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## Aspirin resistance is not a common biochemical phenotype explained by unblocked cyclooxygenase-1 activity.

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# Outpatient treatment of pulmonary embolism is feasible and safe in a substantial proportion of patients<sup>1</sup>

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Dear Sir,

Asymptomatic pulmonary embolism (PE) is frequent in proximal deep vein thrombosis (DVT) and DVT patients are now largely treated as outpatients [1–4]. It was therefore tempting to treat at least some patients with symptomatic PE in an outpatient setting. The aim of the present study was to evaluate the proportion of symptomatic PE patients with a low predicted risk (according to Wicki *et al.* [3] who could effectively be treated in an outpatient setting). Patients ( $n = 255$ ; median age 69 years, range 18–96) were diagnosed during the period from May 1999 to December 2001 with symptomatic, objectively confirmed PE in the two participating centers. 150 patients had predefined exclusion criteria, including contraindication for anticoagulants, history or presence of drug addiction, high probability of noncompliance, body weight > 110 kg, psychiatric conditions, clinically relevant impairment of renal function (creatinine clearance < 30 mL min<sup>-1</sup>), thrombocytopenia (< 120 G L<sup>-1</sup>), any contraindications for low molecular weight heparins (LMWHs) or standard heparin, concomitant fibrinolytic therapy, treatment with oral anticoagulants during the 24 h prior to entering the study. Recruitment was not possible on weekends, which excluded 57 additional patients. Thus, 105 patients could be included in the study, and 43 (41%) could in fact be treated entirely as outpatients (Fig. 1). Treatment consisted of a single daily subcutaneous (s.c.) injection of a weight-adjusted dose of nadroparin calcium [Fraxiforte<sup>®</sup>, 171 UI anti-factor Xa (FXa) kg<sup>-1</sup>, Sanofi-Synthelabo, France], and phenprocoumon until the international normalized ration was in the therapeutic range of 2–3 for two consecutive days (LMWH for a minimum of 5 up to 10 days). Oral anticoagulation was continued for 6–12 months. Among the 62 patients treated in hospital, only 11 had a high risk score [3], while the main reasons for the inpatient treatment was either the patient's choice or the presence of comorbidity (Fig. 1). All patients were followed up for 3 months for clinical manifestation of thromboembolism or

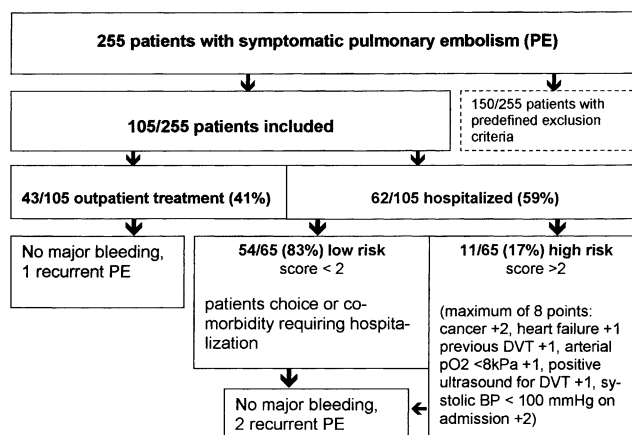


Fig. 1. Study flow chart.

bleedings. During the 3-month follow-up, clinically suspected recurrent DVT or PE was objectively confirmed in three patients (two in the inpatient and one in the outpatient group). No patients died, and there were no major bleeding incidents, although two minor bleedings occurred in the inpatient group. In conclusion, a substantial proportion of patients with symptomatic PE at low predicted risk of adverse outcome could be treated in an outpatient setting by means of a once daily s.c. injection of LMWH nadroparin. These data, along with an earlier report from Canada [5], call for a large-scale randomized study, with particular emphasis on safety and cost-effectiveness in PE patients with a predicted low risk of adverse outcome.

## Acknowledgements

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## Immunoabsorption for the treatment of a patient with severe thrombotic thrombocytopenic purpura resistant to plasma exchange: kinetics of an inhibitor of ADAMTS13

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Dear Sir,

Thrombotic thrombocytopenic purpura (TTP) [1] is characterized by a decreased activity of ADAMTS13 [von Willebrand factor (vWF)-cleaving metalloprotease], caused either by a congenital deficiency (in cases of familial TTP) or by inhibition by an autoantibody (in acquired TTP) [2–4]. ADAMTS13 has been characterized and cloned [5–7]. Plasma exchange is considered to be the treatment of choice to remove unusually large vWF multimers (UL vWF-MM) and inhibitors of ADAMTS13 and to substitute normal vWF and ADAMTS13 [8]. However, sometimes the response is slow despite intensive plasma exchange. We present the case of a patient with severe TTP, resistant to plasma exchange, and show the kinetics of a high titer inhibitor of ADAMTS13 during plasma exchange, immunoabsorption and follow-up.

The 38-year-old female patient with a history of systemic lupus erythematosus was admitted after a grand mal seizure which occurred after a short period of cephalgia, fatigue and 'restless legs'. On admission, dysarthria and a right-sided hemiparesis were obvious. A cranial computerized tomography showed no signs of bleeding, hypoperfusion or vasculitis. Red blood cell count was  $2.7 \text{ TL}^{-1}$ , hemoglobin  $8.0 \text{ g dL}^{-1}$ , platelet count  $13 \text{ GL}^{-1}$ , lactate dehydrogenase (LDH)  $745 \text{ UL}^{-1}$ , creatinine  $1.3 \text{ mg dL}^{-1}$ . Fragmented red cells were visible in the blood smear. Tests of plasmatic coagulation were normal. Antibodies to platelet antigens could not be detected. Coombs-negative hemolytic anemia with red cell fragmentation, together with severe thrombocytopenia, neurological disorder and elevated serum creatinine were highly suggestive for TTP.

Serial determinations of vWF antigen (vWF:Ag), vWF-collagen-binding activity (vWF:CB), factor VIII (FVIII) activity and the multimeric composition of vWF were performed as described elsewhere [9]. The activity of ADAMTS13 was measured according to Gerritsen *et al.* [10], modified by using

a purified recombinant vWF (Baxter BioScience, Vienna, Austria) [11] as substrate, and a different ELISA plate (Exiqon, Vedbaek, Denmark) to measure the residual vWF:CB activity [9]. The titer of the inhibitor of ADAMTS13 was measured as follows: the inhibitor-containing plasma samples undiluted and diluted with physiological saline up to 1:32 were mixed 1:1 with normal plasma and subsequently incubated for 30 min at  $37^\circ\text{C}$ . After incubation, the samples were diluted 1:10 and 1:20 with  $1.5 \text{ mol L}^{-1}$  urea,  $5 \text{ mol L}^{-1}$  Tris, pH 8.0 and the residual ADAMTS13 activity was measured. The amount of inhibitor was calculated according to the assay principle of the Bethesda method initially described for FVIII inhibitors [12] and expressed as inhibitor units per milliliter (InhU  $\text{mL}^{-1}$ ). One inhibitor unit per milliliter was defined as the amount of the inhibitor which decreases the ADAMTS13 activity to 50% in the assay mixture in 30 min compared with a control negative sample.

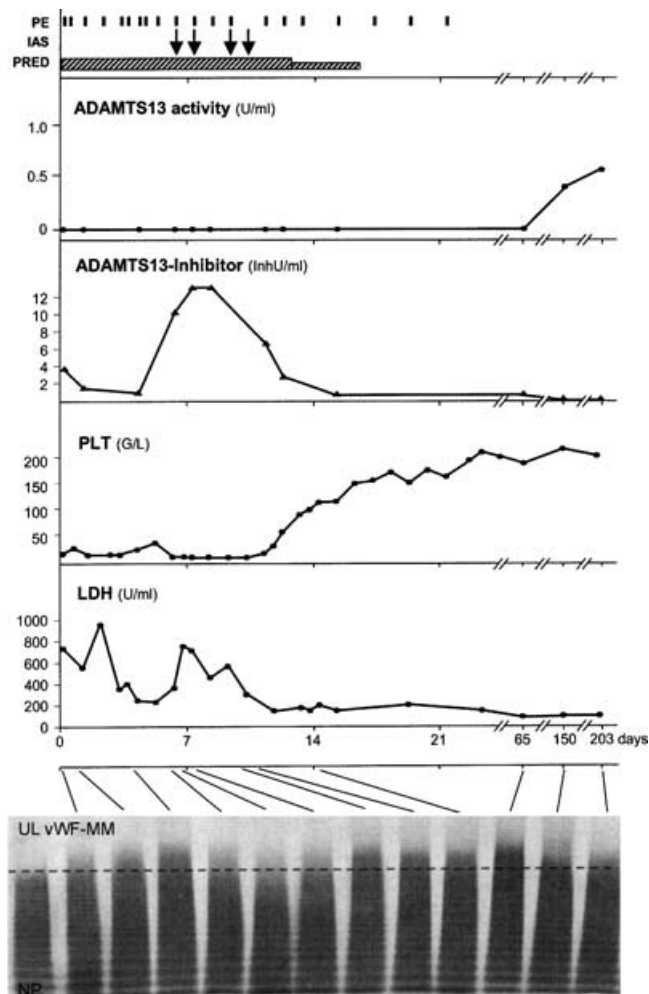
At admission, analysis of the vWF-multimer pattern showed the presence of UL vWF-MM (Fig. 1), vWF:Ag was slightly elevated ( $2.1 \text{ U mL}^{-1}$ ), vWF:CB and F VIII:C were in the normal range ( $1.18$  and  $0.82 \text{ U mL}^{-1}$ , respectively). The activity of ADAMTS13 was below the detection limit of our assay, and an inhibitor against the metalloprotease could be detected at an initial titer of  $3.7 \text{ InhU mL}^{-1}$ . These findings were consistent with the current hypothesis of the pathophysiology of TTP [3,4].

Plasma therapy was initiated by daily exchange of  $50 \text{ mL kg}^{-1}$  body weight of plasma with fresh frozen plasma according to a standard procedure. Additionally, prednisone ( $100 \text{ mg}$  daily) was given. Red blood cells were transfused at hemoglobin values below  $8.0 \text{ g dL}^{-1}$ . No platelet concentrates were given.

During the first days of plasma exchange the patient's platelet count increased and her neurological state improved (Fig. 1). On the sixth day, however, the platelet count decreased from  $34$  to  $6 \text{ GL}^{-1}$ , and LDH increased from  $235$  to  $763 \text{ U mL}^{-1}$  despite continuing plasma exchange. The patient's neurological state deteriorated, and she was intubated and mechanically ventilated to avoid aspiration. Because the patient had an underlying autoimmune disease that had probably triggered the thrombotic microangiopathy, extracorporeal immunoabsorption on anti-human immunoglobulin-Sepharose columns (Ig-Therasorb<sup>®</sup>) was initiated. It was carried out using a continuous extracor-

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**Fig. 1.** Treatment modalities, ADAMTS13 activity and inhibitor, platelet count, LDH, and vWF multimer pattern of a patient with TTP poorly responding to plasma exchange are shown. The lanes of the multimer analysis are assigned to the respective days of blood sampling. PE, plasma exchange; IAS, immunoabsorption; PRED, prednisone; InhU mL<sup>-1</sup>, inhibitor units per milliliter; PLT, platelet count; LDH, lactate dehydrogenase; NP, normal plasma; vWF-MM, multimer pattern of von Willebrand factor; bands above the dotted line indicate unusually large (UL) vWF-MM.

poreal system at a flow rate of 40 mL min<sup>-1</sup> as described previously [13]. The blood was anticoagulated with citrate dextrose and the plasma separated with a Fenwal Autopheresis-C system (Baxter, Unterschleißheim, Germany). The plasma was loaded alternately onto one of two columns filled with Sepharose-coupled with goat anti-human immunoglobulins (Ig-Therasorb<sup>®</sup>, Plasmaselect, Teterow, Germany), while the other column was regenerated. After immunoabsorption, the plasma was reinfused together with the separated blood cells. A total of 7000 mL of plasma was processed in one session lasting about 4 h.

Additionally, 500 mg of cyclophosphamide were given for immunosuppression. Daily plasma exchange and prednisone were continued. Four immunoabsorption sessions were carried out within 5 days. After the third immunoabsorption, the platelet count began to increase and LDH concentrations returned to normal. The patient's neurological state improved rapidly. She

could be extubated on day 13 and discharged without residues after seven further plasma exchanges on the day 24 after admission with a platelet count of 200 GL<sup>-1</sup>.

Prospective serial determinations of vWF-MM, ADAMTS13 activity and ADAMTS13 inhibitor were performed (Fig. 1). During the first days of intensive plasma exchange the inhibitor titer decreased from the initial 3.7 InhU mL<sup>-1</sup> to approximately 1 InhU mL<sup>-1</sup>. On day 6, ADAMTS13 inhibitor titer increased suddenly to 13 InhU mL<sup>-1</sup>, concomitant with an increase of UL vWF-MM and signs of accelerated formation of platelet microthrombi (LDH increase, drop of platelet count, neurological deterioration) (Fig. 1). The reason for this increase is unclear, but would explain the poor or prolonged response to plasma exchange of some patients with TTP. In their retrospective analysis of a patient with chronic relapsing TTP, Furlan *et al.* [14] described a similar phenomenon during a relapse treated with plasma exchange and plasma infusion.

The inhibitor titer decreased rapidly after initiation of immunoabsorption and immunosuppression, but ADAMTS13 activity was still below the detection limit. The UL vWF-MM disappeared during immunoabsorption (probably due to consumption during the exacerbation of the disease), but returned thereafter (although the inhibitor titer was very low) and were detectable until day 65. At this time the patient's platelet count, LDH, renal function and neurological state were normal, but ADAMTS13 activity was still below the detection limit due to a low titer inhibitor (below 0.5 InhU mL<sup>-1</sup>). This suggests that [1] the inhibitor completely suppresses ADAMTS13 activity, even at low titers; and [2] an additional triggering situation is necessary to induce platelet aggregation when UL vWF-MM are present. Therefore, a patient with TTP in remission may be at risk of relapse when ADAMTS13 activity is low and UL vWF-MM are present. In contrast, in cases of severe hereditary ADAMTS13 deficiency even very low ADAMTS13 levels (about 0.05 U mL<sup>-1</sup>) seem to protect from relapse [3]. On day 150, the inhibitor disappeared and ADAMTS13 activity increased to 0.3 U mL<sup>-1</sup>. By day 203, the ADAMTS13 activity was 0.62 U mL<sup>-1</sup> and no UL vWF-MM could be detected. vWF:Ag, vWF:CB and FVIII were within the upper normal range (1.5, 1.4 and 1.4 U mL<sup>-1</sup>, respectively).

Immunoabsorption is a useful method to effectively deplete the plasma of immunoglobulins, and it has been used successfully for short- and long-term treatment of several immunological disorders [13]. Our patient's good response suggests that different treatment strategies, similar to the therapy of autoimmune diseases (immunosuppression, immunoabsorption, high-dose immunoglobulin infusions) may be more effective than plasma exchange in patients with TTP and inhibitors to ADAMTS13. Before these strategies can be implemented, fast and reliable assays are required to measure ADAMTS13 activity and inhibitor titer as well as studies that prove their effectiveness.

In conclusion, the time course of clinical symptoms, and the changes in ADAMTS13 activity and its inhibitor observed in this patient during and after treatment show some remarkable features of TTP. The determination of ADAMTS13 activity and inhibitor, and the vWF multimer pattern is useful to diagnose

and monitor patients with TTP. These measurements can also be used to justify the inclusion of immunoabsorption or immunosuppression in a treatment protocol, and to identify patients at risk of relapse. Prospective studies are still necessary to verify the effectiveness of these new approaches.

### Acknowledgements

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## Autoimmune disease antigen U1 snRNP neutralizes heparin

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Dear Sir

The RNA-binding protein, U1 (small nuclear ribonucleoprotein-associated 68/70 kDa protein; U1-snRNP) is the major antigen of autoantibodies found in the serum of patients with mixed connective tissue disease and to a lesser extent in serum of patients with systemic lupus erythematosus [1]. A well-established symptom in individuals with autoimmune diseases of this type is an increased risk of thrombosis usually associated with the presence of antiphospholipid antibodies [2].

Because of the arginine-rich regions within snRNP 68/70 [3], we investigated whether the protein has an antiheparin activity

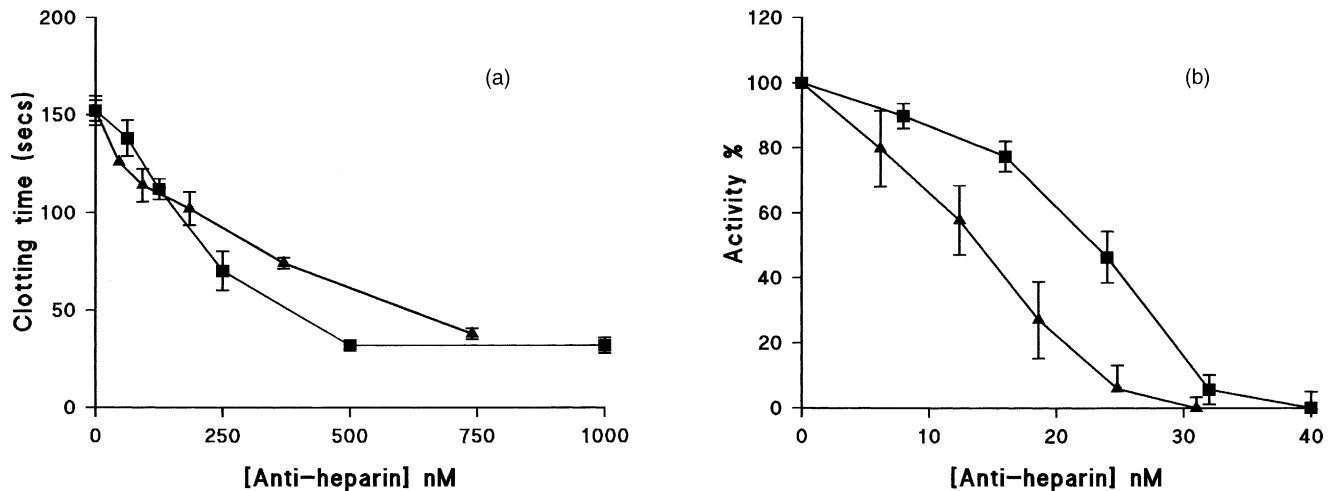
according to the hypothesis that abnormal exposure could also contribute to the increased risk of thrombosis by abrogating the normal functioning of the antithrombin pathway of anticoagulation (blocking the interaction of the serpin with heparan sulfate on the vessel wall). Indeed we have observed that U1-SnRNP has a potent antiheparin activity comparable to that of protamine when observed under two separate experimental conditions. Firstly, the clotting time of heparinized normal human plasma (1.25 IU mL<sup>-1</sup> of plasma, equivalent to around 500 nmol L<sup>-1</sup> heparin) was completely reversed by raising the concentration of snRNP 68/70 (obtained from Diarect AG, Freiburg, Germany) to 750 nmol L<sup>-1</sup> (Fig. 1a; a similar concentration of protamine was required). Secondly, the accelerated rate of inhibition of thrombin by antithrombin in the presence of heparin was reduced by U1-snRNP, as compared to that observed in the absence of heparin by U1-snRNP (Fig. 1b, IC<sub>50</sub> 13 nmol L<sup>-1</sup> compared to 22 nmol L<sup>-1</sup> for protamine).

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**Fig. 1.** (a) The APTT of heparinized human pooled plasma ( $1.25 \text{ IU}$  of unfractionated heparin  $\text{mL}^{-1}$ ) was determined in the presence of an increasing concentration of U1-snRNP ( $\blacktriangle$ - $\blacktriangle$ ) or protamine ( $\blacksquare$ - $\blacksquare$ ). Results are shown as the mean  $\pm$  SEM,  $n = 3$ . (b) The rate of inhibition of thrombin by antithrombin in the presence of heparin ( $10 \text{ nmol L}^{-1}$ , unfractionated heparin) was determined according to a modification of a previously described method [7] in the presence of an increasing concentration of U1-snRNP ( $\blacktriangle$ - $\blacktriangle$ ) or protamine ( $\blacksquare$ - $\blacksquare$ ). Results are calculated as the percentage of heparin-dependent activity and shown as the mean  $\pm$  SEM,  $n = 3$ .

Recently it has been shown that proteins of the nuclear matrix of which U1-snRNP is one example segregate into discrete structures following transcriptional arrest [4]. These structures are observed to move from the nucleus onto the surface of cells during apoptosis and necrosis. We have also reported similar heparin binding structures forming within the nucleus of apoptotic cells, which are subsequently transferred onto the surface or released, into the medium [5,6]. The exposure of U1 snRNP through this mechanism may initiate the generation of antibodies and may also, we propose, enhance the risk of thrombosis by inhibition of the antithrombin pathway of anticoagulation.

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## Multi-therapeutic approach to manage delivery in an alloimmunized patient with type 3 von Willebrand disease

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Dear Sir,

Type 3 von Willebrand disease (VWD) is characterized by a virtually complete deficiency of von Willebrand factor (VWF)

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associated with markedly reduced factor VIII (FVIII) procoagulant activity (FVIII:C) [1]. This combined deficiency of both VWF and FVIII results in severe mucosal bleeding as well as hemophilia-like symptoms, requiring multiple transfusions and leading to development of alloantibodies to VWF in about 10% of patients [2]. The presence of these alloantibodies not only renders replacement therapy with VWF concentrates ineffective, but may even be dangerous, because of severe allergic reactions [3]. Recombinant activated factor VII (rFVIIa) has

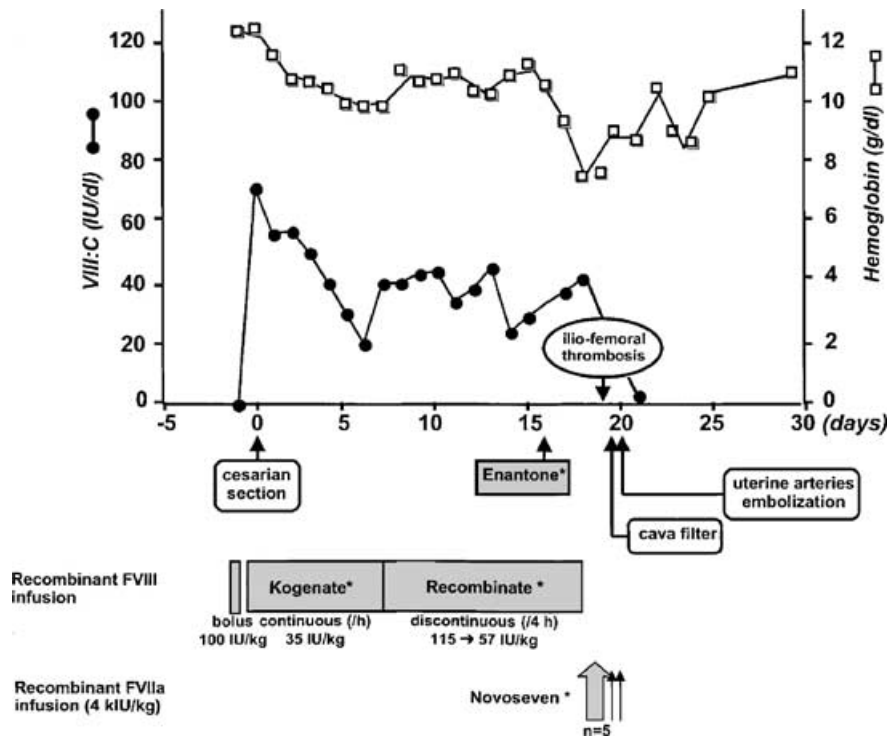
been classically proposed for the treatment of patients with severe hemorrhagic diseases with alloantibodies, mainly hemophilia and Glanzmann's thrombasthenia [4]. Up to now, few reports on the use of rFVIIa in alloimmunized type 3 VWD patients have been published [5,6]. Alternatively, recombinant factor VIII (rFVIII) has been successfully used in a type 3 VWD woman with precipitating alloantibodies to VWF during abdominal surgery [3,7]. However, none of the latter treatments has ever been experienced in obstetrical situations. We here report, for the first time, the successful management of cesarean section and postpartum period using the sequential combination of recombinant FVIII and FVIIa in an alloimmunized type 3 VWD patient.

This patient was a 29-year-old Algerian woman with a life-long bleeding history from puberty, requiring multiple transfusions. Familial inquiry revealed parental consanguinity (first cousins) and the death, related to severe bleeding, of two sisters and two brothers among seven siblings. At the time of her first visit to France, FVIII coagulant activity (FVIII:C), VWF antigen (VWF:Ag) and VWF ristocetin cofactor activity (VWF:RCo) levels were undetectable in plasma and molecular analysis showed a total deletion of VWF gene. Because of prolonged menorrhagia leading to severe anemia, she received VWF/FVIII concentrates (Innobrand<sup>®</sup>; LFB, Les Ulis, France). This transfusion was ineffective and complicated by lumbar pains and chills. Laboratory analysis using classical mixing studies demonstrated the presence of an inhibitor to VWF:RCo (titer of 16 Bethesda Units [BU] per mL) and also to FVIII:C (titer of 4 BU mL<sup>-1</sup>). The inhibitor was identified as an antibody of the IgG class and induced, as previously described, a nonspecific but steric hindrance-linked inhibition of FVIII [8].

A few months later, the patient became pregnant and was referred to our center for obstetrical follow-up and hematological management (Fig. 1). At day 0, just prior to the cesarean section (performed with a tubal ligation after informed and signed consent), a central venous line was inserted after a starting bolus of 100 IU kg<sup>-1</sup> of recombinant FVIII (Kogenate<sup>®</sup>; Bayer, Puteaux, France) immediately followed by a continuous infusion of 35 IU kg<sup>-1</sup> h<sup>-1</sup>. This treatment resulted in a rise of FVIII:C levels up to 60 IU dL<sup>-1</sup>, allowing the cesarean section without any bleeding, and the birth of a healthy full-term boy whose VWF phenotype in venous blood at day 1 of life was as follows: VWF:Ag: 94 IU dL<sup>-1</sup>, VWF:RCo: 93 IU dL<sup>-1</sup>, FVIII:C: 107 IU dL<sup>-1</sup>. The same dose regimen was effective in obtaining FVIII:C levels close to 50 IU dL<sup>-1</sup>, and elicited no side-effects until day 3. At this time, the patient developed cutaneous urticaria, which was treated with antihistamine drugs and corticosteroids. Despite this treatment, the allergy persisted and at day 7, Kogenate<sup>®</sup> was switched to Recombinate<sup>®</sup> (Baxter, France). In addition, as the recovery of FVIII:C levels decreased with levels close to 20 IU dL<sup>-1</sup>, we decided to start discontinuous infusions of 115 IU kg<sup>-1</sup> per 4 h, leading to a 40 IU dL<sup>-1</sup> peak of VIII:C (10 min after infusion) and a 10 IU dL<sup>-1</sup> residual FVIII:C (just before the following infusion). This treatment improved both the allergic reaction and FVIII recovery. Moreover, the absence of gynecological

bleeding allowed the progressive decrease of Recombinate<sup>®</sup> (from 100 IU 4 h<sup>-1</sup> at day 9 to 57 IU 4 h<sup>-1</sup> at day 14). At day 15, treatment by Recombinate<sup>®</sup> was tentatively interrupted, but the onset of gynecological bleeding 12 h later led us to reintroduce Recombinate<sup>®</sup> infusions together with oral methylergometrin, without any clinical improvement. As the uterine bleeding was thought to be related to an early resumption of menstruation, it was decided to perform a subcutaneous injection of gonadotrophin-releasing hormone agonist (Enantone<sup>®</sup>) at day 16 [9]. Unfortunately, 36 h later, despite the addition of aprotinin, gynecological hemorrhage dramatically worsened, with the emission of large clots. This persistent uterine bleeding could be partially explained by a 'flare-up' effect of Enantone<sup>®</sup> [9] and forced us to stop both Recombinate<sup>®</sup> and aprotinin and to begin a treatment by recombinant FVIIa (Novoseven<sup>®</sup>; Novo Nordisk, Denmark), as previously used in alloimmunized type 3 VWD patients by Ciavarella *et al.* [5]. At day 18, an infusion of 4 kIU Novoseven\* kg<sup>-1</sup> 4 h<sup>-1</sup> was started through a femoral central line. Clinical improvement was obvious already after the second infusion of Novoseven<sup>®</sup> with a sharp decrease of uterine bleeding. However, at day 19, after the fifth infusion of Novoseven<sup>®</sup>, biological follow-up revealed a five-fold increase of D-dimer levels leading to the discovery of an ilio-femoral venous thrombosis close to the femoral central line. Thus, NovoSeven<sup>®</sup> had to be stopped and a pulmonary embolism was excluded by tomodesitometry. A few hours later, uterine bleeding dramatically reappeared, requiring multiple red cell transfusions. This dramatic therapeutic deadlock led the medical staff to place a cava filter as a first step and to embolize the uterine arteries as a second step, using in both cases a single NovoSeven infusion (4 kIU kg<sup>-1</sup>). The embolization of the uterine artery was remarkably successful on bleeding, with no additional requirement for erythrocyte transfusions nor any hemostatic drugs. Interestingly, ultrasonography performed 24 h after uterine artery embolization demonstrated the unexpected complete dissolution of the iliofemoral thrombus. In less than 48 h, uterine bleeding definitively stopped allowing the patient's discharge 1 week later.

The retrospective analysis of this complex case led us to discuss several critical points. In addition to the severity of the congenital disease, the presence of a high titer of alloantibodies to VWF constituted a major risk of bleeding, which had to be considered for both the baby and his mother. Regarding the baby, a cesarean section was decided on, because the late passing of IgG alloantibodies through the placenta could have accounted for a transitory acquired VWD. Regarding the mother, management of delivery was extensively discussed with a multidisciplinary staff. The removal of alloantibodies by immunoadsorption was first considered [10], but this therapeutic option was finally discarded for two main reasons: insufficient experience in the obstetrical context and the allergic history of our patient, requiring a total removal of alloantibodies, which could not be guaranteed by the immuno-adsorption. In cases of treatment with VWF concentrates, even minimal residual alloantibody levels have been demonstrated to be capable of inducing the formation of immune complexes that



**Fig. 1.** Clinical, biological and therapeutic follow-up from cesarean section to about one-month postpartum of the alloimmunized type 3 von Willebrand disease patient. The evolution of both hemoglobin level (Hb) and factor VIII clotting activity (FVIII:C) are shown on the graph. Cesarean section was performed at day 0. The drugs used for the therapeutic management are indicated in gray and mainly include recombinant factor VIII, recombinant activated factor VII and one Enantone<sup>®</sup> injection. Ilio-femoral thrombosis occurred at day 19 leading to placement of a cava filter and to embolization of the uterine arteries.

activate the complement system responsible for anaphylactic reactions [3,11]. The use of recombinant FVIIa was also discussed but we decided to reserve this treatment as a last resort in reason of the well-known thrombotic risk associated with pregnancy and postpartum. Thus, in the current case, the lack of *in vitro* inhibitory effect of the alloantibody against FVIII:C prompted us to choose rFVIII (completely devoid of VWF). Although rFVIII infusion has never been used in type 3 vWD pregnant patients, it has been successfully experienced in a type 3 vWD patient with alloantibodies during surgery [7]. We therefore decided to use for the first time rFVIII for the management of delivery and postpartum in this type 3 alloimmunized VWD patient. In this patient, the efficacy of recombinant FVIII alone was clearly demonstrated during the cesarean section, delivery and the following 2 weeks, as previously reported in an alloimmunized type 3 VWD patient during abdominal surgery [7]. Nevertheless, the predictable resumption of menstruation, which was quite early in our patient, was dramatically hemorrhagic and was only overcome by recombinant FVIIa. However, the onset of thrombosis must lead to cautious use of recombinant FVIIa in the peripartum period.

The current observation emphasizes the complex therapeutic management of alloimmunized type 3 VWD patients in any surgical and especially obstetrical situation. It also clearly demonstrates that pregnancy in such patients remains a risky challenge.

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# Serum concentration of $\alpha$ 1-proteinase inhibitor and $\alpha$ 2-macroglobulin correlates with late lumen loss following coronary stent implantation

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Dear Sir,

Restenosis is a well-appreciated limitation of coronary intervention. Fibrin deposition becomes histologically detectable around stent struts soon after implantation [1]. Smooth muscle cell attachment and migration into fibrin is considered an important initiating event in stent restenosis [2,3]. Integrins participate in this process [4,5]. Furthermore, plasma proteinase inhibitors such as  $\alpha$ 1-proteinase inhibitor ( $\alpha$ 1PI),  $\alpha$ 1-antichymotrypsin ( $\alpha$ 1ACT), and  $\alpha$ 2-macroglobulin ( $\alpha$ 2M) are essential for smooth muscle cells to attach, migrate and survive in fibrin [6,7]. These *in vitro* data suggested that plasma proteinase inhibitors may participate in the mechanism of stent restenosis.

In 88 elective patients, 107 *de novo* lesions were treated with stent implantation. Serum concentrations of proteinase inhibitors were measured by nephelometry. Reagent kits for  $\alpha$ 1PI and  $\alpha$ 2M were obtained from Dade Behring Marburg (Germany). The kit for  $\alpha$ 1ACT was obtained from Hoechst Japan (Tokyo, Japan). Follow-up angiography was performed at  $170 \pm 38$  days. Quantitative coronary angiography was performed using QCA-CMS (MEDIS, Leiden, the Netherlands) by an expert cardiologist who was kept unaware of clinical or blood test data.

The average age of the patients was  $64 \pm 9$  years; 85% were male; 13% of patients had a history of bypass surgery; and 6% were hemodialysis patients. Diabetes was found in 34%. Cilostazol was prescribed for 44% of patients while probucol was prescribed only for 5%. Serum concentrations of the proteinase inhibitors at 24 h after stent implantation were within the normal range. Of all the lesions, 46% were located in left anterior descending arteries; type B2 and C accounted for more than 60% of lesions. The lesion length was  $11.7 \pm 5.0$  mm, but stent length was  $19.5 \pm 8.1$  mm. Atherectomy was performed prior to stenting in 16 lesions, rotational atherectomy in 15 and directional atherectomy in one. Stents were selected at the discretion of individual operators. The minimum luminal diameter was  $1.11 \pm 0.49$  mm before the procedure,  $2.60 \pm 0.52$  mm after stent implantation, and  $1.97 \pm 0.60$  mm at 6 months follow-up. The reference diameter was  $2.70 \pm 0.70$  mm before the procedure,

$3.00 \pm 0.47$  mm after stent implantation, and  $2.80 \pm 0.51$  mm at 6 months follow-up. Average acute gain was  $1.48 \pm 0.57$  mm, average late loss was  $0.63 \pm 0.44$  mm, and average loss index was  $0.44 \pm 0.30$ . Binary restenosis determined to be more than 50% by QCA was observed in 14 lesions (13%). Target lesion revascularization (TLR) was performed in six lesions (6%) based on clinical recurrence of angina due to restenosis. The serum concentrations of  $\alpha$ 1PI,  $\alpha$ 1ACT and  $\alpha$ 2M were compared according to TLR. Serum concentrations of  $\alpha$ 1PI and  $\alpha$ 2M were significantly higher with TLR than that without TLR, but no difference was found in  $\alpha$ 1ACT [ $\alpha$ 1PI,  $2.14$  ( $2.03$ – $2.51$ ) vs.  $1.94$  ( $1.80$ – $2.12$ )  $\times 10^{-5}$  mol L $^{-1}$ ,  $P = 0.0312$ ;  $\alpha$ 2M,  $0.36$  ( $0.23$ – $0.44$ ) vs.  $0.18$  ( $0.16$ – $0.23$ )  $\times 10^{-5}$  mol L $^{-1}$ ,  $P = 0.0016$ , respectively]. Scatter plots are shown in Fig. 1. Loss index showed linear correlation with  $\alpha$ 1PI ( $R = 0.2501$ ,  $P = 0.0094$ ) and with  $\alpha$ 2M ( $R = 0.2154$ ,  $P = 0.0259$ ), but no correlation was observed with  $\alpha$ 1ACT. As shown in Table 1, we prepared a set of candidate variables from previous known factors that predict restenosis: diabetes, left anterior descending lesion, type C lesion, lesion length, reference diameter, prior bypass surgery, calcification and hemodialysis. Stepwise regression analysis selected reference diameter, diabetes and hemodialysis as significant predictors. Using these three variables as a basal model,  $\alpha$ 1PI,  $\alpha$ 1ACT, and  $\alpha$ 2M were additionally included in the model. This second model found  $\alpha$ 1PI and  $\alpha$ 2M as significant predictors of loss index, independent of the three variables in the basal model.

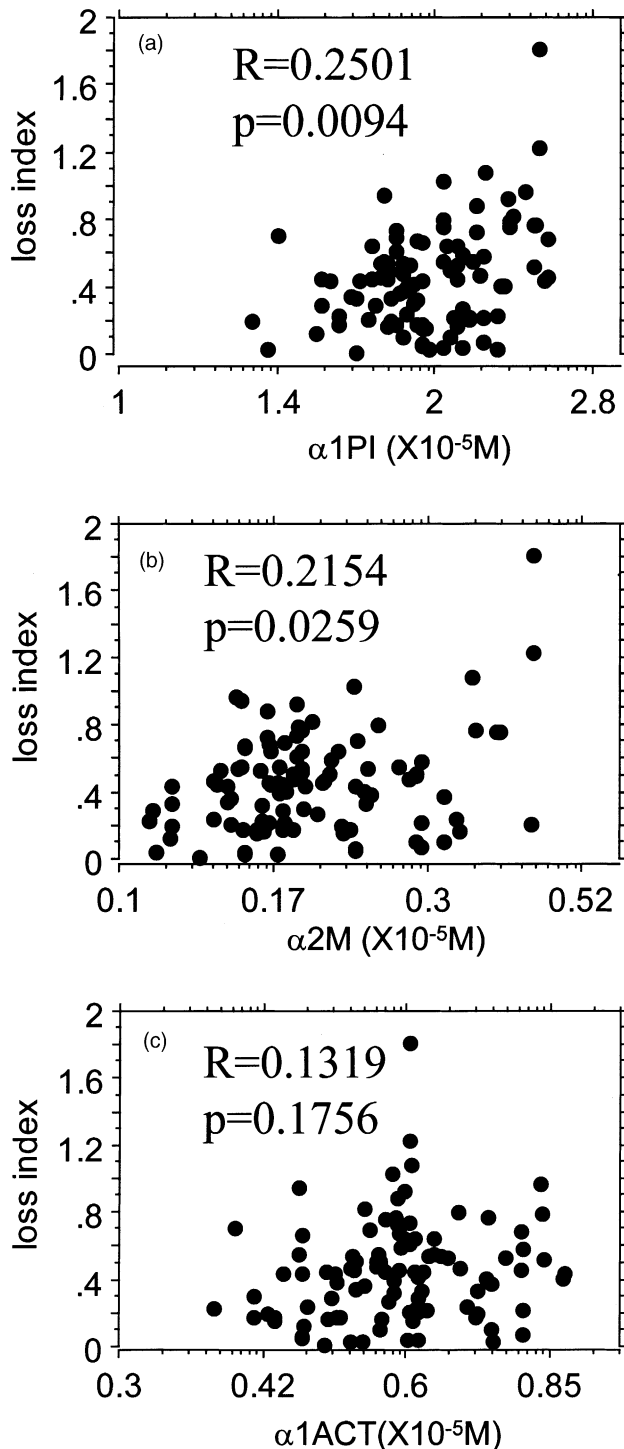
Clinical factors to predict restenosis have been reported, however, many serum factors failed to predict restenosis such as C-reactive protein, tumor necrosis factor- $\alpha$ , interleukin-6 or lipoprotein(a) [8]. Although plasma fibrinogen may predict restenosis [9], no association was found in another report [8]. The weak but significant correlation between plasma proteinase inhibitors and late loss may imply that  $\alpha$ 1PI and  $\alpha$ 2M are involved in the complex mechanism of restenosis. Proteinase inhibitors are the third most abundant proteins in plasma, next to albumin and globulin. Since  $\alpha$ 2M is a highly conserved protein found even in invertebrates, it may be essential to animal survival. An obvious question concerns the target enzymes of  $\alpha$ 1PI and  $\alpha$ 2M.  $\alpha$ 1PI specifically inhibits all serine proteinases, while  $\alpha$ 2M is a universal inhibitor of proteinases. Previous *in vitro* data suggest that the major target enzyme(s) is(are) one or more serine proteinases. Unfortunately, the key serine proteinases produced by vascular smooth muscle cells

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**Fig. 1.** Scatter plots of lumen loss index and concentrations of three proteinase inhibitors. (a)  $\alpha$ 1-proteinase inhibitor ( $\alpha$ 1PI); (b)  $\alpha$ 2-macroglobulin ( $\alpha$ 2M); and (c)  $\alpha$ 1-antichymotrypsin ( $\alpha$ 1ACT).

have not been identified. Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) have received much attention with respect to the pathophysiology of atherosclerosis. However, MMPs are not central to

**Table 1** Results of stepwise regression analysis

Parameter	Partial R [2]	Model R [2]	P-value
<b>Model 1*</b>			
Reference diameter	0.1041	0.1041	0.0007
Diabetes mellitus	0.0247	0.1288	0.0887
Hemodialysis	0.0181	0.1469	0.1421
<b>Model 2†</b>			
Basal model	0.1469	0.1469	0.0009
$\alpha$ 1PI	0.0930	0.2399	0.0006
$\alpha$ 2M	0.0413	0.2812	0.0178

\*Model 1 includes the following variables as candidate predictors for loss index: diabetes, left anterior descending lesion, AHA/ACC type C lesion, lesion length, reference diameter, prior bypass surgery, calcification and hemodialysis. †Model 2 tests the additional explanatory power of  $\alpha$ 1PI,  $\alpha$ 2M and  $\alpha$ 1ACT over model 1.

investigations because only  $\alpha$ 2M would correlate with the loss index if MMPs were the target.

In conclusion,  $\alpha$ 1PI and  $\alpha$ 2M have correlation with late loss following stent implantation.  $\alpha$ 1PI,  $\alpha$ 2M and their unidentified serine proteinase targets are likely to be involved in the pathogenesis of restenosis.

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# Protein Z, a vitamin K-dependent protein in patients with renal failure

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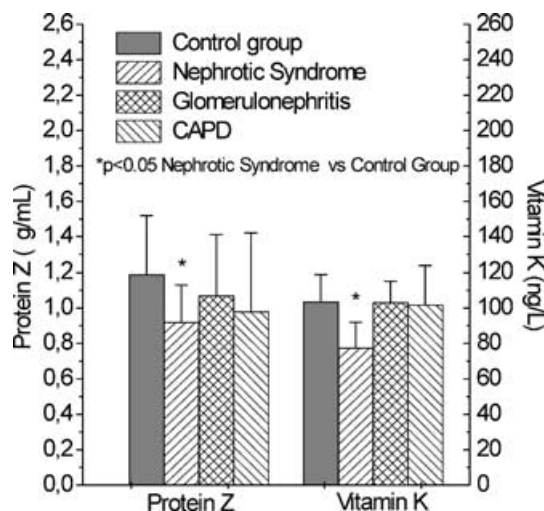
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Dear Sir,

Disturbances in hemostasis are common complications of kidney diseases. Both bleeding diathesis and thromboembolism may complicate the course of chronic uremia [1]. The pathogenesis of uremic bleeding and prothrombotic state in dialyzed patients is multifactorial [1,2]. Results of both *in vitro* and *in vivo* studies suggest that protein Z, a vitamin K-dependent plasma glycoprotein, plays an important role in dampening coagulation [3]. In the presence of phospholipid vesicles and calcium ions, protein Z serves as a cofactor for the inhibition of activated factor X (FX) by protein Z-dependent protease inhibitor. Deficiency of protein Z might therefore result in a prothrombotic state [3,4]. As far as we know, there is limited data about protein Z in kidney diseases. Usalan *et al.* [5] have reported high plasma levels of protein Z in hemodialyzed patients. The aim of our work was to examine soluble plasma protein Z and vitamin K concentrations in patients with kidney diseases.

The study was performed on 22 patients with chronic renal insufficiency (10 patients with nephrotic syndrome, 12 patients with glomerulonephritis without nephrotic syndrome), 22 peritoneally dialyzed (continuous ambulatory peritoneal dialysis; CAPD), and 27 age–sex-matched healthy controls. Inclusion criteria were no diabetes mellitus, no inflammation (C-reactive protein within normal range) and no liver dysfunction (prothrombin time, alanine aminotransferase within normal range). We evaluated protein Z with commercially available kit (Protein Z, Diagnostica, Stago, France). Vitamin K concentration was analyzed by HPLC as described previously [6]. ANOVA and linear regression was used in statistical analysis employing computer software STATISTICA 5.0. Data are presented as means  $\pm$  SD in Fig. 1.

Protein Z was highest in CAPD patients when compared with any other group. In nephrotic syndrome, protein Z was significantly lower when compared with the healthy volunteers, but it did not differ significantly between two groups of patients with chronic renal failure (both with and without nephrotic syndrome). Protein Z correlated negatively with fibrinogen in CAPD ( $r = -0.68$ ,  $P < 0.05$ ) and nephrotic patients ( $r = -0.79$ ,  $P < 0.05$ ). Vitamin K was significantly lower in nephrotic syndrome when compared with non-nephrotic patients



**Fig. 1.** Protein Z and vitamin K concentrations in patients with nephrotic syndrome (NS), glomerulonephritis (GN), on continuous ambulatory peritoneal dialyses (CAPD) and in the healthy volunteers (CG).

( $P = 0.03$ ). In nephrotic patients, vitamin K concentration was low and almost reached statistical significance when compared with the patients maintained on CAPD ( $P = 0.05$ ). Concentrations of vitamin K were not significantly related to either age or gender in patients on renal replacement therapy (CAPD), but in CRF vitamin K correlated positively with age ( $r = 0.73$ ,  $P = 0.017$ ).

Protein Z is highly homologous to FVII, FIX, FX, proteins C and S, and prothrombin [3], which are lost in urine and their plasma concentrations are lowered in nephrotic syndrome. In our study, we found that in patients with nephrotic syndrome, protein Z concentration was significantly lower than in the healthy volunteers and in CAPD patients and it was significantly higher than in the control group. To our knowledge, this is the first report concerning protein Z levels and vitamin K status in patients with nephrotic syndrome. CAPD appears to mimic the metabolic abnormalities that account for the hypercoagulability in the nephrotic syndrome [7]. In our previous study, we observed a hypercoagulable state in CAPD patients when compared with either hemodialyzed patients or healthy volunteers [8]. It has been suggested that due to a peritoneal ultrafiltration, CAPD patients usually exhibit a reduced plasma volume and hemoconcentration. Significant protein losses through the peritoneum may be counterbalanced by the possible increase in protein synthesis in CAPD as a result of a non-

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specific stimulation of the liver to protein production due to a lowered oncotic pressure. Thus, protein Z synthesis in CAPD patients may be increased via this mechanism, however, there was only negative correlation between protein Z and fibrinogen in nephrotic syndrome and CAPD. It is known that vitamin K is needed for the carboxylation of osteocalcin, matrix Gla protein in bone, prothrombin, and proteins S and Z [9]. However, the specific function of these proteins is still unknown. In our study, none of the patients investigated was on oral anticoagulants and all of them had international normalized ratio within normal ranges. As protein Z is one of the vitamin K-dependent proteins, we attempted to find any correlations between vitamin K status and protein Z.

In conclusion, the most interesting finding of our study is the observation that Z concentration is low in nephrotic syndrome protein. It may be due to the urinary losses and/or vitamin K deficiency. Protein Z deficiency might contribute to the enhanced risk of thromboembolic complications in nephrotic syndrome.

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# Aspirin resistance is not a common biochemical phenotype explained by unblocked cyclooxygenase-1 activity

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Dear Sir,

The term aspirin resistance has been used to describe patients who, despite being on therapeutic doses of aspirin, experience thrombotic vascular events. Aspirin resistance can also be a biochemical phenotype based on an inadequate platelet inhibition response. Previous studies involving platelet reactivity or aggregation tests have estimated that between 8 and 45% of patients with ischemic stroke or cardiovascular disease are aspirin resistant [1–6]. Recently, it was shown that urinary concentrations of 11-dehydrothromboxane B<sub>2</sub>, a stable marker of thromboxane A<sub>2</sub> (TXA<sub>2</sub>) production, can be used to predict the risk of future cardiovascular events [7]. Although the causative factor of aspirin resistance has not been elucidated, the observed aspirin-resistant biosynthesis of thromboxane could imply that the platelets are incompletely blocked.

The aspirin-sensitive cyclooxygenase pathway in platelets is initiated when arachidonic acid is released from membrane phospholipids. TXA<sub>2</sub>, an important activator of platelets, is produced from arachidonic acid by sequential action of the enzymes cyclooxygenase-1 (Cox-1) and thromboxane synthetase.

Aspirin blocks the platelet production of TXA<sub>2</sub> by irreversible acetylation of serine 529 of the Cox-1 molecule. The serine residue is not directly involved in the cyclooxygenase activity but its acetylation leads to blocked access of arachidonic acid in the active site [8]. We have now investigated 122 patients and healthy individuals in order to see if aspirin resistance, defined as arachidonic-induced whole blood aggregation and/or ATP release, is a common finding after treatment with low-dose aspirin.

Blood was collected in tubes containing 3.8% citrate from 41 healthy individuals (21 female, 19 men; mean age 40 years; range 23–57 years), 34 patients with ischemic stroke (12 female, 22 male; mean age 72 years; range 50–89 years) and 47 patients with myocardial infarction (9 female, 38 male; mean age 70 years; range 40–86 years). The healthy individuals were analyzed before and after taking aspirin (one tablet of 75 mg aspirin 24 and 1 h before blood sampling). The two groups of patients were all taking 75–160 mg day<sup>-1</sup> of aspirin for various lengths of time (range 1–310 months). Whole blood aggregation using impedance technique [9,10] was performed using a Chronolog 550 Lumi-Aggregometer (Chronolog Corp., Haverstown, USA). Citrated blood was analyzed after dilution 1 : 1 with 0.9% NaCl with 0.5 mmol L<sup>-1</sup> arachidonic acid as agonist. Aggregation recorded as impedance response (in ohms) was measured 6 min after addition of agonist. Release of ATP (in nanomoles) from dense granules

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was detected with luminescence from an added luciferin–luciferase reagent.

The group of healthy individuals showed a mean aggregation of  $26.0 \pm 9.0\%$  and ATP release of  $0.49 \pm 0.33$  nmol. The responses to arachidonic acid are similar to what have been reported by others [10]. The same group of healthy individuals was analyzed after intake of low-dose aspirin and none of the subjects showed any sign of platelet aggregation or ATP release with arachidonic acid as agonist. A completely inhibited response was also observed for the patients with myocardial infarction and, with the exception of a single patient, in the stroke group. The deviating patient in the stroke group showed a weak aggregation response together with an ATP release within the normal range for individuals not taking aspirin. This patient was tested at a later occasion and was then found to be completely blocked by aspirin. Thus, non-compliance cannot be ruled out in this case.

Taken together, aspirin resistance, due to an unblocked Cox-1, appears to be rare in this group of patients and in healthy control subjects. We found only a single patient out of 122 investigated (0.8%) with reactive platelets after intake of low-dose aspirin. This is by far less than the previous reports of 8–45% of aspirin resistance in patients with ischemic stroke or cardiovascular disease [1–6]. In these studies, the definition of aspirin resistance is based on various biochemical definitions based on the platelet reactivity test, optical aggregation with various agonists or using an *in vitro* bleeding time test. However, none of these studies have revealed the mechanism(s) behind aspirin resistance. With our approach, using arachidonic acid-induced whole blood platelet aggregation, we aimed to investigate if the phenomenon of aspirin resistance can be a biochemical phenotype derived from an aspirin insensitive Cox-1 molecule. It has been hypothesized that variants of the Cox-1

gene could result in an aspirin resistant Cox-1 molecule leading to thromboxane generation [7]. However, such a phenotype cannot be identified in this setting. With only a single exception, all samples showed a completely blocked Cox-1 activity indicating that low-dose aspirin is efficient of blocking the Cox-1 activity.

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## Only excessive III concentrations release prostacyclin in human dermal microvascular cells

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Dear Sir,

Antithrombin (AT) is an important inhibitor of serine proteases that are part of the coagulation cascade. Recent interest has

focused on the anti-inflammatory properties of AT. One mechanism suggested for AT's anti-inflammatory effect is the release of prostacyclin (PGI<sub>2</sub>). AT has been shown to induce the release of PGI<sub>2</sub> in several *in vitro* and *in vivo* studies. It stimulated PGI<sub>2</sub> production by cultured bovine aortic endothelial cells which was inhibited by anti-AT antiserum and heparin [1]. AT increased the release of PGI<sub>2</sub> in human umbilical vein endothelial cells. This effect was abolished by addition of heparin and heparinase [2]. The intravenous administration of AT in rats significantly increased the plasma levels of

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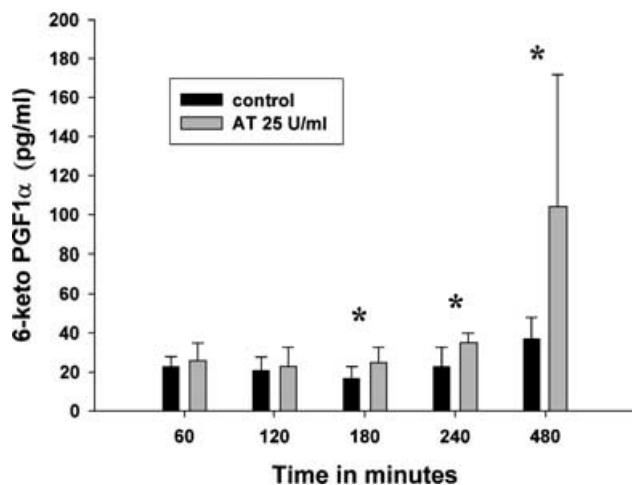
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PGI<sub>2</sub>. Treatment with heparin and AT had no effect on the release of PGI<sub>2</sub> [3]. These observations suggested that AT promotes the release of PGI<sub>2</sub> by interacting with endothelial heparin-like glycosaminoglycans. However, recent results from Uchiba and Okajima [4] did not support these previous findings; incubation of cultured human umbilical vein endothelial cells (HUVECs) and cultured bovine aortic endothelial cells (BAECs) did not induce PGI<sub>2</sub> release. Thus it seems likely that the release of PGI<sub>2</sub> by AT is not common to all endothelial cells and the exact mechanism, therefore, is still unclear. At present, AT-induced PGI<sub>2</sub> formation and release has not been studied in endothelial microvascular cells. Thus, we incubated primary cultures of human dermal microvascular cells (HDMEC), which are derived from human foreskins (for details see [5]) with AT (1, 5, 10 and 25 U mL<sup>-1</sup> Kybernin P<sup>®</sup>) for 60, 120, 180, 240 and 480 min, both without as well as with indomethacin (10 μmol L<sup>-1</sup>). PGI<sub>2</sub> release was estimated by measuring its stable analog 6-keto-prostaglandin-F<sub>1α</sub> (6-keto-PGF<sub>1α</sub>) with an enzyme-immunoassay (EIA) system (Biotrack, Amersham, Bucks, UK)

Incubations of HDMEC with AT at 1, 5 and 10 U mL<sup>-1</sup> had no effect on the release of 6-keto-PGF<sub>1α</sub> in the culture supernatant. Compared with untreated controls, incubation of HDMEC with 25 U mL<sup>-1</sup> AT resulted in an increase of 6-keto-PGF<sub>1α</sub> after 180 min (25.5 ± 8.3 pg mL<sup>-1</sup> vs. 17.1 ± 6.0 pg mL<sup>-1</sup>; *P* = 0.006; *n* = 6), after 240 min (35.6 ± 12.6 pg mL<sup>-1</sup> vs. 23.5 ± 10.1 pg mL<sup>-1</sup>; *P* = 0.003; *n* = 6) and after 480 min (104.8 ± 68.7 pg mL<sup>-1</sup> vs. 37.7 ± 11.4 pg mL<sup>-1</sup>; *P* = 0.008; *n* = 6) (Fig. 1). Coincubation of 25 U mL<sup>-1</sup> AT with indomethacin completely abolished the release of 6-keto-PGF<sub>1α</sub>.

AT has been suggested to act as an anti-inflammatory agent. In several studies, administration of high concentration of AT improved organ failure and mortality in animals exposed to lipopolysaccharides [6,7]. Clinical studies showed a beneficial effect of high concentrations of AT on the incidence of multiple organ failure in patients with severe sepsis [8,9]. However, a



**Fig. 1.** Time-dependent release of PGI<sub>2</sub> in controls and in AT-treated group (25 U mL<sup>-1</sup>). Data are expressed as mean ± SD. \**P* < 0.05 vs. control as compared with corresponding times (Student's *t*-test).

recent study with septic patients maintaining high plasma concentration of AT (150%) by substitution of AT did not result in a reduction of 28-day mortality [10]. One mechanism suggested for the anti-inflammatory effect of AT is the release of prostacyclin (PGI<sub>2</sub>). AT (250 U kg<sup>-1</sup>) reduced coagulation abnormalities and pulmonary vascular injury in endotoxin-exposed rats, and this was accompanied by elevated plasma levels of PGI<sub>2</sub>. These effects were completely inhibited by indomethacin [11]. In a recent study, AT had beneficial effects on ischemia/reperfusion injury of the liver by releasing PGI<sub>2</sub> [12]. In contrast with others [1,2], we cannot demonstrate an increase in PGI<sub>2</sub> by AT treatment of cultured cells in concentrations which are of clinical relevance. Only by applying excessive doses of AT (25 U mL<sup>-1</sup>) could a significant increase of PGI<sub>2</sub> in the culture supernatant be measured. The reasons for these differences are not clear at present. It seems that there are cell-type specific mechanisms involved in AT-induced PGI<sub>2</sub> formation. HDMEC are microvascular endothelial cells derived from human foreskins and are different with regard to cell morphology, proliferation and stimulation by proinflammatory agents as compared with other endothelial cells, e.g. HUVEC, BAEC [13]. We used early passages (3–6) in order to avoid later dedifferentiation. Whether the release of PGI<sub>2</sub> is in fact the crucial mechanism for some of the anti-inflammatory properties of AT seems to be questionable. Dschietzig *et al.* [14] demonstrated that AT treatment failed to increase PGI<sub>2</sub> levels in isolated endotoxin-exposed rat lungs, but augmented release of endothelin 1. Thus, it is possible that mechanisms other than the release of PGI<sub>2</sub> are responsible for the anti-inflammatory effects of AT.

In conclusion, this study demonstrates that only excessive concentrations of AT release PGI<sub>2</sub> in human dermal microvascular cells. Clinically relevant concentrations had no impact. In synopsis with recent data [4], it seems questionable whether the release of PGI<sub>2</sub> by AT is a uniform mechanism in the vascular system.

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## A procyanidin extract prolongs bleeding time but does not prevent thrombosis in rats

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Red wine and derived products have been tested for their capacity to interfere with molecular and cellular mechanisms relevant to the pathogenesis of thrombosis and vascular occlusive disease [1]. Polyphenolic flavonoids present in wine, vegetables, fruits and tea are known for their ability to inhibit platelet aggregation and polymorphonuclear leukocyte function *in vivo*, both in humans and in animal models [2].

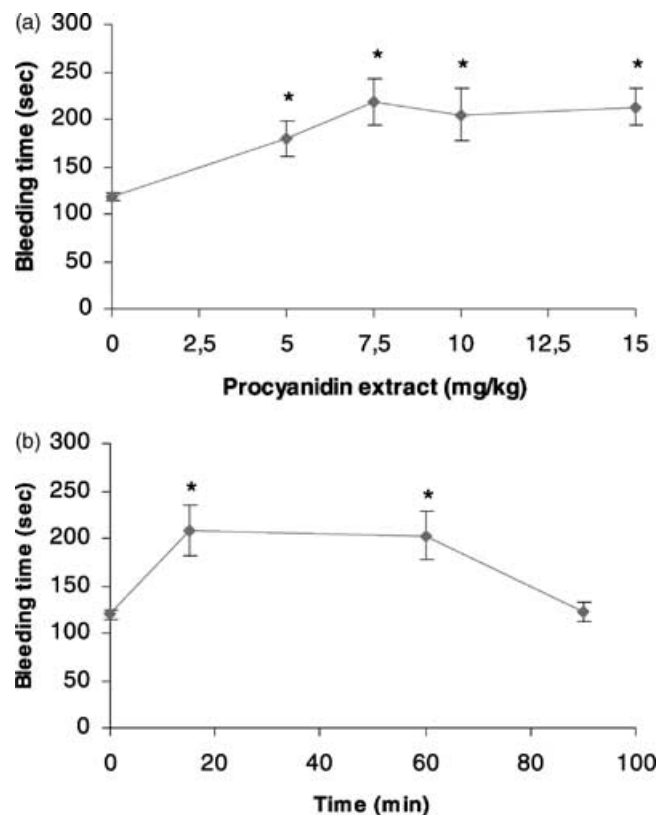
In this study the effects of a procyanidin extract from seed of *Vitis vinifera* (Leucoselect™, Indena, Milan, Italy) [3,4] were studied on template bleeding time (BT), platelet adhesion to fibrillar collagen, and venous thrombosis in rats [5] after both intravenous (i.v.) injection and long-term oral administration with the diet.

The compound, dissolved in isotonic saline at pH 7.3, and administered i.v. at a dose range between 5 and 15 mg kg<sup>-1</sup>, induced a significant prolongation of BT in a dose and time-dependent manner. A statistically significant difference was observed after 5 mg kg<sup>-1</sup> injection of the procyanidin extract and reached a plateau at 7.5 mg kg<sup>-1</sup> (Fig. 1a) and 15 min after i.v. injection (Fig. 1b). An increase in BT was still measurable after 60 min but disappeared after 90 min.

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**Fig. 1.** Effects of a procyanidin extract on bleeding time in rats: (a) dose-response; (b) time-course at 75 mg kg<sup>-1</sup> (means ± SE, n = 15; \*P = 0.05 vs. controls).

No modification of prothrombin time (PT) and activated partial thromboplastin time (APTT) or platelet adhesion to fibrillar collagen was observed after injection (not shown).

Thirty days of a diet ( $3.4 \text{ g day}^{-1}$  per rat) enriched in procyanidin extract (Leucoselect<sup>TM</sup> Phytosome, Indena, Milan, Italy; 34% mixed with 66% lipoid S 30 carrier) also determined a significant prolongation of BT ( $205 \pm 25 \text{ s}$ ) compared with control animals (only lipoid S 30 carrier) ( $123 \pm 11 \text{ s}$ ,  $P < 0.05$ ). Procyanidin-enriched diet did not affect either thrombus formation (73% vs. 70% in control animals) or thrombus weight ( $4.5 \pm 1.4 \text{ mg}$  vs.  $4.2 \pm 1.8 \text{ mg}$  in control animals), in a model of venous thrombosis, induced by inferior vena cava ligation [5]. Procyanidin-enriched diet did not change PT, APTT or platelet adhesion to fibrillar collagen (not shown).

Our results show that procyanidins, similar to de-alcoholated red wine [5], prolong BT but, at variance with wine, they do not inhibit platelet adhesion to collagen or thrombosis. It is possible that the prolongation of BT in our test model might be due to a vasodilatory effect of the extract [6] rather than to reduced platelet–vessel wall interactions. We speculate that procyanidins contribute to the protective effect of red wine or its derived total polyphenols [5,6] by a vasomodulatory role [6] without directly affecting factors of the hemostatic system.

## Acknowledgements

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# Rebuttal to: Effect of heparin on TAFI-dependent inhibition of fibrinolysis

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Dear Sir,

We read with interest the recent paper by Colucci *et al.* [1], who studied the effect of unfractionated heparin on TAFI-dependent inhibition of fibrinolysis. The conclusion of their study was that heparin has no effect on TAFI-dependent clot lysis, as heparin only inhibits thrombin in solution and not clot-bound thrombin. The authors postulate that clot-bound thrombin plays a pivotal role in TAFI-dependent down-regulation of fibrinolysis, and that localized generation of activated TAFI by clot-bound thrombin is sufficient for inhibition of clot lysis.

The conclusion by Colucci *et al.* that heparin, at concentrations which completely inhibit thrombin generation in

solution, has no effect on clot lysis, is in sharp contrast with results obtained in our laboratory. Using a plasma-based clot lysis assay, which has been extensively used to study TAFI-dependent down-regulation of fibrinolysis in a variety of pathologies including factor XI deficiency [2], hemophilia [3,4], liver cirrhosis [5], and hemostatic changes associated with the use of oral contraceptives [6], we also studied the effect of heparin on TAFI-mediated down-regulation of fibrinolysis.

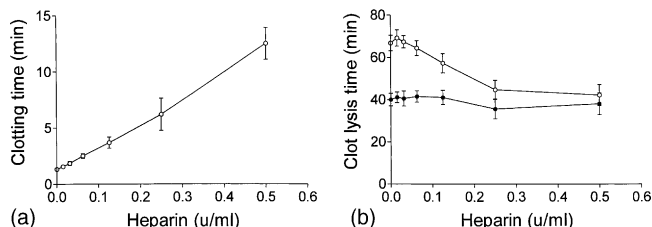
In short, in a 96-wells microtitre plate, pooled normal plasma to which different concentrations of unfractionated heparin (Leo pharmaceutical products, Weesp, The Netherlands) was added, was allowed to clot by the addition of tissue factor (Innovin, Dade Behring, 1 : 1000 times diluted), calcium chloride ( $17 \text{ mmol L}^{-1}$ ), and phospholipid vesicles ( $10 \mu\text{mol L}^{-1}$ ), fibrinolysis was induced by addition of tPA ( $30 \text{ U mL}^{-1}$ ; Chromogenix, Mölndal, Sweden). Coagulation and clot lysis were monitored by continuous turbidity measurements at a wave-

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**Fig. 1.** The effect of heparin on coagulation (panel A) and clot lysis (panel B). Pooled normal plasma containing different concentrations of heparin was allowed to clot by addition of tissue factor, calcium chloride, and phospholipid vesicles. Fibrinolysis was initiated by addition of tPA. Clot formation and subsequent lysis was monitored by turbidity measurements at 405 nm in time. Experiments were performed in absence (○) and presence (●) of CPI. Shown are mean of three independent experiments performed in duplicate. Error bars indicate standard deviation.

length 405 nm, and clotting times and clot lysis times were determined as described [4].

As shown in Fig. 1(A), clotting times dose-dependently increased on addition of heparin. No clot formation was observed at concentrations above  $0.5 \text{ U mL}^{-1}$ . Clot lysis times dose-dependently decreased on addition of heparin (Fig. 1B, open circles). When carboxypeptidase inhibitor from potato (CPI, Calbiochem, La Jolla, CA), a specific inhibitor of activated TAFI, was added to the plasma, clot lysis time was significantly decreased compared to control values, indicating that TAFI activation contributes to inhibition of fibrinolysis in this assay. Addition of heparin did not affect clot lysis time in the presence of CPI (Fig. 1B, closed circles), indicating that the decrease in clot lysis time was attributable to decreased TAFI activation.

The discrepancy between the experiments of Colucci *et al.* and our own, can probably be explained by substantial differences in experimental set-up. In the experiments performed by Colucci *et al.*, first, a fibrin clot is made by adding thrombin and calcium to citrated plasma, which is subsequently incubated for 1 h at  $37^\circ\text{C}$ . Next, the clot is washed with saline, and transferred to defibrinated plasma containing vehicle or heparin. Clot lysis is induced by adding tPA and calcium chloride. In contrast, in our assay, heparin is already present before clot formation.

Thus, in the Colucci assay, the effect of heparin on lysis of a fully matured clot is investigated, whereas in our experiments, the effect of heparin on resistance against fibrinolysis on a developing clot is examined. In the assay performed by Colucci, the TAFI activation in the first part of the assay will already contribute in part to down-regulation of fibrinolysis in the second part of the assay, as some C-terminal lysine and arginine residues will become available during fibrinolysis induced by endogenous tPA. Moreover, and probably of more importance, the thrombin generated in the first part of the assay will bind to the clot, and will contribute to TAFI activation in the second part of the assay. TAFI activation via clot-bound thrombin formed in the first part of the assay, presumably will occur by factor XI-dependent enhancement of thrombin generation [7]. In fact, heparin has been shown to enhance factor XI activation by fibrin-bound thrombin [8]. If this process remains associated to

the clot, the generated thrombin will remain unsusceptible to inhibition by heparin. Enhancement of thrombin generation via clot-bound thrombin is required for TAFI activation, as absence of calcium- or vitamin K-dependent factors enhances clot lysis (Fig. 3 in the paper by Colucci *et al.*) [1].

In contrast, in our assay, the presence of heparin in plasma before clot formation suppresses thrombin generation throughout the process of clot formation and subsequent lysis, and consequently diminishes the amount of clot-bound thrombin.

In conclusion, we want to modify the conclusion drawn by Colucci *et al.* that heparin does not affect TAFI-mediated down-regulation of fibrinolysis *in vitro*. In our opinion, heparin does not affect resistance against fibrinolysis in a mature clot, but significantly affects clot stability of a developing clot by means of diminishing TAFI activation. If these data can be translated into the *in vivo* situation, this would mean that heparin administered after venous or arterial thromboembolism does not facilitate thrombolysis, as TAFI activation by clot-bound thrombin provides optimal lysis resistance. However, heparin would contribute to the prevention of reocclusion, as TAFI activation in a newly developing clot is efficiently inhibited, making the new thrombus more susceptible to breakdown by endogenous fibrinolysis.

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# Rebuttal to: Effect of heparin on TAFI-dependent inhibition of fibrinolysis

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Dear Sir,

We agree with Lisman and de Groot [1] that heparin, under certain conditions, may promote fibrinolysis by inhibiting TAFI activation. In fact, it is also our experience that heparin, when added prior to clot formation (in a microplate assay similar to that reported by Lisman and de Groot), shortens the lysis time through a TAFI-dependent mechanism. This finding, however, is quite predictable, considering that in this model heparin exerts a strong thrombin inhibitory effect before fibrin formation, and is not totally new; de Fouw *et al.* [2] reported similar results several years ago, although at that time the thrombin-dependent antifibrinolytic factor was still unknown. As pointed out in our paper [3], the goal of our study was to see whether heparin had a profibrinolytic effect under conditions in which thrombin can be generated on the clot surface. In particular we wished to assess to which extent fibrin-associated thrombin contributed to TAFI-mediated inhibition of fibrinolysis in order to gain new insights on the mechanism of action of heparin in thrombolytic regimens. For this reason the experimental model was set up to mimic the *in vivo* condition during thrombolytic therapy, in which heparin and t-PA are administered after a thrombus has already formed. Quite surprisingly, we observed that, under our experimental conditions, heparin failed to stimulate clot lysis despite complete inhibition of thrombin generation and TAFI activation in the plasma bath in which the blood clot was immersed. We provide evidence suggesting that activation of small amounts of TAFI by the sole fibrin-bound thrombin (formed on the clot surface after recalcification of defibrinated plasma, i.e. after addition of heparin) was sufficient to make the clot resistant to a pharmacologic concentration of t-PA and hypothesize the existence of a localized phenomenon probably involving TAFI cross-linked

to fibrin. Obviously, the conclusion that in our model heparin does not stimulate fibrinolysis through a TAFI-dependent mechanism does not imply that heparin is unable to stimulate fibrinolysis under different experimental conditions. Accordingly, any speculation as to the possible clinical implication must be strictly limited to the *in vivo* condition that the *in vitro* model attempts to simulate (in our case, thrombolytic therapy). If TAFI proves to play a role in the *in vivo* regulation of fibrinolysis in humans [4], we, too, believe that heparin might function as a profibrinolytic through the inhibition of TAFI activation under certain conditions such as reocclusion by a newly formed clot or even accretion of an existing thrombus (in our model thrombus accretion did not occur because we used defibrinated plasma). However, if our data are confirmed in more physiological conditions, the suggested use of anticoagulants capable of inhibiting clot-bound thrombin will probably grant a wider profibrinolytic effect that might also influence the resistance of a mature clot.

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