Hormones, haemostasis, and the risk of thrombosis
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Chapter 6

Thrombin-activatable fibrinolysis inhibitor in hyperthyroidism and hypothyroidism

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Abstract
Introduction: Endocrine disorders affect both the coagulation and fibrinolytic systems, and have been associated with the development of cardiovascular diseases. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a link between the coagulation and fibrinolytic system. The aim of this study was to determine the effect of hyperthyroidism and hypothyroidism on TAFI levels and function.

Methods: The effect of hyperthyroidism on TAFI was studied in healthy volunteers who were treated with levothyroxine for 14 days. The effect of hypothyroidism on TAFI was studied in a multicentre observational cohort study. Blood was drawn before treatment of patients with newly diagnosed hypothyroidism and when euthyroidism was achieved. Plasma clot-lysis times, TAFIa-dependent prolongation of clot-lysis and TAFI levels were measured.

Results: Hyperthyroidism resulted in a hypofibrinolytic condition and in an enhanced TAFIa-dependent prolongation of clot-lysis. A trend towards decreased plasma TAFI levels was observed in healthy volunteers who used levothyroxine. Hypothyroidism resulted in hyperfibrinolysis and a reduced TAFIa-dependent prolongation of clot-lysis.

Discussion: Alterations of TAFIa-dependent prolongation of clot-lysis in patients with thyroid disorders may cause an impaired haemostatic balance. The disturbed haemostatic balance in patients with hyperthyroidism might make them more prone for thrombosis, while the risk for bleeding may increase in patients with hypothyroidism.

Introduction
Endocrine disorders are common diseases in the general population and affect both the coagulation and fibrinolytic systems. Patients with hypothyroidism as well as hyperthyroidism have an increased risk for cardiovascular diseases, however it is related to different underlying mechanisms[9-16]. The mechanism for the development of cardiovascular disease in patients with hypothyroidism is mainly based on the development of atherosclerosis caused by weight gain, hypertension and increased lipid levels[16], while patients with hyperthyroidism are more prone to atrial fibrillation and have increased plasma levels of procoagulant factors[9-12]. The prothrombotic condition in patients with hyperthyroidism is illustrated by elevated plasma levels of von Willebrand factor (VWF), fibrinogen, factor VIII, factor IX and factor X, and possibly by enhanced platelet function[9-14]. Moreover, a reduced fibrinolytic activity was observed in these patients due to increased levels of plasminogen activator inhibitor-1 (PAI-1)[9]. In contrast, patients with overt hypothyroidism have an increased bleeding tendency. The most common coagulation disorder in these patients is acquired von Willebrand’s syndrome, which is characterised by decreased levels of factor VIII and von Willebrand factor[9,12-13]. Evidence on other coagulation parameters is less robust, but reduced levels of the coagulation factors IX, X and XI have been reported in hypothyroid patients[9,12-13]. In addition, several changes in plasma levels of antiplasmin, t-PA, PAI-1 and D-dimer have been described, which suggests that thyroid hormone deficiency affects the fibrinolytic system as well[9-13]. However, the net effect on fibrinolytic activity is still unclear[9-13].

A protein that links the coagulation and fibrinolytic system is thrombin-activatable fibrinolysis inhibitor (TAFI)[16]. TAFI is a glycoprotein that is synthesised in the liver and circulates in plasma as a carboxypeptidase B-like proenzyme. Thrombin activates TAFI and activated TAFI (TAFIa) acts as a fibrinolysis inhibitor. The antifibrinolytic function of TAFI is based on cleavage of C-terminal lysine residues of partly degraded fibrin, the binding sites for plasminogen and its activator tissue-type plasminogen activator. This results in less plasmin formation and thus TAFI attenuates fibrinolysis. Besides its antifibrinolytic function, TAFI is also involved in wound healing, angiogenesis and inflammation[19-21].

Recently, plasma levels of TAFI were measured in patients with thyroid disorders. Elevated plasma TAFI levels were observed in patients with mild and overt hypothyroidism[9,16], while both decreased as well as increased plasma TAFI levels were reported in patients with hyperthyroidism[9,16]. Elevated TAFI levels in patients with hypothyroidism suggest a hypofibrinolytic activity. Given the uncertainty about the fibrinolytic activity in patients with hypothyroidism and contradictory results on TAFI levels in patients with hyperthyroidism, the present study was performed to assess the effects of both hyperthyroidism and hypothyroidism on plasma TAFI levels, and on TAFIa-dependent prolongation of clot-lysis.

Materials and methods
Study design
The effects of hyperthyroidism on TAFI were studied in two single-blinded, crossover, randomised controlled trials in healthy volunteers. Participants received both no medication or levothyroxine...
to induce hyperthyroidism for 14 days and, based on the half-life of levothyroxine, exposures were separated by a wash-out of at least 28 days. Randomisation was performed by an independent physician who had no information about the participants, nor responsibility for determining eligibility of the participants. For safety reasons, we started with inducing mild hyperthyroidism by treating the participants with levothyroxine 0.3 mg per day. After performing this study, and none of the participants had shown important side effects, we were allowed to induce more pronounced hyperthyroidism. Participants with a body weight below 80 kg received levothyroxine 0.45 mg per day, while participants with a body weight of 80 kg or more received levothyroxine 0.6 mg per day. Levothyroxine was taken every morning, on awakening, 30 minutes prior to food intake. Blood was drawn at baseline and day 14 of each study period, between 8 and 10 am. These studies were performed at the department of Internal Medicine of the Slotervaart Hospital in Amsterdam, the Netherlands.

The effect of hypothyroidism on TAFI was studied in a multicentre observational cohort study. Patients with a new diagnosis of overt hypothyroidism were included, before or within the first 48 hours of replacement therapy. Overt hypothyroidism was defined as an increase of serum thyrotropin (TSH) above 15 mU/L, with serum free thyroxine (FT4) concentration below the lower limit of the reference range. Blood sampling was performed at inclusion (during hypothyroidism) and repeated after reaching euthyroidism (usually after 3-4 months). Samples were collected at the Slotervaart Hospital and at the Academic Medical Centre, both in Amsterdam, the Netherlands.

Study protocols were approved by the Medical Ethical Review Board of both the Slotervaart Hospital and the Academic Medical Centre in Amsterdam. Written informed consent was obtained in all participants.

Participants and plasma
For the hypothyroidism studies, healthy volunteers aged 18 to 40 years, regardless of gender or race, were recruited by local advertisements. Volunteers were excluded if they had any of the following: history of thyroid disease or venous thrombosis, ongoing medical or psychiatric illnesses, regular use of prescription or non-prescription medications including oral contraceptive agents, illicit drugs or excessive alcohol use, surgery or hospitalisation in the previous 3 months, or non-traditional sleep/wake habits (e.g. night shift work or frequent travel across time zones).

For the hypothyroidism study, patients with a new diagnosis of primary overt hypothyroidism, with a minimum age of 18 years, were enrolled before or within the first 48 hours of replacement therapy. Patients were excluded if they had any of the following: secondary hypothyroidism, subclinical hypothyroidism, more than 48 hours of thyroid hormone replacement therapy, known congenital or acquired von Willebrand’s syndrome, or presence of severe inflammatory disease (e.g. active inflammatory bowel disease, pneumonia).

For all studies, blood was collected into trisodium citrate containing tubes and tubes were centrifuged twice (15 min, 2500 g at 15°C). Plasma was aliquoted and stored at -80°C until further use.

Reagents
Rabbit lung thrombomodulin was obtained from American Diagnostica (Greenwich, CT) and potato carboxypeptidase inhibitor from Calbiochem (La Jolla, CA). Hippuryl-Arginine and H-D-Phe-Pro-Arg-Chloromethylketone (PPACK) was purchased from Bachem (Bubendorf, Switzerland) and phosphoenolpyruvate (PEP), ATP, NADH, pyruvate kinase dehydrogenase (PK/LDH), and recombinant t-PA (Actilyse) from Biopool AB (Umeå, Sweden). Recombinant human tissue factor (Innovin) was from Siemens Healthcare Diagnostics (Marburg, Germany) and thrombin was a gift from Dr. W. Kisiel (University of New Mexico, NM).

Thyroid hormones
Serum levels of thyroid hormones were measured at the local laboratory of the Slotervaart Hospital on the day of sampling. Levels of FT4, tri-iodothyronine (T3) and TSH were assessed using commercially available assays (ADVIA Centaur® immunoassay system, Siemens Healthcare Diagnostics, Marburg, Germany). Levels of thyroid hormones in samples of patients in the hypothyroidism study, recruited at the Academic Medical Centre in Amsterdam, were measured at the local laboratory of the Slotervaart Hospital after storage at -80°C.

Clot-lysis times
The overall fibrinolytic activity was determined by measuring clot-lysis times in citrated plasma as previously described with some minor modifications. Briefly, 47 µL plasma was activated by a mixture of 175 U/mL Actilyse, 17 mM CaCl₂, 10 µM phospholipids and recombinant human tissue factor (Innovin, 10ⁿ dilution). Volumes were adjusted to 100 µL with hepes-buffered saline. Turbidity was measured in time at 37°C at 405 nm for 3 hours in a Thermomax microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). The clot-lysis times were defined as the time difference between half-maximal lysis and half-maximal clotting. Normal pool plasma that was used as a reference, was derived from a donor pool of at least 200 healthy volunteers.

TAFIa-dependent prolongation of clot-lysis time
TAFIa-dependent prolongation of clot-lysis time was determined by measuring clot-lysis times in the absence or presence of potato carboxypeptidase inhibitor (CPI, 12 µM). The TAFIa-dependent prolongation was defined as the difference in clot-lysis time in the absence and presence of CPI.

TAFI activity
Plasma TAFI levels were determined in TAFI activity assay as previously described, except that plasma was diluted 30 times.

All assays were performed by technicians who were unaware of the thyroid status of the samples.

Statistical analysis
Statistical analysis was performed using SPSS software, version 15.0 (SPSS Inc, Chicago, IL, USA). In the hyperthyroidism studies, relative changes per parameter for each individual
were calculated by subtracting the baseline value from the day 14 value, dividing it by the baseline value and multiplying the result by 100%. Change from baseline values were compared between levothyroxine treatment and no medication by Wilcoxon tests. In the hypothyroidism study, values during hyperthyroidism and euthyroidism were compared by Wilcoxon tests. Results are presented as medians with 95% confidence intervals (95% CI).

**Results**

**Hyperthyroidism: levothyroxine 0.3 mg per day**

Sixteen participants (9 men and 7 women) completed the study with 0.3 mg per day levothyroxine. The mean age was 30 (range 25 to 39) years, with a body mass index of 24 (range 19 to 33) kg/m². (Table 1).

Treatment with levothyroxine 0.3 mg per day for 14 days resulted in significantly increased levels of FT4 and T3, and reduced levels of TSH, compared to the control situation. Median thyroid hormone values in participants treated with levothyroxine 0.3 mg per day were 28.5 pM (95% CI 20.0 to 33.0 pM) for FT4, 2.50 nM (95% CI 2.20 to 3.10 nM) for T3 and 0.09 mU/L (95% CI 0.02 to 0.22 mU/L) for TSH. A considerable number of individuals was still in the subclinical range (T3 levels within the reference range and TSH below the lower limit of the reference range). In the control situation, without levothyroxine treatment, thyroid hormone values remained within the normal reference range. (Table 2).

Although a number of individuals was still in the subclinical range, a trend towards prolonged clot-lysis times was observed after levothyroxine exposure compared to the control situation; median change 7% (95% CI 2 to 12%) vs 2% (95% CI -2 to 3%), p=0.14. In addition, plasma TAFI levels and TAFIa-dependent prolongation of clot-lysis were determined. No effect of levothyroxine exposure compared to no medication; median change 12% (95% CI 5 to 24%) vs 7% (95% CI -7 to 10%), p=0.09. (Table 2).

**Table 1. Baseline characteristics.**

<table>
<thead>
<tr>
<th></th>
<th>Mild hyperthyroidism (n=16)</th>
<th>More pronounced hyperthyroidism (n=12)</th>
<th>Hypothyroidism (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30 (25; 39)</td>
<td>29 (26; 40)</td>
<td>52 (25; 86)</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>56.3</td>
<td>50.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Body-mass index (kg/m²)</td>
<td>24.2 (19.2; 33.0)</td>
<td>23.3 (21.1; 26.2)</td>
<td>27.4 (17.9; 40.0)</td>
</tr>
</tbody>
</table>

N indicates number. Data are presented as mean (range).

**Table 2. Healthy volunteers: mild hyperthyroidism.**

<table>
<thead>
<tr>
<th></th>
<th>No medication</th>
<th>Levothyroxine 0.3 mg per day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t=0 days</td>
<td>t=14 days</td>
</tr>
<tr>
<td>FT4 (pM)</td>
<td>15.0 (13.0; 16.0)</td>
<td>15.0 (14.0; 16.0)</td>
</tr>
<tr>
<td>T3 (nM)</td>
<td>1.85 (1.70; 1.90)</td>
<td>1.80 (1.80; 2.10)</td>
</tr>
<tr>
<td>TSH (mU/L)</td>
<td>1.75 (1.21; 1.94)</td>
<td>2.15 (1.73; 2.27)</td>
</tr>
<tr>
<td>Clot-lysis time (% pooled plasma)</td>
<td>94 (88; 105)</td>
<td>97 (86; 109)</td>
</tr>
<tr>
<td>TAFIa-dependent prolongation of clot-lysis (% pooled plasma)</td>
<td>96 (84; 113)</td>
<td>100 (83; 125)</td>
</tr>
<tr>
<td>TAFI concentration (% pooled plasma)</td>
<td>89 (79; 97)</td>
<td>87 (75; 101)</td>
</tr>
</tbody>
</table>

FT4 indicates free thyroxine; T3, tri-iodothyronine; TSH, thyrotropin; and TAFI, thrombin-activatable fibrinolysis inhibitor. Data are presented as medians (95% confidence intervals).

**Hyperthyroidism: levothyroxine 0.45 mg / 0.6 mg per day**

To induce more pronounced hyperthyroidism, a second study was performed including 12 participants, 6 men and 6 women. Based on their body weight, 3 participants received levothyroxine 0.6 mg per day, while 9 participants received levothyroxine 0.45 mg per day. The mean age was 29 (range 26 to 40) years, with a body mass index of 23 (range 21 to 26) kg/m². Five of these participants had also participated in the first hyperthyroidism study. (Table 1).

As expected, treatment with these higher doses of levothyroxine resulted in more pronounced hyperthyroidism. Median thyroid hormone values after levothyroxine exposure were 40.0 pM (95% CI 37.5 to 47.0 pM) for FT4, 3.80 nM (95% CI 3.35 to 4.05 nM) for T3 and 0.02 mU/L (95% CI 0.02 to 0.22 mU/L) for TSH. As expected, treatment with these higher doses of levothyroxine resulted in more pronounced hyperthyroidism. Median thyroid hormone values after levothyroxine exposure were 40.0 pM (95% CI 37.5 to 47.0 pM) for FT4, 3.80 nM (95% CI 3.35 to 4.05 nM) for T3 and 0.02 mU/L (95% CI 0.02 to 0.22 mU/L) for TSH. A considerable number of individuals was still in the subclinical range (T3 concentrations 40.0 pM (95% CI 37.5 to 47.0 pM) for FT4, 3.80 nM (95% CI 3.35 to 4.05 nM) for T3 and 0.02 mU/L (95% CI 0.02 to 0.22 mU/L) for TSH. A considerable number of individuals was still in the subclinical range (T3 concentrations 40.0 pM (95% CI 37.5 to 47.0 pM) for FT4, 3.80 nM (95% CI 3.35 to 4.05 nM) for T3 and 0.02 mU/L (95% CI 0.02 to 0.22 mU/L) for TSH. In the control situation, without treatment of levothyroxine, thyroid hormone values remained within the normal reference range. (Table 3).

Levothyroxine exposure significantly prolonged clot-lysis times compared to the control situation; median change 14% (95% CI 9 to 17%) vs 2% (95% CI -4 to 4%), p<0.01. Although not statistically significant, a trend towards reduced plasma TAFI levels was observed after levothyroxine exposure compared to the control situation; median change -3% (95% CI -10 to 0%) vs 4% (95% CI -2 to 13%), p=0.11. In addition, levothyroxine exposure significantly enhanced TAFIa-dependent prolongation of clot-lysis times compared to no medication; median change 19% (95% CI 5 to 33%) vs 7% (95% CI -6 to 13%), p=0.03. (Table 3).
Table 3. Healthy volunteers: more pronounced hyperthyroidism.

<table>
<thead>
<tr>
<th></th>
<th>No medication</th>
<th>Levothyroxine 0.45/0.6 mg per day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t=0 days</td>
<td>t=14 days</td>
</tr>
<tr>
<td>FT4 (pM)</td>
<td>15.0 (12.5; 15.0)</td>
<td>14.0 (13.5; 15.0)</td>
</tr>
<tr>
<td>T3 (nM)</td>
<td>1.90 (1.70; 2.20)</td>
<td>2.10 (1.95; 2.40)</td>
</tr>
<tr>
<td>TSH (mU/L)</td>
<td>1.64 (1.19; 3.07)</td>
<td>1.65 (1.49; 2.97)</td>
</tr>
<tr>
<td>Clot-lysis time (% pooled plasma)</td>
<td>89 (79; 95)</td>
<td>88 (83; 95)</td>
</tr>
<tr>
<td>TAFIa-dependent prolongation of clot-lysis (% pooled plasma)</td>
<td>82 (70; 106)</td>
<td>94 (74; 108)</td>
</tr>
<tr>
<td>TAFI concentration (% pooled plasma)</td>
<td>85 (66; 90)</td>
<td>86 (73; 90)</td>
</tr>
</tbody>
</table>

FT4 indicates free thyroxine; T3, tri-iodothyronine; TSH, thyrotropin; and TAFI, thrombin-activatable fibrinolysis inhibitor. Data are presented as medians (95% confidence intervals).

Hypothyroidism

To evaluate the effect of hypothyroidism on TAFI, 20 patients with newly diagnosed overt hypothyroidism were studied, of whom 7 men and 13 women. All patients reached euthyroid state after substitution therapy. Patients had a mean age of 52 (range 25 to 86) years, with a body mass index of 27 (range 18 to 40) kg/m2. (Table 1).

The median thyroid hormone levels at inclusion were consistent with hypothyroidism; 6.5 pM (95% CI 4.5–15.0) for FT4 and 58.37 nM (95% CI 45.76–91.09) for TSH. Levels returned to normal values after treatment with levothyroxine. (Table 4).

Patients showed significantly reduced clot-lysis times during hypothyroidism compared to euthyroidism; median 89% (95% CI 71 to 108%) vs 114% (95% CI 92 to 125%), p<0.01. Plasma TAFI levels did not differ between the hypothyroid and the euthyroid state; median 98% (95% CI 93 to 115%) vs 96% (95% CI 90 to 105%), p=0.20, while TAFIa-dependent prolongation of clot-lysis was reduced during hypothyroidism compared to euthyroidism; median 89% (95% CI 71 to 108%) vs 114% (95% CI 92 to 125%), p<0.01. (Table 4).

TAFI in hyper- and hypothyroidism

Table 4. Hypothyroidism.

<table>
<thead>
<tr>
<th></th>
<th>Hypothyroidism</th>
<th>Euthyroidism</th>
<th>Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT4 (pM)</td>
<td>6.5 (5.0; 8.0)</td>
<td>17.0 (16.0; 18.0)</td>
<td>10.5 (11.3; 20.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TSH (mU/L)</td>
<td>58.37 (45.76; 91.09)</td>
<td>1.94 (0.87; 3.29)</td>
<td>-56.43 (0.08; 92.35)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Clot-lysis time (% pooled plasma)</td>
<td>93 (88; 102)</td>
<td>106 (96; 110)</td>
<td>13 (5; 16)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TAFIa-dependent prolongation of clot-lysis (% pooled plasma)</td>
<td>89 (71; 108)</td>
<td>114 (92; 125)</td>
<td>25 (9; 24)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TAFI concentration (% pooled plasma)</td>
<td>98 (93; 115)</td>
<td>96 (90; 105)</td>
<td>-2 (14; 3)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

FT4 indicates free thyroxine; TSH, thyrotropin; and TAFI, thrombin-activatable fibrinolysis inhibitor. Data are presented as medians (95% confidence intervals).

Discussion

This study showed that hyperthyroidism results in hypofibrinolysis and enhanced TAFIa-dependent prolongation of clot-lysis times, while a trend towards reduced plasma TAFI levels was observed. A hyperfibrinolytic condition with reduced TAFIa-dependent prolongation of clot-lysis times was found in hypothyroidism, despite unaltered TAFI levels.

Our findings corroborate previous reports describing reduced plasma TAFI levels in patients with hyperthyroidism or in patients receiving thyroid hormone substitution therapy. Reduced plasma TAFI levels suggest a hyperfibrinolytic state, however, this is inconsistent with the overall hypofibrinolytic condition observed in hypothyroid patients. Although the fibrinolytic condition in patients with hyperthyroidism is best explained by simultaneous alterations in levels of plasminogen activator inhibitor (PAI), TAFI activity may play an additional role.

To our knowledge, this study investigated for the first time a role of TAFI in an in vitro fibrinolysis assay during hyper- or hypothyroidism. In these experiments, coagulation was initiated by tissuefactor and thereby TAFI activation occurs only via thrombin that has been generated by the coagulation cascade. We observed increased TAFIa-dependent prolongation of clot-lysis times in individuals with hyperthyroidism, indicating that TAFI may shift the fibrinolytic balance towards a hypofibrinolytic condition. A hyperfibrinolytic condition is a risk factor for cardiovascular disease or thrombosis, and TAFI may thereby contribute to the development of thrombotic events in patients with hyperthyroidism. Conversely, patients with hypothyroidism showed a reduced TAFIa-dependent prolongation of fibrinolysis, suggesting that TAFI may be involved in the increased bleeding tendency as is observed in these patients.
Interestingly, in individuals with thyroid hormone excess or deficiency, the changes in plasma TAFI levels are conflicting with respect to the antifibrinolytic activity of TAFI. This might be explained by TAFI activation via thrombin. Since the procoagulant changes observed in patients with hyperthyroidism may result in enhanced thrombin generation, this may subsequently give rise to increased TAFI activation, independent of the plasma TAFI concentration, and vice-versa in patients with hypothyroidism. An alternative explanation for the paradoxical combination of TAFI levels and its antifibrinolytic function may be that other biological mechanisms are affected by TAFI. Since TAFI is known to play a role in inflammation processes\(^{1,12}\), it would be interesting to investigate inflammatory markers in future studies.

In conclusion, alterations of TAFIa-dependent prolongation of clot-lysis in patients with thyroid disorders may cause an impaired haemostatic balance. The disturbed haemostatic balance in patients with hyperthyroidism might make them more prone for thrombosis, while the risk for bleeding may increase in patients with hypothyroidism.

Acknowledgements

We thank all volunteers for their willingness and enthusiasm to participate in this trial. We owe many thanks to Eric Fliers, professor at the department of Endocrinology and Metabolism of the Academic Medical Centre, Amsterdam, for his advice on the levothyroxine doses administered. We also thank Matthijs van Wissen, Jiri Wagenaar, Patrick Smit and Sara Rafi for their efforts as independent physicians, and Huib Bout and Natasja Huisman for their help in blood processing and laboratory procedures.

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