The hormonal influence on the haemostatic system and the risk of thrombosis
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The effect of thyroid hormone analogue on coagulation and fibrinolysis: a double-blind randomized controlled trial

Danka JF Stuijver, Fleur M van der Valk, Bregje van Zaane, Suzanne Battjes, Joost CM Meijers, Erik S Stroes, Harry R Büller, Victor EA Gerdes
ABSTRACT

Background
Recently, the development of synthetic thyroid hormone mimetic compounds and metabolites have received attention as potential treatment modalities for hypercholesterolemia and obesity. As thyroid hormone treatment has been found to induce a hypercoagulable state, we wondered whether treatment with a thyroid hormone metabolite, 3,5-diiodothyronine (T2) leads to activation of coagulation and inhibition of fibrinolysis.

Methods
A double-blind, multicentre trial was performed in which 40 male patients with the metabolic syndrome were randomised to receive either the thyroid hormone metabolite TRC150094 (at a dose of 50mg per day) or placebo for 28 days. For the present study, parameters of thyroid function and haemostasis were assessed before and after treatment.

Results
Treatment with TRC150094 (T2) did not affect parameters of coagulation or fibrinolysis. After treatment, levels of thyroid hormone free thyroxine (FT4) increased by 11% and triiodothyronine (T3) by 21%. Levels of thyroid stimulating hormone were not affected.

Conclusions
In this 4 week trial, treatment with the thyroid hormone derivate (T2) did not lead to activation of coagulation and inhibition of fibrinolysis, however, it does appear to affect thyroid hormone metabolism.
INTRODUCTION

The potential beneficial metabolic effects of thyroid hormones have long intrigued investigators and pharmaceutical companies as they could be of use to treat obesity and its related co-morbidities. Recently, the development of both synthetic thyroid hormone mimetic compounds and metabolites have received attention as potential treatment modalities for lowering cholesterol, increasing peripheral insulin sensitivity and promoting fat loss. Most thyroid hormone mimetic compounds, or analogues, are designed to selectively bind and activate the β1 isoform of the nuclear thyroid hormone receptor (TRβ), which is highly expressed in the liver, and have hardly any affinity for the α1 isoform, which is expressed in the cardiac system. This way, the favourable metabolic effects of thyroid hormone without concurrent adverse effects are mimicked. Short-term trials have confirmed the efficacy of TRβ agonists in lowering levels of atherogenic lipoproteins or body weight, without eliciting adverse effects. Other promising therapeutic options are the naturally occurring thyroid hormone metabolites, such as the thyronamines, 3,3-diiodotyronine (3,3-T2) and 3,5-diiodothyronine (T2). These metabolites are thought to have lesser affinity towards the thyroid hormone receptor and therefore, possibly, exert fewer thyrotoxic effects than T3. Indeed, T2 has been proposed to effectively and safely improve the metabolic profile at relatively low concentrations.

Although these beneficial effects of thyroid hormone analogues and metabolites on lipids and body weight are thought to protect against cardiovascular disease, thyroid hormones have been suggested to induce a hypercoagulable state. Several studies have described an increase in coagulation factor levels after start of thyroid hormone therapy, both in hypothyroid patients and in euthyroid subjects treated with levothyroxine (T4). Since the target subjects for these novel therapeutic agents are mostly patients with the metabolic syndrome, whose overweight has already put them at high risk for both arterial and venous thrombosis, a rise in coagulation factors may have clinical implications, especially in the beginning of treatment. Nevertheless, despite the increasing amount of evidence on the prothrombotic effects of thyroid hormone, none of the currently available trials have evaluated whether the same is true for thyroid hormone analogues and metabolites.

Fortunately, we were recently given the opportunity to partake in a phase 2A trial on TRC150094, a T2 metabolite, in male subjects with the metabolic syndrome. The aim of the present analysis was therefore to assess whether treatment with TRC150094 (T2) leads to activation of coagulation and inhibition of fibrinolysis.
Study design

The primary aim of this phase 2A, double-blind, placebo-controlled trial was to study the safety and efficacy of TRC150094 for 28 days in increasing insulin sensitivity in male patients with increased cardiometabolic risk. In the present analysis, we assessed parameters of coagulation and fibrinolysis before and after treatment.

Twenty subjects were enrolled at Veeda Clinical Research, India, and another 20 subjects at the Academic Medical Centre, the Netherlands. The maximum duration of participation in the study for each subject was 9.5 weeks including an approximately 4-week screening period, 4 weeks of treatment and a 10-day post-treatment follow-up evaluation period. All participants provided written informed consent. The study protocol was approved by the local institutional review board in the Netherlands and India.

Patient selection

Caucasian patients were recruited from the outpatient clinic of the Academic Medical Centre, Amsterdam, the Netherlands, and Indian patients from the outpatient clinic of Veeda, India. Eligible patients were male subjects, aged 30 to 65 years, with a diagnosis of metabolic syndrome based on the following criteria: waist circumference ≥ 102 cm, triglycerides ≥ 150 mg/dL, blood pressure ≥130/85 mm Hg or use of medication for hypertension, fasting glucose > 5.8 mmol/l and < 9.0 mmol/l at screening or on day -1. All patients had to have a stable weight during 3 months prior to inclusion.

Subjects were excluded if they had a history of arterial thrombosis or clinically relevant abnormalities on the 12-lead electrocardiogram at screening; history of thyroid disease; current use of antidiabetic treatment; renal disease; liver disease; abuse of alcohol or drugs; or a history of significant blood loss due to any reason, including blood donation, in the 12 weeks prior to the first dose of study drug.

Study procedures

Each subject attended the study centre in a fasting state for a total of 5 visits during the study period: one screening visit, 2 study visits (one baseline and one end of treatment), 1 intermediate safety visit and 1 post-study follow-up visit. Physical examination, vital signs, safety biochemistry and laboratory investigations were performed at each visit by a physician blinded for treatment allocation. After screening, eligible subjects were randomized to receive TRC150094 or placebo in a ratio of 1:1. Participants were instructed to take a fixed dose of 50 mg once daily (morning) under fasting conditions. Dosing took place on days 1 up to 28. Subjects received labeled bottles with entericoated tablets containing either active treatment or placebo. For further details see the study protocol.
Randomization, compliance and withdrawal
Randomization was performed via computer-generated sequences corresponding to either the study drug or placebo. In case of any adverse drug reaction, the randomisation was disclosed and the subject was withdrawn from the study. In all cases, the reasons for withdrawal were recorded. Participants who were withdrawn were replaced by new patients and were not included in the analysis. At study completion, all participants were asked to report the number of doses missed.

Laboratory investigations.
Fasting venous blood samples for tests of coagulation and fibrinolysis were drawn at baseline and day 28. Blood samples were collected in 0.5 mL plain tubes for assessment of thyroid function, and in 5 mL trisodium citrate containing tubes for tests of coagulation and fibrinolysis (BD Vacutainer, Plymouth, UK). Citrated blood was immediately centrifuged, and the supernatant re-centrifuged, for 15 minutes at 2500x g at 15 °C to obtain platelet poor plasma. Plasma was aliquoted and stored at -80 °C until further use. Serum levels of thyroid hormones and tests of coagulation and fibrinolysis were performed in batch after all participants had completed the study, at the Academic Medical Centre, Amsterdam. Prothrombin time (PT), activated partial thromboplastin time (aPTT), and functional assays of coagulation factor VIII (FVIII:C), IX (FIX) and von Willebrand factor ristocetin cofactor activity (VWF:RiCo) were performed using an automated coagulation analyzer (Behring Coagulation System, Dade Behring, Marburg, Germany) with reagents and protocols of the manufacturer (Siemens Healthcare Diagnostics). D-dimer (Innovance, Siemens Healthcare Diagnostics) was also determined on the Behring Coagulation System. For the measurement of von Willebrand factor antigen (VWF:Ag) we used in-house enzyme linked immunosorbent assays (ELISA) with antibodies from DAKO (Glostrup, Denmark). Prothrombin fragment 1+2 (Enzygnost monoclonal F1+2, Dade Behring), plasmin-antiplasmin complexes (PAP, DRG diagnostics, Marburg, Germany) and plasminogen activator inhibitor type-1 antigen (PAI-1:Ag, Hyphen BioMed, Andrésy, France) were determined by ELISA. Endogenous thrombin potential (ETP) was determined with a calibrated automated thrombogram (CAT). The CAT assays the generation of thrombin in cloting plasma using a microtiter plate reading fluorometer (Fluoroskan Ascent, ThermoLab systems, Helsinki, Finland) and Trombinscope software (Thrombinscope BV, Maastricht, the Netherlands). Three parameters were derived from the thrombin generation curve: lag time, peak height and ETP (area under the curve). Reference values for thyroid hormones, and coagulation and fibrinolytic parameters are shown in the Tables.
Statistical analysis

For the primary outcome of this study, a sample size of 20 in each group was calculated to be sufficient to detect an absolute difference in peripheral insulin sensitivity, before and after treatment.\textsuperscript{12} As we were able to detect significant differences in our previously performed randomized cross-over study in which 16 volunteers were treated with high dose levothyroxine for 28 days, we hoped this number to be sufficient for the present analysis as well.\textsuperscript{11,12} Data for qualitative variables are presented as incidence rates (N, number and percent). The data of continuous variables were summarised using measures of central tendency (i.e. mean, median) and dispersion (i.e. standard deviation, range). Continues data were analysed with parametric or non-parametric tests depending on the data distribution verified by the Shapiro-Wilk test. Within-group comparisons of pre- and post-treatment values were performed using the paired samples t-test or Wilcoxon signed ranks test. We calculated relative changes per parameter for each individual by subtracting the pre-treatment value from the post treatment value and multiplying by 100%. Between-group comparisons of the relative change were performed using the unpaired samples t-test or Mann–Whitney U test.

Statistical analysis was performed using SPSS 16.0.1 (statistical software).

Table 1. Baseline characteristics of participants.

<table>
<thead>
<tr>
<th></th>
<th>TRC150094 (n = 20)</th>
<th>Placebo (n = 20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean, years</td>
<td>48.2 (31-66)</td>
<td>49.6 (32-64)</td>
<td>0.67</td>
</tr>
<tr>
<td>BMI, mean, eenheid</td>
<td>33.2 (27.1-43.1)</td>
<td>33.7 (27.5-45.8)</td>
<td>0.71</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>140.4 (117-160)</td>
<td>137.9 (119-160)</td>
<td>0.42</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>87.3 (74-93)</td>
<td>86.9 (70-100)</td>
<td>0.85</td>
</tr>
<tr>
<td>Pulse frequency, min</td>
<td>71 (53-94)</td>
<td>70.4 (52-93)</td>
<td>0.85</td>
</tr>
<tr>
<td>Haemoglobin, mmol/l</td>
<td>9.0 (7.6-10.2)</td>
<td>8.9 (7.6-10.9)</td>
<td>0.74</td>
</tr>
<tr>
<td>Platelets, x10^9/L</td>
<td>276 (172-512)</td>
<td>260 (119-424)</td>
<td>0.49</td>
</tr>
<tr>
<td>Leukocytes, x10^9/L</td>
<td>6.4 (1.2-10.6)</td>
<td>7.5 (5.4-9.6)</td>
<td>0.86</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>67.0 (39.7-101)</td>
<td>68 (39.98)</td>
<td>0.87</td>
</tr>
<tr>
<td>Alanine aminotransferase, U/l</td>
<td>39.9 (20.4-106.4)</td>
<td>34.2 (19-61)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

BMI indicates body mass index.
Baseline characteristics are presented as means (range).

RESULTS

Between September 2011 through February 2012 we randomly assigned 40 men to TRC150094 (n = 20) or placebo (n = 20). Mean age was 48.2 years in the TRC150094 group and 49.6 years
in the control group. Hematology and clinical chemistry showed normal bloodcount, liver, and renal functions at baseline. All participants completed the study protocol. No patients reported to have missed a daily treatment dose. Table 1 shows the baseline characteristics.

**Thyroid function**

Results are summarized in Table 2. Mean levels of FT4 and T3 markedly increased in the treatment group with 11% and 21%, compared to an 3% increase and 5% decrease in the placebo group, respectively. The mean levels of FT4 and T3, however, remained within their respective reference ranges. No change in the level of TSH was detected in the patients on either TRC150094 or placebo.

**Coagulation and fibrinolytic parameters**

Median baseline values for haemostatic parameters were slightly higher for TRC150094 group compared to the placebo group. However, no significant changes were observed for any of the haemostatic parameters, neither between pre- and post-treatment values within groups, nor in post-treatment values between groups. In Table 2 the details are depicted. In the TRC150094 group, the individual relative change of FT4 was related to the change in levels of PAI-1 ($\beta 0.49 \ p=0.03$), F1+2 ($\beta 0.57, \ p=0.01$) and APTT ($\beta -0.58, \ p<0.01$). A significant correlation between change in haemostatic parameters and change in T3 was not found.

**DISCUSSION**

In this 4 week trial, treatment with the naturally occurring metabolite 3,5-diiodothyronamine (T2) TRC150094 did not result in an activation of coagulation or inhibition of fibrinolysis, even though a significant increase in levels of the thyroid hormones FT4 and T3 was found. This study is the first to assess the effect of a thyroid hormone derivate on the coagulation and fibrinolytic system. Thyroid hormone excess has been found to induce a hypercoagulable and hypofibrinolytic state, both in patients with hyperthyroidism, and in healthy subjects after administration of levothyroxine (synthetic T4).$^{13-15}$ How thyroxine influences the coagulation system is not well known, however *in vitro* studies have shown a direct effect of T3 on receptor-mediated protein synthesis of coagulation and fibrinolytic proteins at the hepatic and endothelial level.$^{16,17}$ We could not detect an effect of administration of T2 in the current dose on the haemostatic system. This may be explained by the suggested nongenomic effects of T2 due to a direct interaction with the mitochondria, while the effects of T3 and T4 are initiated via the nucleus.$^{7,18,19}$ The rapid effects of T2 are independent of protein synthesis, due to an affinity with the thyroid receptor which is 40- to 500-fold lower than that of T3.$^{20,21}$ Another possibility is that the dose of T2 was too low. In this study, administration
Table 2. Parameters of thyroid function, coagulation and fibrinolysis before and after treatment.

<table>
<thead>
<tr>
<th>Parameter (reference range)</th>
<th>Placebo ($n = 20$) Baseline</th>
<th>Day 28</th>
<th>Relative change (%)</th>
<th>TRC150094 ($n = 20$) Baseline</th>
<th>Day 28</th>
<th>Relative change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thyroid function</strong></td>
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<tr>
<td>FT4 (10-23 pmol/L)</td>
<td>12.11 (11.25; 12.97)</td>
<td>12.32 (11.41; 13.23)</td>
<td>2.53 (-4.00; 9.07)</td>
<td>11.73 (10.74; 12.72)</td>
<td>12.57 (11.65; 3.49)</td>
<td>10.57 (-2.48; 23.62)</td>
</tr>
<tr>
<td>T4 (60 – 140 nmol/L)</td>
<td>88.75 (80.47; 97.03)</td>
<td>90.75 (82.12; 99.38)</td>
<td>2.76 (-2.31; 7.84)</td>
<td>84.00 (77.85; 90.15)</td>
<td>81.75 (75.90; 87.60)</td>
<td>-1.97 (-7.60; 3.65)</td>
</tr>
<tr>
<td>T3 (1.2-2.8 nmol/L)</td>
<td>2.04 (1.89; 2.19)</td>
<td>1.93 (1.78; 2.09)</td>
<td>-4.69 (-10.67; 1.29)</td>
<td>1.93 (1.74; 2.11)</td>
<td>2.25 (2.07; 2.43)</td>
<td>20.81 (6.90; 34.70)</td>
</tr>
<tr>
<td>TSH (0.35-4.7 mIU/L)</td>
<td>1.86 (1.55; 2.17)</td>
<td>2.01 (1.51; 2.51)</td>
<td>10.92 (-11.5; 33.33)</td>
<td>2.10 (1.63; 2.57)</td>
<td>2.23 (1.70; 2.76)</td>
<td>10.57 (-2.48; 23.62)</td>
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<tr>
<td><strong>Coagulation</strong></td>
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<tr>
<td>PT (10.7-12.9 sec)</td>
<td>12.5 (11.5; 13.3)</td>
<td>13.2 (11.5; 14.0)</td>
<td>3.44 (-1.6; 6.2)</td>
<td>12.3 (11.4; 13.0)</td>
<td>12.4 (11.5; 13.2)</td>
<td>0.0 (-2.6; 3.1)</td>
</tr>
<tr>
<td>aPTT (25.0-38.0 sec)</td>
<td>33.5 (30.2; 36.2)</td>
<td>32.1 (28.5; 35.6)</td>
<td>0.0 (-8.20; 3.48)</td>
<td>32.7 (30.0; 36.4)</td>
<td>30.6 (28.6; 33.3)</td>
<td>-2.15 (-8.93; 2.16)</td>
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<tr>
<td>VWF:Ag (50-150%)</td>
<td>107.5 (91.6; 129.3)</td>
<td>108.0 (86.0; 140.0)</td>
<td>-2.1 (-11.3; 13.8)</td>
<td>102.0 (75.3; 125.8)</td>
<td>93.5 (82.0; 120.0)</td>
<td>0.0 (-14.2; 9.8)</td>
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<td>VWF:RCo (58-172%)</td>
<td>109 (94; 126.3)</td>
<td>101.0 (95.0; 129.0)</td>
<td>-4.9 (-12.3; 4.8)</td>
<td>103.0 (65.3; 122.5)</td>
<td>89.5 (66.5; 120.8)</td>
<td>-6.8 (-11.6; 3.2)</td>
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<tr>
<td>Parameters</td>
<td>Mean (95% CI)</td>
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<td><strong>Thyroid hormone analogue (T2) and coagulation</strong></td>
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<td>Thyroid hormone analogue (T2)</td>
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<td>T3</td>
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<td>FT4</td>
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<tr>
<td>FT4 indicators</td>
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</table>
| FT4 indicates free thyroxine; T3, tri-iodothyronine; TSH, thyroid stimulating hormone (thyrotropin); PT, prothrombin time; aPTT, activated partial thromboplastin time; VWF, von Willebrand factor; Ag, antigen; RiCo, ristocetin cofactor activity; ETP, endogenous thrombin potential; and PAI-1, plasminogen activator inhibitor-1. Data are presented for thyroid hormones as means (95% confidence interval) and for haemostatic parameters as medians (interquartile range).
of TRC150094 did induce a rise in T3 and free T4 levels which could theoretically lead to an upregulation of coagulation factors, however the rise in thyroid hormone levels was subtle and may be too small to detect any differences in coagulation parameters as compared to studies in which synthetic T4 is given.

The most attractive thyromimetic actions of thyroid hormone derivates, lipid lowering and calorigenesis, are primarily exerted in the liver. Short-term trials with newly developed TRβ selective agonist that bind and activate the β1 isoform of the nuclear thyroid hormone receptor and have hardly any affinity for the α1 isoform expressed in the cardiac system, have confirmed the efficacy of TRβ agonists in lowering levels of atherogenic lipoproteins or body weight, without eliciting adverse effects. Given the idea of a thyroid hormone-mediated upregulation of coagulation factors at the hepatic level, TRβ selective agonist might be able to affect the regulation of liver-synthesized clotting factors. The fact that the dose of T2 used in the present study did not show effects does not rule out the possibility that TRβ selective agonists may have an effect on coagulation or fibrinolysis.

Whether the slight changes in free T4 and T3 are a chance finding, or an indirect effect of the T2 administration needs to be investigated. Intuitively, one would expect a decrease of T3 or free T4 unless the assays for free T4 or T3 do pick up some of the administered TRC150094. This is also of importance for the observation that changes in free T4, but not T3, were associated to changes in PAI-1, F1+2 and APTT in the TRC150094 group.

The limitations of this study merit some considerations. First, this study is likely to be underpowered due to a relatively small sample size and short trial period. However, the power analysis was based on other primary outcomes which will be analysed and published separately. Also, we found baseline characteristics for haemostatic parameters to slightly differ for both study groups. We know that these parameters show a high inter-person variability within the healthy population, which makes it difficult to compare between individuals. Although modest effects might have gone undetected due to this variability, the relative changes did not show marked differences, which make it unlikely that major effects of TRC150094 on haemostasis were missed.

In conclusion, we could not detect an effect of T2 on coagulation and fibrinolysis. Since thyroid hormone derivates deserve further study as potential agents in the treatment of dyslipidemia and other risk factors for atherosclerosis, we would urge future prospective clinical trials to assess the effect on coagulation and fibrinolysis, both on laboratory parameters and clinical endpoints, of this class of agents.
REFERENCES