



Full Length Article

Monitoring of heparins in antithrombin-deficient patients[☆]

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ABSTRACT

Introduction: Heparins exert their anticoagulant effect through activation of antithrombin. Whether antithrombin deficiency leads to clinically relevantly reduced anti-Xa activity of heparins is unknown. We investigated the relation between antithrombin deficiency and anti-Xa activity measurements of plasma samples spiked with unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH).

Materials and methods: Plasma samples from 34 antithrombin-deficient subjects and 17 family controls were spiked with UFH and LMWH (nadroparin) aimed to correspond with an anti-Xa activity of 0.8 IU/mL. Antithrombin, β -antithrombin and anti-Xa activities were measured.

Results: Mean anti-Xa activity with LMWH was 0.55 IU/mL (0.30–0.74) (recovery 69%, 38–93%) in antithrombin-deficient subjects and 0.82 (0.71–0.89) IU/mL in controls (recovery 103%, 89–111%). Expected anti-Xa measurements after LMWH spiking were found in 17/17 non-deficient subjects and in 8/34 antithrombin-deficient subjects. Anti-Xa measurements in the expected range (0.6–1.0 IU/mL) after UFH spiking were found in 17/17 non-deficient subjects and in 1/22 antithrombin-deficient subjects. Antithrombin activity correlated with anti-Xa activity of UFH ($R = 0.77$) and LMWH ($R = 0.66$). Mixing studies of pooled normal plasma and antithrombin-deficient plasma showed that anti-Xa recovery was linearly reduced with antithrombin activity decreasing below 100%.

Conclusions: Reduced antithrombin activity causes significantly reduced anti-Xa levels. Standard LMWH- or UFH-doses are likely to lead to under treatment in antithrombin-deficient individuals.

1. Introduction

Heparins are widely used anticoagulant drugs that exert their effect by activation of antithrombin. Antithrombin is a slow-working anticoagulant molecule circulating in a repressed state in the blood. Its anticoagulant function is greatly enhanced after activation by heparin. Different types of heparin molecules, such as unfractionated heparin (UFH) or low-molecular weight heparin (LMWH), exert their anticoagulant effects in different ways, either predominantly via factors Xa, IXa and thrombin (UFH) or via factors Xa and IXa (LMWH) [1].

Antithrombin deficiency (OMIM 613118) is an inherited autosomal dominant defect, clinically characterized by a fourteen-fold increased risk of a first venous thromboembolism (VTE) [2]. In high-risk periods such as pregnancy and puerperium, thrombotic risk is even higher, with current risk-estimates as high as 7.3% for the antepartum period and 11.1% for the postpartum period. Antithrombin deficiency is therefore

a recognized indication for prophylactic treatment with LMWH [3]. However, despite prophylactic treatment with LMWH, VTE events have been described in pregnant antithrombin-deficient women [4–6]. We hypothesized that antithrombin-deficient individuals may have less therapeutic effect of heparins due to the antithrombin deficiency itself.

A subdivision of antithrombin deficiency is made in type I deficiency (quantitative defect) and type II deficiency (qualitative defects) [7]. Antithrombin consists of two isoforms, 90% of circulating antithrombin being α -antithrombin and 10% β -antithrombin. The N-glycan at Asn135 is not present in β -antithrombin. β -antithrombin has a higher affinity for heparin, and binds better to the endothelium [8] and may therefore serve as the major inhibitor of coagulation in vivo, and as the major target for heparins [9,10]. In the setting of inherited antithrombin deficiency the interplay between antithrombin deficiency types, β -antithrombin levels and types of heparins and activity measurements of these drugs is unknown.

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Table 1
Measurements in antithrombin-deficient and non-antithrombin-deficient subjects.

Group	Antithrombin activity (%)	β -antithrombin activity (%)	Antithrombin antigen (%)	Anti-Xa activity with UFH	Anti-Xa activity with LMWH
No antithrombin deficiency (n = 17)	108.7, 89.8–131	105.9, 85.0–137.6	111.4, 87.8–140.0	0.79, 0.64–0.93 (n = 17)	0.81, 0.71–0.89
Antithrombin deficiency (n = 34)	58.1, 44.0–72.0	71.9, 42.2–115.7	72.6, 40.5–135.8	0.51, 0.32–0.63 (n = 22)	0.57, 0.30–0.74
Type I (n = 20)	58.1, 44.0–72.0	63.7, 42.2–94.2	57.3, 40.5–76.6	0.52, 0.42–0.63 (n = 14)	0.57, 0.45–0.65
Antithrombin Cambridge II (n = 1)	51.0	105.7	103.0	0.53	0.74
Type II RS (n = 1, antithrombin Glasgow)	70.0	98.0	135.8	0.32	0.30
Type II HBS (n = 5)	68.0, 56.0–72.0	98.9, 92.5–111.9	105.8, 96.0–128.3	0.50, 0.48–0.52 (n = 2)	0.59, 0.45–0.62
Type II PE (n = 7)	55.7, 49.0–63.0	79.8, 49.3–115.7	75.9, 70.2–93.0	0.48, 0.43–0.59 (n = 4)	0.55, 0.42–0.64
Type II PE and type II HBS (n = 12)	58.1, 49.0–72.0	94.9, 49.3–115.7	86.2, 70.2–128.3	0.49, 0.43–0.59 (n = 6)	0.56, 0.42–0.64

Measurements in antithrombin-deficient and non-antithrombin-deficient subjects. Data are presented as median and range. UFH: unfractionated heparin. LMWH: low-molecular-weight heparin. For the anti-Xa measurements after addition of UFH less samples were available, the numbers are shown in the UFH column. Type II HBS: type II heparin binding site. Type II RS: type II reactive site. Type II PE: type II pleiotropic effects.

The aim of this study is to investigate the relation between antithrombin deficiency, subtypes of antithrombin deficiency, β -antithrombin levels and anti-Xa activity measurements of plasma samples spiked with UFH or LMWH.

2. Materials and methods

2.1. Participants

In a cross-sectional and retrospective family cohort study we included patients with inherited antithrombin deficiency and their first-degree relatives. Inclusion of participants is described elsewhere in more detail [11]. Eighty participants in the study did not use LMWH/UFH at inclusion. From these 80, we had blood samples available from 51 subjects for the present study. From these 51 subjects, 17 were non-antithrombin-deficient family members. Of the 51 subjects, all samples were spiked with LMWH, and for 39 subjects enough plasma remained for spiking with UFH. This study was approved by the institutional medical ethics committee of the University Medical Center Groningen (ABR nr. NL41324) and is in accordance with the 2013 Helsinki Declaration.

Clinical features, blood collection, specialized antithrombin measurements and *SERPINC1* gene mutation analyses have been described in detail elsewhere [11,12]. Standard antithrombin activity measurements were performed with an assay based on anti-FXa inhibition (INNOVANCE Antithrombin assay; Siemens Healthcare Diagnostics Products) (normal levels > 80%). Measurements of β -antithrombin activity were performed using a previously published protocol that was adapted by V. Karlaftis [13]. Anti-Xa assay measurements were performed using the Hemosil Liquid anti-Xa assay (this test does not contain exogenous antithrombin) and APTT measurements were performed using the Hemosil (SynthASil) Liquid activated partial thromboplastin time (APTT), both according to the protocols from the manufacturer on a ACLTOP 500, both from Werfen. APTT measurements were also performed using the actinFS APTT test from Siemens on a CS2100i.

2.2. Addition of anticoagulants to plasma

UFH (LEO Pharma BV, Amsterdam, The Netherlands) and LMWH (nadroparin, Aspen Pharma, Dublin, Ireland) were added to plasma samples of the participants. The final concentrations in plasma were aimed to correspond with anti-Xa levels of 0.8 IU/mL, in order to perform the study in the therapeutic anti-Xa levels of 0.6–1.0 IU/mL that are seen with twice daily dosing of LMWH [14]. In the APTT mixing

studies the UFH levels were aimed to prolong the APTT 2–2.5 time in the normal pool plasma, corresponding to a final heparin concentration of 0.3 IU/mL.

2.3. Mixing studies

In addition to the studies in patient samples, we performed mixing studies of pooled normal plasma and antithrombin-deficient plasma to analyse the relation between anti-Xa activity measurements, APTT measurements and antithrombin levels. Antithrombin-deficient plasma was obtained from Diagnostica Stago (Asnières sur Seine, France). Antithrombin-deficient plasma was mixed with pooled normal plasma to obtain a range of antithrombin concentrations from 0 to 100%. Pooled normal plasma was obtained from 60 healthy donors and immediately aliquoted and frozen at -80°C after processing.

2.4. Statistical analysis

Data are expressed as mean \pm standard deviation (SD), medians (with ranges), or numbers (with percentages) as appropriate. Medians of two groups were compared by the independent samples median test. Spearman's rho correlation coefficient was used to assess correlation between continuous variables. *P*-values of 0.05 or less were considered statistically significant. IBM SPSS version 21 (New York, USA) was used for all analyses. Figures were created using GraphPad PRISM version 5.01 (San Diego, USA).

3. Results

Of 51 subjects available for this study, 18 participants had type I antithrombin deficiency, 1 had antithrombin Cambridge II, 5 had type II heparin binding site (type IIHBS) antithrombin deficiency, 1 had type II reactive site (type IIRS) antithrombin deficiency, 9 had type II pleiotropic effect (type IIPE) antithrombin deficiency, and 17 had no antithrombin deficiency. At the study visit, of the 51 subjects, 31 used no anticoagulant drugs (of which 3 with previous VTE), 1 subject used a platelet inhibitor (no previous VTE) and 19 used vitamin K antagonists (of 17 with previous VTE). The antithrombin activity, β -antithrombin activity, antithrombin antigen levels and anti-Xa activity were not normally distributed. Antithrombin and anti-Xa measurements according to subtypes are shown in Table 1.

In antithrombin-deficient individuals, spiking with LMWH resulted in lower median anti-Xa activity than in non-deficient individuals (0.57 IU/mL, range 0.30–0.74 vs. 0.81 IU/mL, 0.71–0.89, $p < 0.001$).

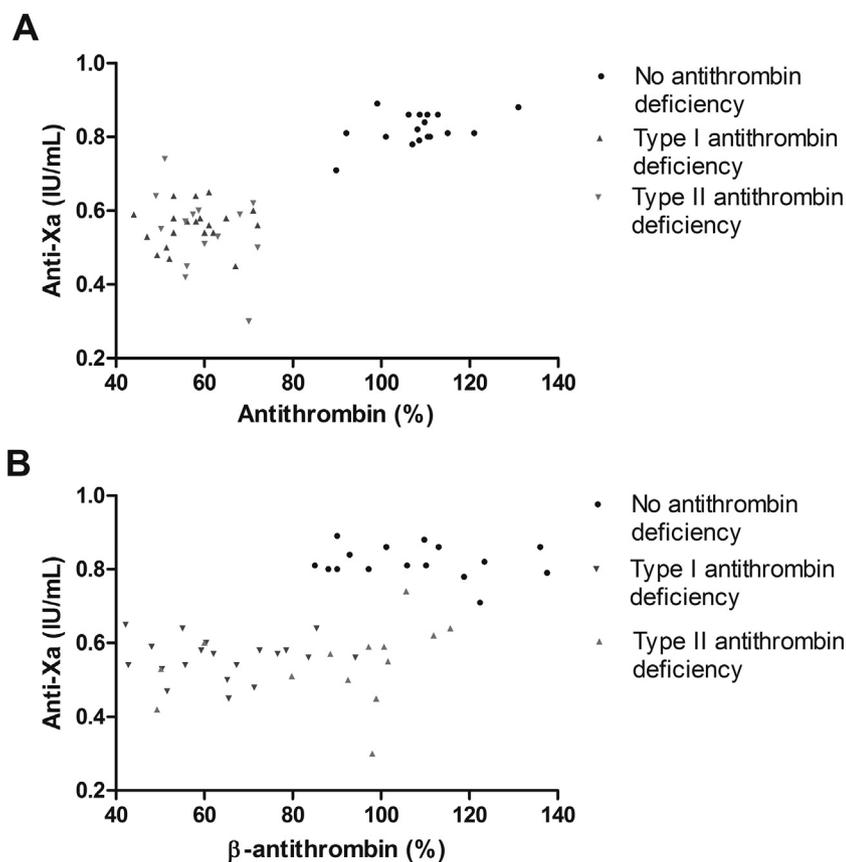


Fig. 1. Scatterplots of antithrombin activity and anti-Xa activity measurements.

Panel A: antithrombin and anti-Xa activity measurements after spiking with low-molecular-weight heparin (LMWH). Panel B: β -antithrombin and anti-Xa activity measurements after spiking with LMWH. Type II HBS: type II heparin binding site antithrombin deficiency. Type II RS: type II reactive site antithrombin deficiency. Type II PE: type II pleiotropic effects antithrombin deficiency.

Mean anti-Xa recovery with LMWH was 103% (89–111%) in controls and 69% (38–93%) in antithrombin deficient individuals. In antithrombin-deficient individuals, after spiking with UFH, the median anti-Xa was also lower than in non-deficient individuals (0.51 IU/mL, 0.32–0.63, vs. 0.79 IU/mL, 0.64–0.93, $p < 0.001$). Between type I and type II antithrombin-deficient individuals the mean anti-Xa was not significantly different for both heparins. Expected anti-Xa levels (0.6–1.0 U/mL) after UFH spiking were found in all non-deficient subjects and in 1/22 (5%) of antithrombin-deficient subjects. Expected anti-Xa levels after LMWH spiking were found in all non-deficient subjects and in 8/34 (24%) of the antithrombin-deficient subjects.

Scatterplots of antithrombin- and β -antithrombin activity and anti-Xa measurements of all investigated samples are shown in Fig. 1.

Using Spearman's rho correlation analysis, antithrombin activity was correlated with anti-Xa activity of UFH ($R = 0.77$, $p < 0.001$) and of LMWH ($R = 0.66$, $p < 0.001$). β -antithrombin activity also correlated with anti-Xa activity of UFH ($R = 0.59$, $p < 0.001$) and of LMWH ($R = 0.56$, $p < 0.001$), but less strongly.

In order to investigate if anti-Xa measurements were linked to specific *SERPINC1* mutations underlying the antithrombin deficiency, we related anti-Xa measurements to the underlying mutations. With all mutations, mean anti-Xa levels were lower than in the non-antithrombin-deficient family members. Only 2 mutations differed from the other mutations: the anti-Xa level in the c.1246G > T mutation ($n = 1$) (antithrombin Cambridge II) was somewhat higher with LMWH, and in the type IIRS c.1274G > A mutation ($n = 1$) (antithrombin Glasgow) the anti-Xa level was lower with both UFH and LMWH, as compared to the other mutations. These results are shown in Fig. 1.

In order to further investigate the relation between antithrombin levels and anti-Xa measurements, we performed a similar spiking experiment in mixtures with increasing concentrations of antithrombin (0–100%) of commercially available antithrombin-deficient plasma with pooled normal plasma. Anti-Xa levels after spiking these samples

with either UFH or LMWH are clearly related to antithrombin concentrations (Fig. 2).

These results prompted us to perform the same experiment with two different APTT tests (as differential heparin sensitivity of different APTT tests is common). In the very low ranges of antithrombin activity, decreased APTT measurements were found. The antithrombin levels below which APTT measurements decreased varied between tests, as shown in Fig. 2.

4. Discussion

Our experiments show that anti-Xa measurements in the presence of LMWH or UFH are clearly correlated with antithrombin activity. Mean anti-Xa levels were about 35% lower than expected in antithrombin-deficient subjects as compared to non-deficient subjects. Of antithrombin-deficient subjects, 76–95% had levels that would be classified as subtherapeutic in clinical setting, whereas all controls had expected anti-Xa levels.

The correlation of antithrombin activity with anti-Xa measurements is stronger than the correlation of β -antithrombin activity with anti-Xa measurements. These data therefore do not support the theory of β -antithrombin being the strongest inhibitor of coagulation in vivo, although the natural activator of antithrombin is not currently known [9,10]. The weaker correlation of β -antithrombin activity with anti-Xa measurement may be explained by either β -antithrombin not being a stronger inhibitor than overall antithrombin, or due to limitations of the β -antithrombin measurement itself. Either way, antithrombin activity measurements are easier to perform, and have a stronger correlation with anti-Xa measurements than β -antithrombin measurements. However, it cannot be excluded that using different antithrombin activity assays would lead to different correlations, as no available antithrombin activity test is able to detect all types of antithrombin deficiency [15–17].

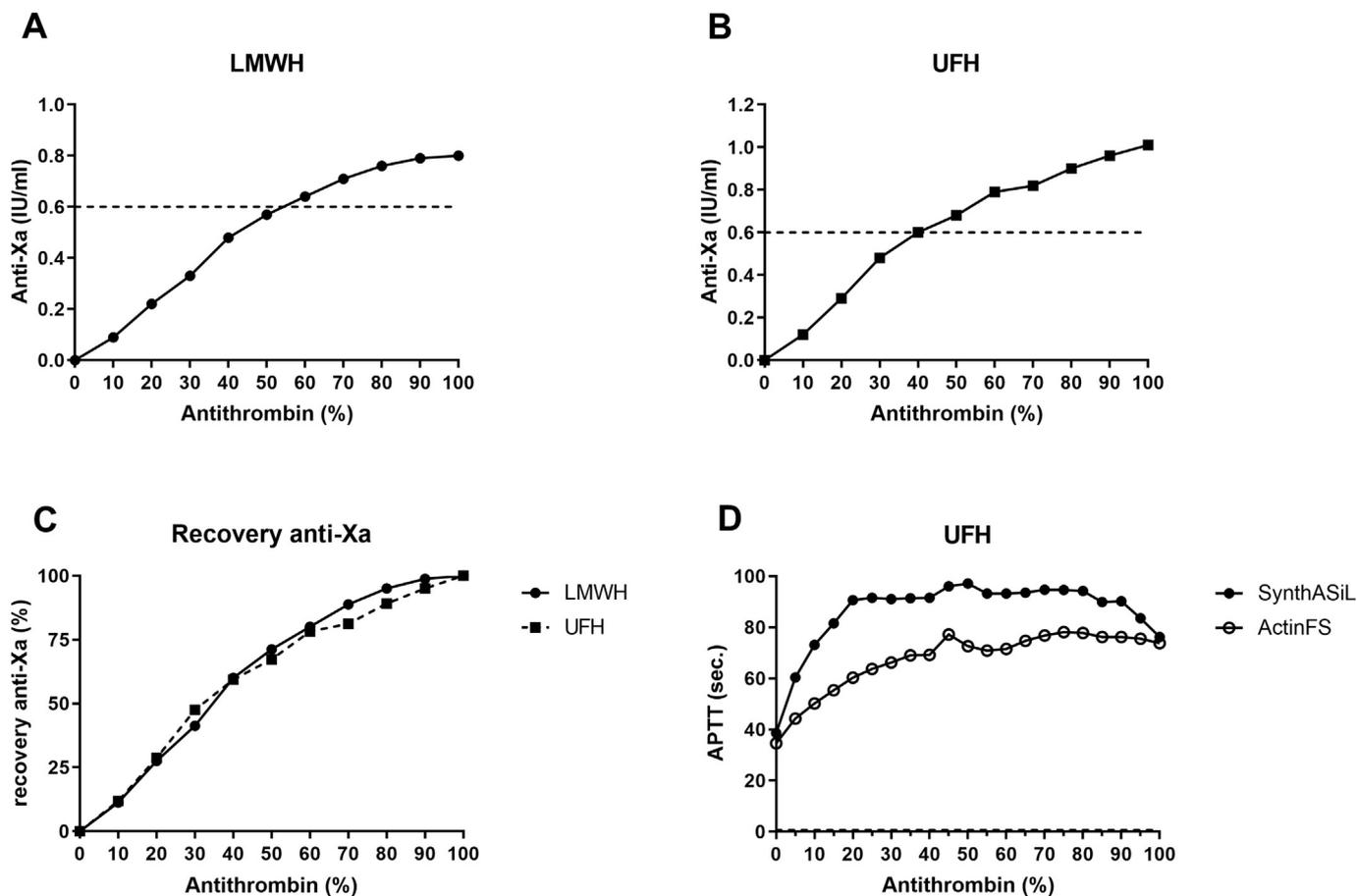


Fig. 2. Anti-Xa and APTT measurements in mixing studies of antithrombin-deficient and pooled normal plasma.

Panel A: anti-Xa activity according to antithrombin levels after adding nadroparin. Panel B: anti-Xa activity according to antithrombin levels after adding unfractionated heparin (UFH). Panel C: Recovery of anti-Xa according to antithrombin level, normalized for maximum anti-Xa activity. UFH: unfractionated heparin. Panel D: APTT measurements with two different tests, according to antithrombin levels after adding UFH.

In the mixing studies, a sharp contrast was seen between the anti-Xa measurements and APTT measurements after spiking with UFH with respect to its sensitivity to reduced antithrombin activity. Whereas in samples with reduced antithrombin activity anti-Xa measurements were reduced as well, APTT measurements were only influenced very low levels of antithrombin. APTT measurements differ considerably from anti-Xa measurements, as they do not only measure the anti-Xa effect of UFH, but also the inhibition of thrombin by antithrombin. Therapeutic reference ranges of APTT tests are based on their correspondence with anti-Xa activity levels. However; given the variability of heparin sensitivity of various APTT tests, it has been shown to be exceedingly difficult to translate therapeutic reference ranges of one APTT test to another. This could result in the use of very much different UFH doses from centre to centre depending on the APTT test used [18]. In our mixing studies with UFH and APTT measurements, the different heparin sensitivity between two different tests is also shown (Fig. 2). In clinical practice, too low APTT measurements (whether resulting from antithrombin deficiency or not) would lead to physicians increasing heparin doses.

Different types of LMWH may have different profiles regarding the relation between antithrombin- and β -antithrombin concentrations, different *SERPINC1* mutations and anti-Xa measurements. Our study was limited to only one type of LMWH, as nadroparin is a commonly used LMWH in The Netherlands.

The lower anti-Xa activity found in the patient with the type IIRS c.1274G > A mutation (antithrombin Glasgow) may be explained by a different conformation of the antithrombin Glasgow molecule than the normal heparin-activated conformation of normal antithrombin,

resulting in an increased heparin affinity [19]. Although we had only this one type IIRS patient in our cohort, this may be a class effect: Antithrombin London is another type IIRS variant that has been described to have abnormally strong binding of inactive antithrombin to heparin. In this way antithrombin London acts as a competitive antagonist of normal antithrombin [20]. Similar findings were found in antithrombin Pescara [19].

The normal heparin response that was found in the patient with the Cambridge II mutation is in agreement with the findings of Mushunje et al., who also found a normal response to heparins. The thrombophilic predisposition was explained by perturbations by glycosaminoglycans localized in the vessel walls [21].

The relation between antithrombin levels and anti-Xa measurements has been found before in subjects with liver cirrhosis [22], ICU patients and patients with sepsis or nephrotic syndrome [23] and in children during cardiopulmonary bypass [24]. These are all situations with multiple haemostatic abnormalities. In contrast, in our study antithrombin deficiency is the single major abnormality in the haemostatic system. In most of our antithrombin-deficient subjects anti-Xa measurements after spiking with UFH and LMWH were below the expected range of 0.6–1.0 U/mL, whereas in all anti-Xa measurements non-deficient subjects were in the expected range. Therefore, it is possible that in practice, antithrombin-deficient subjects are under-treated with regular doses of UFH/LMWH. This may explain the occurrence of thrombotic events in antithrombin-deficient individuals despite treatment with LMWH [4–6]. Antithrombin-deficient VTE patients who were identified because of requiring high doses of heparin have been described before [25]. However, it is unknown if the

interaction of antithrombin and anti-Xa activity is a cause of the high risk of recurrent VTE in antithrombin deficiency [2], and in patients with mildly reduced antithrombin levels (< 87%) [26,27]. Although the use of LMWH generally requires no routine monitoring [14], the knowledge of inherited antithrombin deficiency causing a reduced anticoagulant effect of heparins, as measured by anti-Xa assays, in combination with the clear prothrombotic phenotype of antithrombin deficiency supports measuring anti-Xa activity in antithrombin-deficient subjects who are treated with heparins. Doses of LMWH should be adjusted to achieve the target range in order to achieve a sufficient antithrombotic effect. Alternatively, co-administration of antithrombin concentrates may be considered: co-administration of antithrombin concentrates in children receiving UFH has been shown to increase anti-Xa factor levels [28]. However, data on efficacy of antithrombin concentrates are limited [29], and no clinical studies evaluating safety and/or efficacy of increasing LMWH doses according to anti-Xa levels in antithrombin-deficient subjects have been performed, nor are likely to be performed because of the rarity of antithrombin deficiency.

Since most anti-Xa assays used in clinical laboratories (including the test used in our study) do not contain exogenous antithrombin, anti-Xa activity assays are often fully dependent on the patient's own antithrombin. This situation reflects the *in vivo* situation. Adding exogenous antithrombin to anti-Xa activity assays may lead to overestimation of the anticoagulant effect of heparins in patients with antithrombin deficiency.

In conclusion, we have found that anti-Xa activity measurements of LMWH and UFH are clearly correlated with antithrombin activity, and that even mildly reduced antithrombin activity may result in a reduced anti-Xa recovery. In most antithrombin-deficient plasma samples anti-Xa measurements were below the expected range of 0.6–1.0 U/mL. Antithrombin-deficient individuals, already at an increased risk for recurrent venous thrombosis, may therefore be undertreated using standard doses of UFH/LMWH.

Author contribution

FNC wrote the manuscript; all other co-authors commented on previous versions of the manuscript and agreed with the final content. FNC, MVL and RM performed the study. FNC, MVL, ABM, MPMM and KM designed the study.

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