosed acute TTP and found a severe deficiency in 86% (26/30) of them. All TTP patients (37/37) had a severe VWF-cleaving protease deficiency in the other study published by Tsai and Lian[9]. In a prospective cohort study of 66 acute TTP cases conducted by Veyradier *et al*.[29], 89% (59/66) of the patients had decreased VWF-cleaving protease activity, which was severely reduced in 71% (47/66) of them. Similar results were found by Mori *et al*. in 18 TTP patients[30]. By contrast, in the Oklahoma TTP-haemolytic uraemic syndrome (HUS) registry[31] only 33% (16/48) of patients with TTP were severely deficient in ADAMTS13 activity. Thirty-one of 46 TTP patients (67%) had a severe VWF cleaving protease deficiency in a study by Coppo *et al*.[32] Finally, Peyvandi *et al*.[33] and Zheng *et al*.[34] found severely reduced protease activity in 48% (48/100) and 80% (16/20) of patients with acute TTP, respectively.

Figure 2 summarises the rates of ADAMTS13 deficiencies in reported case series of TTP. A severe protease deficiency is present in the majority but not in all patients diagnosed with acute TTP (range, 33% - 100%), thus challenging the previous observations[35] that severely deficient activity of VWF-cleaving protease is a specific diagnostic marker to discriminate TTP from other microangiopathies. On the other hand, some authors have found severe ADAMTS13 deficiency in microangiopathies other than TTP[36-38]. It is possible that these discrepancies reflect differences in case definitions for TTP or assay methodologies[39].

Short-term prognostic value of ADAMTS13 testing in acute TTP

Since the development of ADAMTS13 assays[8,9], several investigators have evaluated whether or not protease activity testing is useful to predict short-term outcomes (remission and mortality rate). With regards to the prognostic value of ADAMTS13 activity testing in predicting remission in acute TTP, data from the Oklahoma TTP-HUS Registry showed a slightly higher remission rate in TTP patients with severe ADAMTS13 deficiency than in those without a severe deficiency (88% versus 75%)[31]. Zheng *et al.*[34] obtained

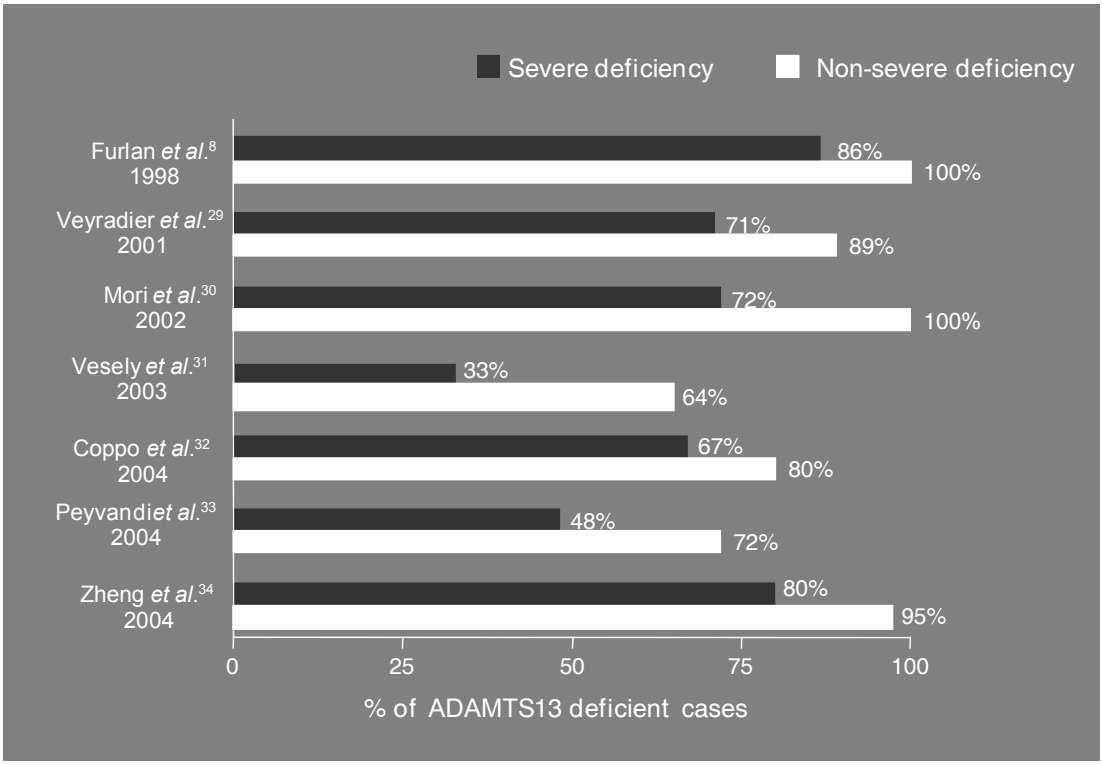

Figure 2 - Rates (%) of ADAMTS13 (partial or severe) in patients with clinically-diagnosed acute TTP

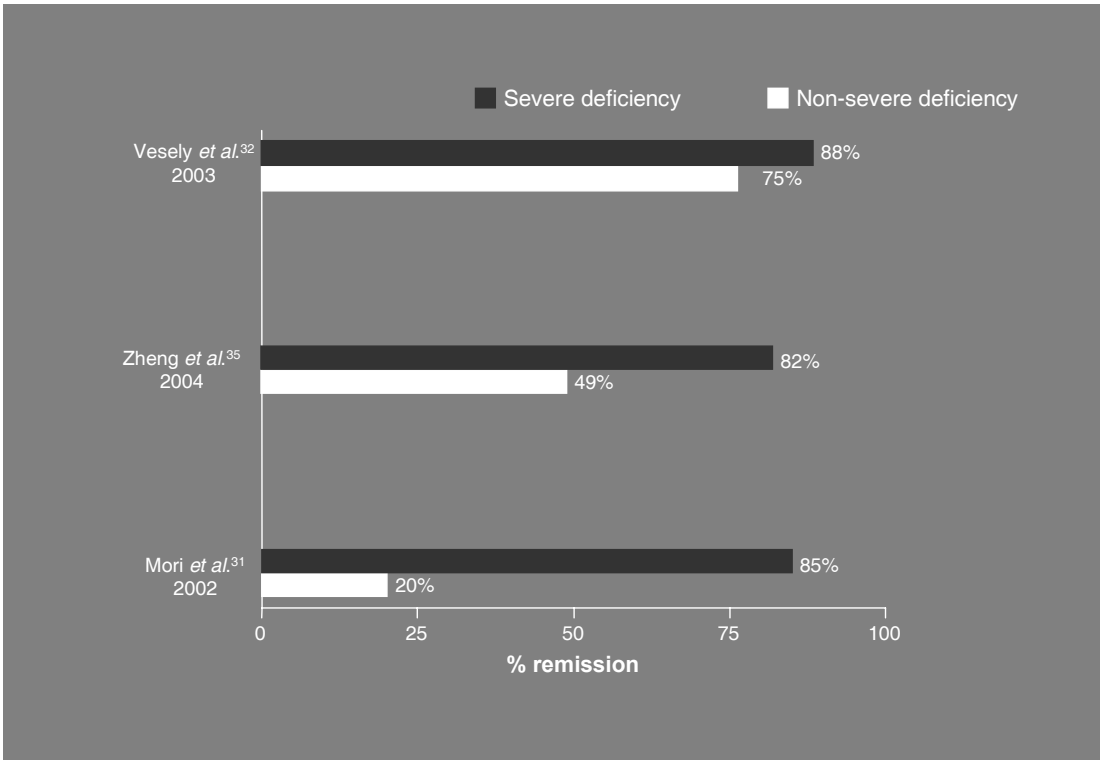

Figure 3a - Remission rates (%) in patients with acute TTP with severe *vs* non-severe ADAMTS13 deficiency

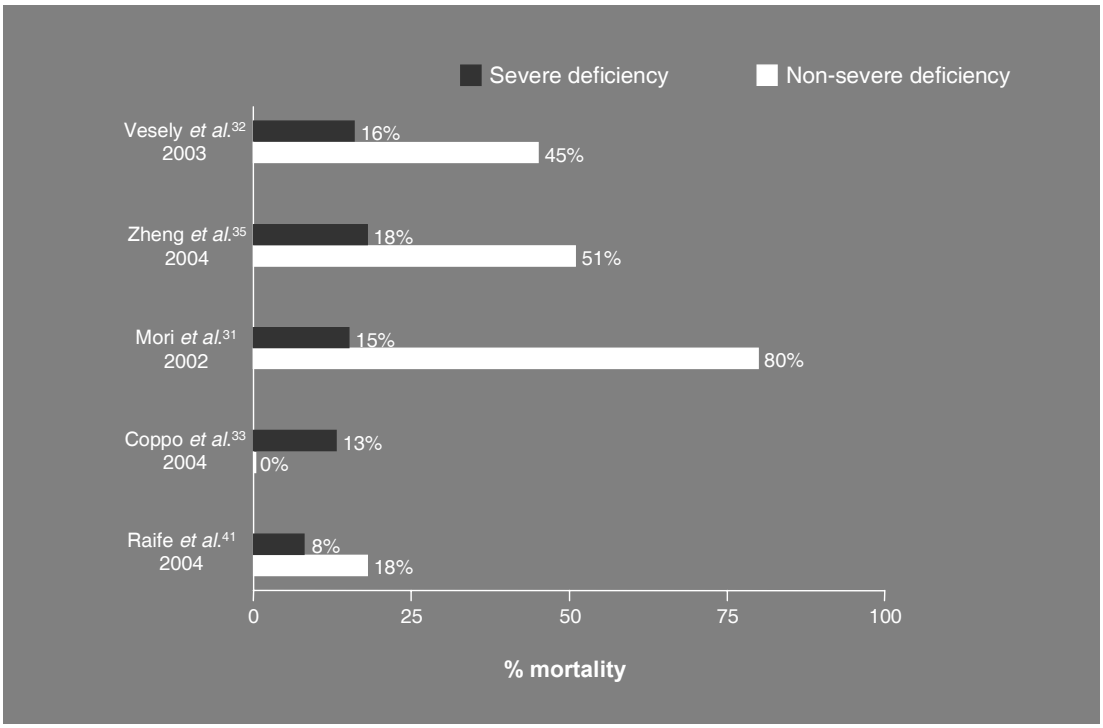

Figure 3b - Mortality rate (%) in patients with acute TTP with severe *vs* non-severe ADAMTS13 deficiency

substantially similar results (82% remission rate in TTP patients with severe ADAMTS13 deficiency versus 49% in patients with non-severe deficiency). A more marked difference in the remission rate between patients with severe or non-severe VWF-cleaving protease deficiency (85% versus 20%) was found by Mori *et al.*[30] (Figure 3a).

The impact of ADAMTS13 activity levels on mortality rate in patients with acute TTP has also been studied. In the Oklahoma TTP-HUS Registry[31], the mortality rate was lower in patients with severe deficiency of ADAMTS13 activity than in those with non-severe deficiency (16% versus 45%). Similar results were reported by Zheng *et al.*[34], Mori *et al.*[30], and Raife *et al.*[40] (Figure 3b). By contrast, Coppo *et al.*[32] found that patients with severe ADAMTS13 deficiency had a higher mortality rate (13% in TTP patients with severe ADAMTS13 deficiency versus 0% in those with non-severe deficiency).

Overall, these data suggest that, during acute TTP, a severe deficiency of ADAMTS13 is associated with a greater likelihood of favourable short-term outcomes (remission and survival rates). Conversely, TTP cases with detectable ADAMTS13 activity are associated with a high mortality rate. It cannot be excluded, though, that the latter finding is simply due to the fact that patients with detectable ADMATS13 develop TTP in association with severe diseases or conditions such as metastatic cancer and organ allotransplantation.

Anti-ADAMTS13 testing

Diagnostic value of anti-ADAMTS13 testing

As for ADAMTS13 activity, also the prevalence of ADAMTS13 neutralising inhibitors in acute TTP patients at presentation varies greatly in the different studies, ranging from 38% to 95%[29-31,33,34,41], with an apparently higher prevalence (67%-87%) in patients with severe ADAMTS13 deficiency[33,42]. This variability could reflect the low reproducibility of anti-ADAMTS13 antibody assays between different studies. Furthermore, it is possible that the assessment of inhibitory ADAMTS13 antibodies *in vitro* may be an incomplete evaluation of ADAMTS13 activity impairment *in vivo*, where inhibitory and non-inhibitory anti-ADAMTS13 antibodies may act in concert.

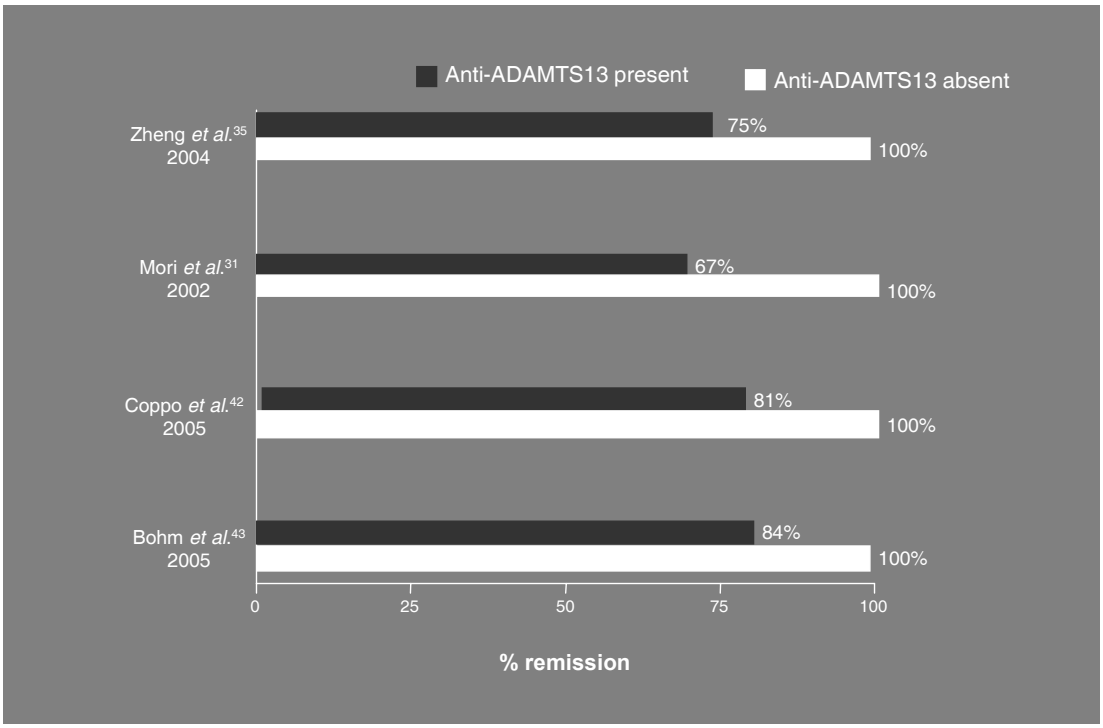

Figure 4a - Remission rates (%) in patients with acute TTP with or without anti-ADAMTS13

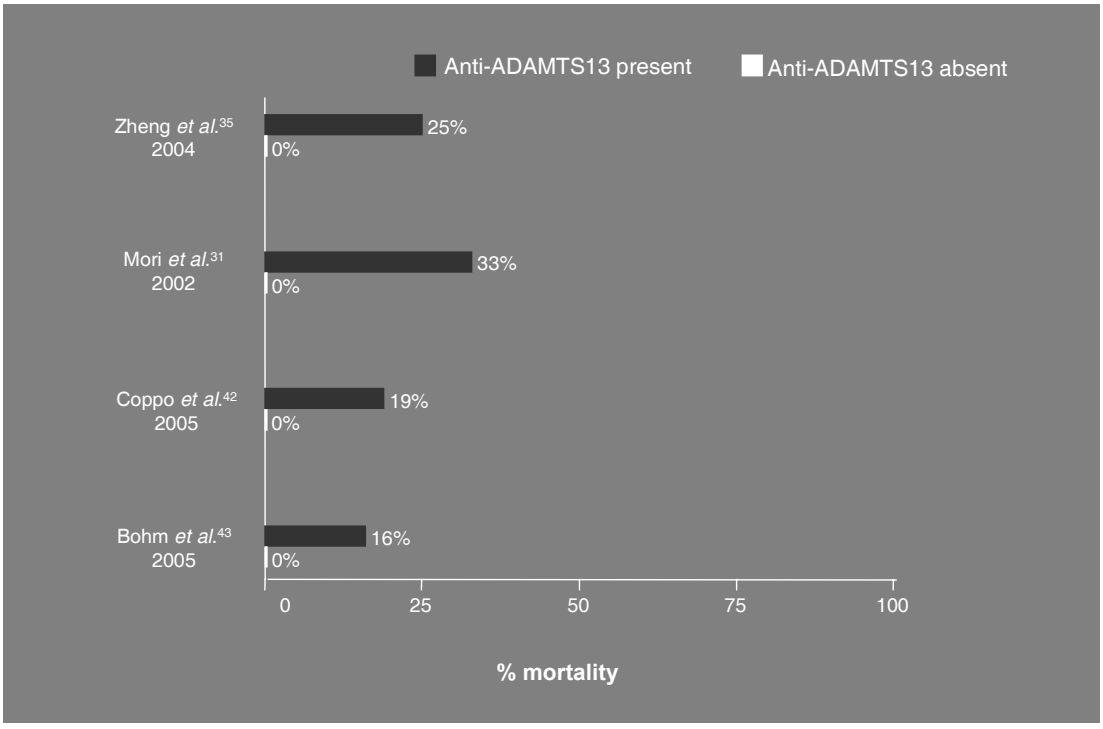

Figure 4b - Mortality rate (%) in patients with acute TTP with or without anti-ADAMTS13

Short-term prognostic value of anti-ADAMTS13 testing

Most of the aforementioned studies also analysed the short-term prognostic value of anti-ADAMTS13 testing (Figure 4a and 4b). In the study by Zheng *et al.*[34], all patients (8/8) with undetectable inhibitory anti-ADAMTS13 achieved a complete remission, while one death was detected among the four patients with detectable inhibitory anti-ADAMTS13. Similarly, Mori *et al.*[30] reported that complete remission was reached in all eight patients with undetectable ADAMTS13 inhibitors, while among six patients with anti-ADAMTS13 antibodies, four (67%) obtained a response and two (33%) died.

In the study conducted by Coppo *et al.*[41], deaths (4/21, 19%) were observed only in patients with detectable inhibitor, while all cases with undetectable inhibitor (12/12) evolved favourably obtaining durable complete remissions. Finally, in a study including both patients with an initial episode of TTP and relapsed patients, Böhm *et al.*[42] found that all TTP cases with absent anti-ADAMTS13 (7/7) achieved a complete response to treatment, while two of the 18 patients with detectable anti-ADAMTS13 died.

Overall, these data indicate that inhibitors are associated with a worse prognosis. The sample sizes of these studies was invariably small, so that all results and conclusions should be interpreted with great caution. While some authors reported a positive correlation between high inhibitor titres and severity of clinical presentation, treatment refractoriness and the rate of deaths[30,41,43], suggesting that the strength of ADAMTS13 inhibitors may be associated with treatment responsiveness and outcome, others failed to demonstrate such correlations[32,43].

ADAMTS13 and anti-ADAMTS13 testing for the prediction of TTP recurrence

Given the higher rate of relapse (20-30% of cases) in patients surviving an acute initial episode of TTP, many investigators have focused on the search for predictors of TTP recurrence[44]. With this aim, a number of studies assessed the value of ADAMTS13 and anti-ADAMTS13 in predicting the likelihood of recurrence in TTP patients during both the acute phase and first remission.

So far no single prospective study has been of adequate size, but pooled data from Veyradier *et al.*[29], Vesely *et al.*[31], and Zheng *et al.*[34] indicate that ADAMTS13 deficiency caused by inhibitors is associated with a relapsing course of TTP (15/35, 43%), while patients without inhibitors during acute TTP rarely relapse (1/19, 5%). Similarly, severe deficiency of ADAMTS13 activity during the acute phase of TTP identifies a subgroup of patients with a higher likelihood of relapse (37% in patients with severe ADAMTS13 deficiency versus 6% in patients with non-severe ADAMTS13 deficiency).

In a prospective cohort study, Ferrari *et al.*[45] investigated 32 patients who had low plasma levels of ADAMTS13 activity at the time of the first acute episode of TTP and subsequently achieved remission. Interestingly, the presence of high levels of inhibitory anti-ADAMTS13 IgG at presentation was associated with the persistence of undetectable ADAMTS13 activity in remission, which was in turn predictive of relapses within 18 months. These results were in accordance with those published by Peyvandi *et al.* in a retrospective cohort study of 109 TTP patients[46]. Survivors of an acute TTP episode with severely reduced ADAMTS13 levels and/or with anti-ADAMTS13 antibodies during remission had an approximately three-fold greater likelihood of having another episode of TTP than patients with higher protease activity and no antibody[46].

Collectively, these data suggest that severe ADAMTS13 deficiency and the presence of anti-ADAMTS13 antibodies during acute TTP or in first remission are associated with a higher risk of recurrence.

Conclusions

In spite of the recent progress in our understanding of the pathophysiology of TTP, a number of issues remain unsolved. First of all, the possibility of managing TTP patients guided by the results of ADAMTS13 tests is jeopardised by the unsatisfactory and limited availability of standardised ADAMTS13 assays suitable for clinical laboratories. From this point of view, the use of the fluorogenic ADAMTS13 testing (FRETS-VWF73 assay) appears to be particularly promising[23]. However, despite these advances in protease testing, increasing evidence has raised questions about the sensitivity of decreased ADAMTS13 activity for the diagnosis of TTP and the specificity of ADAMTS13 deficiency as a means of discriminating TTP from other microangiopathies, thus suggesting that as yet unidentified environmental

or genetic factors contribute to the aetiology of TTP.

Another controversial issue regards the prognostic value of ADAMTS13 testing. Although literature data suggest that the detection of an anti-ADAMTS13 inhibitor is associated with increased treatment refractoriness and a higher mortality rate, the number of patients evaluated is too limited to draw firm conclusions. Additional studies are also required to elucidate the relationship between the inhibitor titre and short-term outcomes. Similarly, the finding that severe ADAMTS13 deficiency and the presence of anti-ADAMTS13 antibodies during acute TTP or first remission are associated with an increased risk of relapses needs to be confirmed by further prospective studies on larger populations of TTP patients.

Key words: ADAMTS13, thrombotic thrombocytopenic purpura, TTP.

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