

**Doctoraat Hendrik B Feys:**

**Insights into thrombotic thrombocytopenic purpura by monoclonal antibody-based analysis of the von Willebrand factor cleaving protease, ADAMTS13**

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**Objectives**

The main objective was to develop an easy and inexpensive assay to accurately determine the exact concentration of the ADAMTS13 metalloprotease in crude extracts like plasma and expression medium. Therefore monoclonal antibodies (mAbs) directed against human ADAMTS13 were needed. mAbs are highly specific and powerful binding tools that are (mainly) generated in mice. Secondly, mAbs can be used in numerous other biochemical applications like western blotting, flow cytometry, immunohistochemistry and -precipitation. Thirdly, certain mAbs can also inhibit the function of the enzyme, thereby mimicking the diseased TTP state *in vitro* or *in vivo* when a suitable recipient animal is found.

**Background**

Von Willebrand factor (VWF) is an indispensable glycoprotein for normal hemostasis at high shear rates. VWF multimers bridge blood platelets with sites of vascular injury, allowing an aggregate to form and to seal off the gaping vessel wall. VWF is synthesized as unusually large VWF (ULVWF) molecules which are then rapidly cleaved in circulation, resulting in the normal VWF multimeric pattern. This process is catalyzed by a circulating metalloprotease, ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin-1 motifs #13).

When ADAMTS13 is dysfunctional or absent, ULVWF build up in circulation and can induce intravascular platelet formation. This disseminated intravascular thrombosis leads to thrombocytopenia and the rare but severe disorder thrombotic thrombocytopenic purpura (TTP). Study of ADAMTS13 and its function will hence contribute to understanding the molecular interactions taking place during aggregate formation in general and in TTP in particular

**Generating mAbs**

Mice were injected with an expression plasmid containing ADAMTS13 cDNA. Prior to hybridoma preparation, mice were boosted with semi-pure recombinant ADAMTS13. The latter was expressed by a transfected heterologous cell line using an inducible system. After DNA immunization two mice responded with a humoral response (Fig 1). The booster injection resulted in a positive response in all mice.

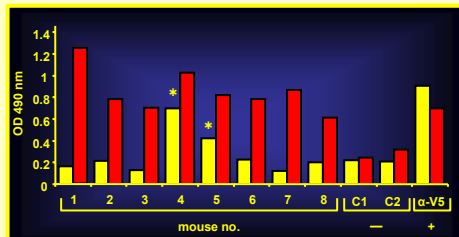


Fig 1: Eight mice and two control mice were subjected to a well-defined immunization scheme. After DNA immunization (yellow) serum was assessed for the presence of anti-ADAMTS13 antibodies. (\*) Indicates serum positive for antibodies. Next, all mice were injected with rADAMTS13 and serum was again assessed (red). A commercial antibody (a-V5) was taken as a positive control for the assay.

All twenty-five mAbs were assessed in western blot to denatured ADAMTS13, they were mapped to the respective ADAMTS13 domains and they were analyzed for their ability to inhibit ADAMTS13 function. Only one mAb (3H9) was found to inhibit the VWF cleaving capacity of the metalloprotease in a dose-dependent way, *in vitro* (Fig 2). This mAb can hence be used to mimic TTP.

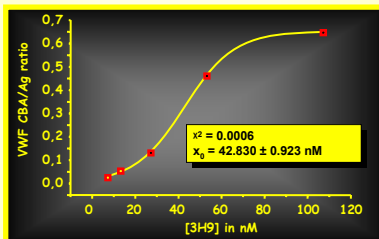


Fig 2: 3H9 dose-dependently inhibits ADAMTS13 function. Residual binding of VWF multimers to collagen, expressed as VWF CBA/Ag ratio is displayed in function of increasing amount of inhibitory 3H9. When ADAMTS13 is active, less VWF can bind to the collagen substrate. (inset) Chi square ( $\chi^2$ ) for sigmoidal fit was calculated and  $x_0$  represents the  $IC_{50}$  of the antibody.

**ADAMTS13 antigen assay**

To measure ADAMTS13 concentrations in crude extracts like plasma and expression medium, a high affinity mAb with high specificity needs to be selected. mAb 2G3 fulfilled this criterion and could bind ADAMTS13 from plasma, pooled from healthy donors. On the other hand, the same setup showed no signal when plasma from patients with TTP (both acquired and congenital) was analyzed (Fig 3).

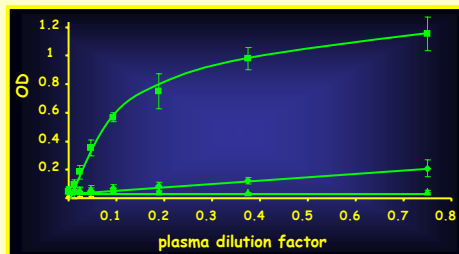


Fig 3: mAb 2G3 can bind ADAMTS13 from normal pooled human plasma (■) but was unable to detect the antigen in plasma from donors with congenital (▲), not with acquired TTP (★). Detection was with two other biotinylated mAbs and peroxidase labeled streptavidin.

We used this antigen determination assay to analyze the amount of protease in plasma from genotyped relatives from a Chinese homozygous congenital TTP patient. We found that relatives carrying the diseased allele had exactly half the protease amount of those family members not carrying the mutation. These non-carrying members had ADAMTS13 levels like normal unrelated Chinese donors, who still had significantly lower amounts than Caucasians (Fig 4).

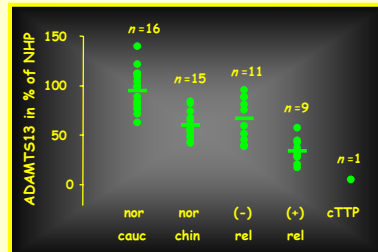


Fig 4: We used our newly designed ADAMTS13 concentration determination assay to analyze the amount of protease in family members of a congenital TTP patient [cTTP] expressed as % to pooled normal human Caucasian plasma (NHP). [(+) rel] Relatives heterozygous for the mutation, [(-) rel] wild-type relatives. [nor chin] Normal Chinese donors [nor cauc] normal Caucasians.

**Investigated conditions**

Several conditions, classified as physiological (newborns, oral contraceptive intake, pregnancy) and pathological (inflammation, liver cirrhosis, cardiac surgery) were investigated using our newly developed antigen assay. No drastic differences were observed in pregnancy, oral contraceptive intake and normal controls. On the other hand, newborns had drastically lowered ADAMTS13 antigen (not shown). Also patients with severe liver cirrhosis (so called Child C) had significantly lower plasma levels than milder forms (Child A and B) (Fig 5). Acute inflammation, as can be measured by C-reactive protein levels, caused a significant drop compared to patients in remission (Fig 5). Patients undergoing cardiac surgery had lower levels than healthy controls and the levels dropped drastically during surgery (not shown).

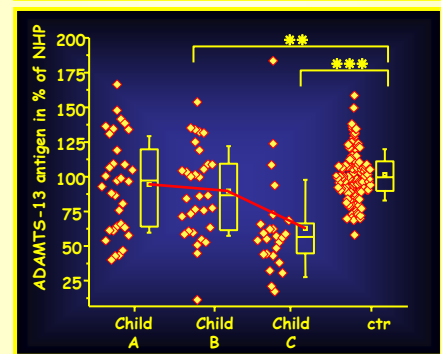
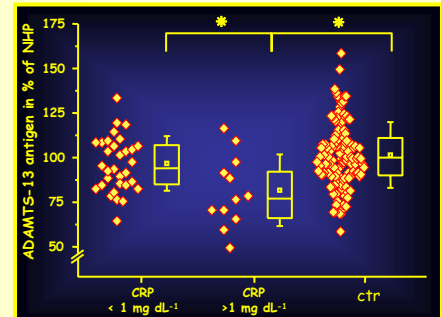


Fig 5: (top) Patients with inflammation were classified according to acute phase (CRP > 1) or not (CRP < 1) and compared to normal controls (ctr). (bottom) Liver cirrhosis patients were divided according to Child score (a medical scoring system to express disease severity with A < B < C). Data are expressed relative as % to normal human pooled plasma.

**Discussion**

During this four years of PhD, 25 mAbs were developed against human ADAMTS13. This was done by DNA immunization supplemented with a minor boost employing recombinant ADAMTS13. All mAbs were characterized in western blot, mapped to their respective domains and assessed for interference with the *in vitro* activity of the enzyme. Antibody 3H9 was found to be inhibitory and could therefore serve as a tool to induce TTP in a recipient laboratory animal. Using the high affinity antibody 2G3, a specific, sensitive and reproducible assay for the determination of ADAMTS13 concentration in crude extracts was set up. The assay could accurately distinguish alterations in plasma levels as carriers from a diseased ADAMTS13 allele contained exactly half the amount of wild-type plasma. Several bodily conditions were investigated and we could show that newborns, patients with acute inflammation and patients with severe liver cirrhosis have lowered ADAMTS13 plasma levels.