The hormonal influence on the haemostatic system and the risk of thrombosis
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Thyroid dysfunction and fibrin network structure: A mechanism for increased thrombotic risk in hyperthyroid individuals

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ABSTRACT

Background
Hyperthyroidism is associated with increased thrombosis risk and fibrin clot structure determines susceptibility to vascular thrombotic events. We aimed to investigate clot formation and lysis in hyperthyroidism using observational and interventional studies.

Methods
Ex vivo fibrin clot structure/fibrinolysis and plasma levels of thrombotic/inflammatory markers were investigated in hyperthyroid individuals (n=24) and matched controls (n=19), using turbidimetric assays, ELISA, and confocal and electron microscopy. The effects of normalizing thyroid function were analyzed (n=19) and the role of short-term exogenous hyperthyroidism in healthy volunteers studied (n=16).

Results
Hyperthyroid subjects displayed higher clot maximum absorbance compared with controls (0.41±0.03 and 0.27±0.01 arbitrary units, respectively; P<0.01), and longer clot lysis time (518±23 and 461±18 sec, respectively; P<0.05), which correlated with freeT4 levels. Plasma levels of fibrinogen and plasminogen activator inhibitor-1 were significantly higher in patients compared with controls. Normalizing thyroid function in 19 subjects was associated with lower maximum absorbance and shorter lysis time, accompanied by reduction in fibrinogen, plasminogen activator inhibitor-1, and D-dimer levels. Complement C3, but not C-reactive protein, levels were higher in hyperthyroid subjects compared with controls (0.92±0.05 and 0.64±0.03 g/liter, respectively; P<0.01), correlated with clot structure parameters, and decreased after intervention. Confocal and electron microscopy confirmed more compact clots and impaired fibrinolysis during hyperthyroidism. Exogenous hyperthyroidism in healthy volunteers had no effect on any of the clot structure parameters.

Conclusions
Endogenous hyperthyroidism is associated with more compact clots and resistance to fibrinolysis ex vivo, related to the degree of hyperthyroidism and C3 plasma levels, and these changes are modulated by achieving euthyroidism. Altered clot structure/lysis may be one mechanism for increased thrombotic risk in hyperthyroidism.
INTRODUCTION

Although a possible link between hyperthyroidism and increased thrombosis potential was described more than a century ago, the reported evidence proposing an increased arterial and venous thrombotic risk in thyrotoxicosis is more recent. Studies have shown that hyperthyroidism is associated with increased plasma levels of procoagulant and antifibrinolytic clotting factors, including fibrinogen and plasminogen activator inhibitor-1 (PAI-1), concluding that hyperthyroidism is associated with a prothrombotic milieu. Studies, however, have generally concentrated on analyzing a limited number of coagulation factors and therefore been unable to fully address the balance between thrombosis and fibrinolysis. It is now well established that structure of the fibrin network can determine predisposition to arterial and venous thrombotic events; clots composed of thin fibers, small pores, and compact structure are resistant to fibrinolysis and associated with increased risk of occlusive vascular disease. Direct analysis of clot structure/fibrinolysis can determine the balance between prothrombotic and fibrinolytic factors, allowing the global assessment of fibrin-related thrombosis risk, which hitherto has not been evaluated in patients with thyroid dysfunction.

Thrombotic events are also associated with chronic low-grade inflammation; both C-reactive protein (CRP) and plasma complement C3 levels having been shown to predict ischemic events. Although the mechanisms linking inflammation to increased thrombosis risk are not fully understood, several pieces of evidence suggest important interactions between the complement system and coagulation proteins. Recent work detected C3 in the fibrin clot, which may modulate neutrophil recruitment to the site of injury and may influence clot lysis. Using purified proteins, we have recently demonstrated that C3 is incorporated into the clot during fibrin network formation, particularly in pathological conditions, thereby directly influencing the fibrinolytic process.

In the current study, we explore, for the first time, clot structure/fibrinolysis and analyze plasma levels of clotting factors/inflammatory markers in subjects with hyperthyroidism compared with a matched control group. In addition to this observational study, we conduct two interventional studies evaluating the effect of normalizing thyroid function and the role of exogenous hyperthyroidism on fibrin-related thrombosis potential.
METHODS

Patient recruitment
We assessed the effects of endogenous and exogenous hyperthyroidism on clot structure/fibrinolysis using a cross-sectional observational and two longitudinal interventional studies. We recruited 24 hyperthyroid subjects from the local endocrine clinic at Leeds Teaching Hospitals Trust. Exclusion criteria included individuals with known cardiovascular disease, stroke, deep vein thrombosis, pulmonary embolus, or hematological or malignant disorder or on any regular treatment at the time of diagnosis. Individuals above the age of 60 yr and below 18 yr were not included, and the study was approved by the local research ethical committee. All participants gave informed consent. This group of patients was compared with 19 age-matched healthy controls, not on any treatment, in the observational part of the study. Individuals with hyperthyroidism were rendered euthyroid through medical treatment; a total of 19 individuals completed this part of the study with repeat samples taken after a mean period of 6.5 ± 0.8 months.

To assess the specific effects of thyroid hormones *per se* on clot structure and fibrinolysis, levothyroxine was administered to healthy volunteers in a cross-sectional design as detailed elsewhere. Briefly, 16 subjects in part A were prescribed 0.3 mg levothyroxine/d or placebo with blood samples taken at baseline and 14 d. In part B, 12 subjects took either 0.45 mg levothyroxine/d (if body mass was ≤80 kg) or 0.6 mg/d (if >80 kg) or placebo. Blood samples were drawn at baseline and 14 days.

Sample collection
Blood samples were collected midmorning into citrated tubes, spun down within 2 h of collection with the plasma being stored at −40 C until analysis. The first 5 ml of blood was used for clinical laboratory diagnosis, and the rest of the blood drawn was used for clot structure analysis. All blood samples were taken without the use of a tourniquet. Immunoassays were employed for estimation of thyroid hormone and TSH plasma levels using Siemens Centaur (Siemens Healthcare Diagnostics, Camberley, UK), whereas TSH-binding inhibitory Ig (TBI) test was used for TSH-receptor antibodies (University Hospital of Wales, Cardiff, UK).

Laboratory investigations

Clot structure analysis
This was undertaken using turbidimetric analysis as described before and the following parameters were recorded. Maximum absorbance (MA) is the maximum OD of the clot, a
measure of fibrin network density. Higher MA of plasma clots has been consistently associated with increased cardiovascular risk in various studies.\textsuperscript{21-23} Lysis time (LT), the time from full clot formation to 50\% lysis, is an indicator of fibrinolytic potential; a longer LT is believed to be associated with increased cardiovascular risk.\textsuperscript{24,25} Lysis area (LA) is area under the curve, a complex broader measure of clot formation time, clot density, and lysis potential. Larger LA is associated with increased cardiovascular risk.\textsuperscript{21}

**Measurement of C3, CRP, PAI-1, and fibrinogen plasma concentrations**

Both plasma complement C3 and CRP were determined using in-house ELISA as previously described.\textsuperscript{14} PAI-1 (Invitrogen, Paisley, UK) and D-dimer (American Diagnostica Inc., Stamford, CT) were assessed by ELISA according to the manufacturer’s protocol, whereas fibrinogen levels were determined using the Clauss method.\textsuperscript{26}

**In vitro assessment of carbimazole effects**

Carbimazole was the standard treatment regimen for thyrotoxic patients, so the effects of methimazole, the biologically active metabolite of carbimazole, on clot structure and lysis were evaluated to ensure it was not responsible for any of the observed changes. Methimazole was added to plasma samples at final dilutions in 0.9\% saline of 0, 0.156, 0.312, 0.625, 1.25, 2.5, 5, and 10 \(\mu\)g/ml to reflect \textit{in vivo} plasma concentrations after drug administration, normally 0.1–1.0 \(\mu\)g/ml.\textsuperscript{27} The same turbidimetric protocol was employed as before, using methimazole-spiked plasma samples from two control and two thyrotoxic subjects.

**Laser scanning confocal microscopy (LSCM)**

Fibrin clots from pooled plasma samples were visualized using confocal microscopy. A pooled plasma of 10 random hyperthyroid subjects and a pool of the same individuals in the euthyroid phase were compared both with each other and with a pool of 10 normal control plasma samples. One 15-\(\mu\)l Ibidi (Applied Biophysics, Troy, NY) slide was used, and 7.5 \(\mu\)l plasma was added in triplicate to a well. The fibrin clot was created by adding 21 \(\mu\)l permeation buffer, 1.5 \(\mu\)l fluorescent-labeled fibrinogen (0.036 g/liter Alexa Flour Dye; Invitrogen, Paisley UK), and 5 \(\mu\)l activation mix [containing 0.05 U/ml human thrombin (Calbiochem, Nottingham, UK) and 5 mmol/liter \(\text{CaCl}_2\) in permeation buffer]. Clots were visualized through an LSM 510 META microscope with a \(\times40\) immersion lens (both Carl Zeiss UK Ltd., Welwyn Garden City, Hertfordshire, UK).

**Scanning electron microscopy (SEM)**

Clots were prepared using pooled plasma samples from healthy controls and patients in the hyperthyroid and euthyroid phase (\(n = 10\) each group) as previously described.\textsuperscript{26} Briefly,
plasma pools from healthy controls, hyperthyroid patients during thyrotoxic and euthyroid phases were diluted 1:1 in permeation buffer, and subsequently, 45 μl of diluted plasma was added to 5 μl of activation mix containing 10 U/ml thrombin and 50 mmol calcium chloride. Samples were incubated in a moist chamber for 2 h, washed with sodium cacodylate buffer, and fixed in 2% glutaraldehyde. Clots were further processed by stepwise dehydration and sputter coated with platinum palladium. Fiber diameters of all clots were measured with image analysis software package ImageJ version 1.23y (National Institutes of Health, Bethesda, MD).

**Statistical analysis**

Between group analysis was performed using T-test or Mann-Whitney test for normally distributed and non-parametric data, respectively. For within group analysis, paired tests were used. Pearson and Spearman Rank correlation coefficients were used as appropriate to elicit underlying relationships between all parameters measured.

Given initial pilot data in hyperthyroid subjects, as well as data in healthy control, power calculations suggested that including 16 subjects will be enough to detect 10% change in clot final turbidity or lysis time, with a power of 80% at p=0.05. Therefore, we planned to recruit 22-24 subjects with the assumption of 10-15% drop-out rate.

**RESULTS**

**Subject characteristics**

Of the 24 hyperthyroid subjects recruited, 19 completed the interventional part of the study (one omitted due to starting additional therapy, two withdrawals for personal reasons, and two lost to follow-up after moving away from the study center). Only individuals who completed the study are included in the data presented below. Basic characteristics of patients and controls are summarized in Table 1. Diagnosis of Graves’ disease (GD) was made in subjects with clinical and biochemical thyrotoxicosis in the presence of at least two of the following: a palpable smooth/uniform goiter, extrathyroidal manifestations of GD, detection of thyroid-specific antibodies, or personal or family history of organ-specific autoimmunity. All patients had symptoms of thyrotoxicosis for more than 2 months (range 2–8 months) before attending the endocrine clinic. Using these criteria, 22 of the original 24 patients had GD, but in one individual with positive thyroid peroxidase and negative TSH receptor antibodies, the diagnosis was subsequently changed from GD to postpartum thyroiditis (PPT). In the interventional study (n = 19, 18 GD, and 1 PPT), 16 were on block and replace therapy at the time the follow-up sample was taken and one was on titration with carbimazole (Table
Table 1. Age, gender, and thyroid status of healthy control subjects and thyrotoxic patients during the hyperthyroid and euthyroid phase.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=19)</th>
<th>Hyperthyroid (n=19)</th>
<th>Euthyroid (n=19)</th>
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<td>Age (yr)</td>
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<td>35.8±2.8</td>
<td>36.4±2.8</td>
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<td>Male/female (n)</td>
<td>9/10</td>
<td>6/13</td>
<td>6/13</td>
</tr>
<tr>
<td>FT4 (10-25 pmol/l)</td>
<td>16.1±0.65</td>
<td>46.7±5.04*</td>
<td>18.8±1.51**</td>
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<td>TSH (0.2-4.0 mIU/l)</td>
<td>1.78±0.16</td>
<td>&lt;0.05*</td>
<td>1.49±0.90**</td>
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<td>Diagnosis</td>
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<td>- PPT</td>
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<td></td>
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<td>- Mild Asthma</td>
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<td>Treatment</td>
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<td>- Propranolol (40-80 mg/d)</td>
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<td>- Carbimazole</td>
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<tr>
<td>- Propylthiouracil</td>
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<td>0</td>
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</tr>
<tr>
<td>- L-T4</td>
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<td>18</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>Achievement of euthyroidism</td>
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<td>- B&amp;R</td>
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<tr>
<td>- Titration with ATD</td>
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<tr>
<td>- Other</td>
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<td>0</td>
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<tr>
<td>TBII positive</td>
<td>0</td>
<td>17</td>
<td>ND</td>
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</table>

GD indicates Graves’ disease; PPT, postpartum thyroiditis; L-T4, levothyroxine treatment; ATD, Antithyroid drugs; B&R, block and replace; FH, family history; ND, not done; PMH, past medical history; PTM, pretibial myxoedema; TED, thyroid eye disease.

* P < 0.05 comparing healthy controls with hyperthyroid subjects.

** P < 0.05 comparing hyperthyroid subjects at baseline and after normalizing thyroid function.
1). Two subjects were on levothyroxine only [one with PPT and one after radioactive iodine therapy (RAI)]. RAI was given secondary to mild neutropenia with carbimazole treatment (antibiotic administration was never required, and a repeat blood sample was taken 6 months after RAI). In patients who completed the interventional part of the study, three had relapsed disease (6–24 months after discontinuation of antithyroid drugs) and 16 had GD de novo. TBII test was positive in 17 of the 18 individuals with a diagnosis of GD.

The interventional study in healthy controls was divided into two parts as described in the method section. The characteristics of healthy volunteers are described elsewhere (22), and summarized in Table 2.

Effects of endogenous hyperthyroidism and subsequent euthyroidism on fibrin network structure

This was investigated using an observational study to compare hyperthyroid individuals with healthy controls, followed by studying the effects of normalizing thyroid hormone levels on clot structure and fibrinolysis.

Hyperthyroidism is associated with reversible changes in clot structure and fibrinolysis

Turbidimetric analysis

Clot MA was raised in hyperthyroid subjects compared with controls [0.41 ± 0.02 and 0.27 ± 0.01 arbitrary units (AU), respectively; \( P < 0.01 \)], LT was longer (518 ± 23 and 461 ± 18 sec, respectively; \( P = 0.03 \)), and LA was larger (218 ± 21 and 181 ± 11 AU, respectively, \( P = 0.05 \)). Normalizing thyroid function resulted in a drop in mean free \( T_4 \) (\( FT_4 \)) in from 46.7 ± 5.0 to 18.9 ± 1.5 pmol/liter (\( P < 0.01 \)), which was associated with reductions in clot MA from 0.43 ± 0.02 to 0.36 ± 0.02 AU (\( P < 0.01 \)), LT from 518 ± 23 to 465 ± 17 sec (\( P = 0.02 \)), and LA from 218 ± 21 to 158 ± 17 AU (\( P = 0.02 \)). Clot MA after normalizing thyroid function remained higher than in healthy controls (\( P < 0.01 \)), but LT and LA were similar (\( P > 0.1 \) for both). Results are summarized in Figure 1, A–C. Analyzing all 24 subjects at baseline gave similar data and showed a significant difference compared with controls (data not shown).

Laser scanning confocal microscopy

Confocal microscopy was used to visualize the fibrin networks and directly assess fibrinolysis of mature, fully formed clots. A 20-μm Z-stack section was generated by taking 36 serial images at 0.55-μm intervals (Fig. 2A). Using pooled samples from each group, a more compact clot was noted in the hyperthyroid subjects compared with controls, which only partially normalized after achieving euthyroidism, consistent with the turbidimetric assay data.
described above. Thicker sections of the clot (50 μm) were also made, which showed a similar pattern (data not shown).

Table 2. Age, gender, and thyroid status of healthy control subjects undergoing thyroid hormone administration using 0.3 mg/d (study A) or 0.45/0.6 mg/d L-T4 according to weight (study B).

<table>
<thead>
<tr>
<th></th>
<th>Study A Baseline (n=16)</th>
<th>Hyperthyroid (n=16)</th>
<th>Study B Baseline (n=12)</th>
<th>Hyperthyroid (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs) mean, SD</td>
<td>30.8±1.0</td>
<td>29.9±1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>9/7</td>
<td>6/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FT4 (pmol/l) mean, SD</td>
<td>16.68±0.82</td>
<td>28.06±2.02*</td>
<td>14.8±0.44</td>
<td>41.5±2.1*</td>
</tr>
<tr>
<td>TSH (mIU/l)</td>
<td>1.97±0.22</td>
<td>0.15±0.04*</td>
<td>2.50±0.42</td>
<td>0.03±0.003*</td>
</tr>
</tbody>
</table>

* p<0.05 comparing baseline and day 14 following daily treatment with LT4.

Lysis of fully formed clots from pooled samples was performed, which took 252 ± 24 sec during the hyperthyroid phase and 185 ± 25 sec when subjects were rendered euthyroid (P = 0.03). Lysis of control clots took 169 ± 18 sec, significantly shorter compared with hyperthyroid but similar to euthyroid clots (P = 0.03 and 0.61, respectively). These results represent the mean of four independent experiments (Figure 3, A and B).

Figure 1. Clot structure parameters and plasma levels of coagulation/inflammatory proteins in subjects with hyperthyroidism at baseline and after normalizing thyroid function compared with age-matched healthy controls. A, Clot MA, a measure of clot density; B, time from full clot formation to 50% lysis, an indicator of fibrinolysis potential; C, LA, a complex measure of clot density, formation, and lysis (n = 19 for each group); D–G, plasma levels of fibrinogen, PAI-1, CRP, and complement C3, respectively (n = 19 for each group).
A. Laser scanning confocal microscopy (LSCM) and scanning electron microscopy (SEM) respectively, showing fibrin networks made from pooled plasma of controls and hyperthyroid subjects (pool of 10 random samples from each group). A denser clot is evident in hyperthyroidism, with partial reversal of the changes following normalisation of thyroid function.

B. Fibre thickness in a total of 240 fibres from control, hyperthyroid and euthyroid clots (30 fibres were measured in 8 different clot areas from each group).

**Figure 2.** Visualisation of ex vivo fibrin clots from healthy controls and subjects with hyperthyroidism at baseline and after normalising thyroid function.
Scanning electron microscopy
Due to higher resolution, SEM has the advantage over LSCM of investigating fibrin fibers in more detail. All clots were prepared in duplicate and 12 separate areas from each condition were studied using ×5,000, ×10,000 and ×30,000 magnifications. Figure 2B shows typical clots from each group using the highest magnification. Again this technique provided evidence consistent with more compact clots in hyperthyroid subjects compared with controls, with formation of a less compact structure after normalization of thyroid hormone levels. Fibrin fiber thickness from eight separate clot areas (n = 30 fibers from each clot) from control, hyperthyroid, and euthyroid subjects were measured (total of 240 fibers from each group). Mean fiber diameter in control, hyperthyroid, and euthyroid subjects was 108.1 ± 2.0, 120.5 ± 2.1, and 113.7 ± 2.2 nm, respectively. The difference between hyperthyroid subjects and controls was significant (P < 0.001), as was the difference between hyperthyroid and euthyroid samples in the same individuals (P = 0.03). However, the difference between euthyroid patients and controls just failed to reach statistical significance (P = 0.054).

Taken together, these data indicate that fibrin clots are denser and more compact with thicker fibers and resistance to fibrinolysis in hyperthyroidism compared with age-matched healthy controls. Rendering patients euthyroid partially reverses these changes, with clots achieving LT similar to controls, although they remain more compact.

Endogenous hyperthyroidism is associated with reversible changes in plasma levels of thrombotic and inflammatory markers
Levels of fibrinogen were higher in hyperthyroid subjects compared with control (2.91 ± 0.21 and 1.98 ± 0.06 g/liter, respectively; P < 0.01), with similar findings for PAI-1 levels (2067 ± 261 and 649 ± 133 pg/ml, respectively; P < 0.01). Of the inflammatory markers studied, C3 levels were higher in hyperthyroid subjects compared with controls (0.92 ± 0.05 and 0.64 ± 0.03, respectively; P < 0.01) and although CRP levels appeared higher, the difference failed to reach statistical significance (1.23 ± 0.46 and 0.54 ± 0.16 mg/liter, respectively; P = 0.08).

Normalizing thyroid function saw a fall in fibrinogen levels from 2.91 ± 0.21 g/liter during hyperthyroidism to 2.2 ± 0.1 g/liter (P < 0.01). Similarly, PAI-1 plasma levels decreased to 847 ± 188 pg/liter (P < 0.01) and C3 levels to 0.76 ± 0.05 g/liter (P < 0.01). In contrast, CRP showed no significant changes. Comparing the euthyroid phase with healthy controls, C3 levels remained lower in healthy controls (P = 0.03), whereas no significant difference was found when comparing fibrinogen and PAI-1 levels (P > 0.1 for both). All results are summarized in Fig. 1, E–G. We have also analyzed D-dimer levels as an indirect measure of activation of the coagulation pathway; during hyperthyroidism levels of 392 ± 47 ng/ml were recorded falling to 238 ± 28 ng/ml during the euthyroid phase (P < 0.01).
Figure 3. Microscopic lysis of fully formed ex vivo fibrin clots from healthy controls and subjects with hyperthyroidism at baseline and after normalising thyroid function.
A. One representative experiment demonstrating lysis of fully formed clots made from pooled plasma of healthy controls or hyperthyroid subjects after the addition of tissue plasminogen activator and plasminogen to the edge of the clot. B. Mean lysis time (4 independent experiments) of clots made from pooled plasma of healthy controls and hyperthyroid subjects (pool of 10 random samples from each group).
Carbimazole has no direct effect on clot structure parameters in vitro
The presence of methimazole had no significant effect on clot structure parameters in the samples analyzed. Using 0 and 1.25 g/liter methimazole in four samples, clot MA was 0.34 ± 0.04 and 0.32 ± 0.02 AU, respectively, and LT 573 ± 13 and 590 ± 19 sec, respectively, whereas LA was 226 ± 17 and 224 ± 19 AU, respectively (P > 0.1 for all).

Clot structure parameters correlate with metabolic, thrombotic, and inflammatory markers in the hyperthyroid phase
In the control group, clot LT correlated with PAI-1 levels, whereas MA and LA showed correlations with fibrinogen levels. In the hyperthyroid group, the correlation between LT and PAI-1 was absent, whereas all clot structure parameters (MA, LT, and LA) correlated with plasma C3 levels. In the same group, fibrinogen levels continued to correlate with MA and LA. The correlation between C3 and LT was absent in patients after normalization of thyroid hormone levels (data not shown). All results are summarized in Table 3. In the patient group, no correlation was detected between TBII titers and any of the clot structure parameters (data not shown).

Effects of exogenous hyperthyroidism on fibrin network structure
To establish whether the above changes are directly related to thyroid hormones, we investigated the effects of levothyroxine treatment on clot structure and fibrinolysis.

Short-term exogenous hyperthyroid has no effect on clot structure or fibrinolysis
In part A of the study, all 16 volunteers completed the study. After 14 d treatment with levothyroxine, FT<sub>4</sub> levels rose from 16.8 ± 0.82 to 28.1 ± 2.02 pmol/liter (P < 0.01) associated with a fall in TSH levels from 1.97 ± 0.22 to 0.15 ± 0.04 mIU/liter (P < 0.01). Regardless of whether individuals received placebo or levothyroxine treatment, no changes were found in clot MA or LT (Figure 4). Similarly, a difference in LA was not demonstrated (data not shown).
In part B, all 12 volunteers completed the study, and FT<sub>4</sub> levels rose from 14.8 ± 0.44 pmol/liter at baseline to 41.5 ± 2.08 pmol/liter after 2 wk levothyroxine treatment (P < 0.05). TSH was 2.5 ± 0.42 mIU/liter at baseline, dropping to 0.03 ± 0.003 mIU/liter (P < 0.05). Similar to low-dose levothyroxine, higher-dose thyroid hormone treatment was not associated with any significant changes in clot MA, LT (Figure 4), or LA (data not shown).

Short-term exogenous hyperthyroidism is associated with no change in inflammatory protein plasma levels
Plasma levels of fibrinogen and PAI-1 for this part of the study are documented elsewhere, and we present here inflammatory protein levels. In part A, CRP and C3 showed no significant
changes compared with baseline levels. Plasma levels of these proteins also failed to show any difference compared with baseline in part B. Results are summarized in Figure 4.

**Table 3.** Correlations between metabolic/thrombotic/inflammatory factors plasma levels and clot structure/lysis parameters in healthy controls and in subjects with hyperthyroidism.

<table>
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<tr>
<th></th>
<th>Fibrinogen</th>
<th>CRP</th>
<th>C3</th>
<th>PAI-1</th>
<th>LT</th>
<th>LA</th>
<th>MA</th>
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<tbody>
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<td>0.32</td>
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<td>Fibrinogen</td>
<td>0.13</td>
<td>0.16</td>
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<td>0.17</td>
<td>0.46*</td>
<td>0.77**</td>
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Significant correlations are shown in bold; * p<0.05, ** p<0.01. CRP, C-reactive protein; C3, complement C3; PAI-1, plasminogen activator inhibitor-1; LT, clot lysis time; LA, clot lysis area; MA, clot maximum absorbance.

**DISCUSSION**

Hyperthyroidism, one of the commonest endocrine conditions, is associated with an increased risk of arterial and venous thrombotic complications. A number of studies have demonstrated increased prothrombotic coagulation protein levels in thyrotoxicosis, including fibrinogen, Factor VIII, Factor X, and von Willebrand factor antigen, together with increased levels of the antifibrinolytic factor PAI-1. It was subsequently postulated that hyperthyroidism is associated with a prothrombotic/hypofibrinolytic milieu. Plasma levels of some prothrombotic factors, however, are decreased, including FV and FX, which may offset, at least partly, the increased thrombosis potential in this condition. A drawback of studies to date is the investigation of a single, or even a group of, clotting factors, which is insufficient to fully illustrate the entire balance between clot formation and fibrinolysis. The advantage of our work is the dynamic analysis of clot structure characteristics, determining the balance between thrombotic and fibrinolytic factors and enabling the global assessment of fibrin-
related thrombotic risk. Furthermore, we compare, for the first time, thrombosis potential in individuals with endogenous and exogenous levothyroxine-induced hyperthyroidism. This ex vivo work specifically investigates the collective role of plasma proteins on fibrin network structure and fibrinolysis in thyroid dysfunction, and therefore, the influence of cellular components (i.e. platelets) is not addressed.

Figure 4. Clot structure parameters and plasma levels of inflammatory proteins in healthy volunteers at baseline and after placebo or levothyroxine administration for 14 days. A and B, clot maximum absorbance after low (0.3 mg/d) and high (0.45 or 0.6 mg/d) levothyroxine administration, respectively. C and D, clot lysis time after low (0.3 mg/d) and high (0.45 or 0.6 mg/d) levothyroxine administration, respectively. E and F, complement C3 plasma levels after low (0.3 mg/d) and high (0.45 or 0.6 mg/d) levothyroxine administration, respectively. G and H, C-reactive protein plasma levels after low (0.3 mg/d) and high (0.45 or 0.6 mg/d) levothyroxine administration, respectively.

This study provides a number of novel findings including 1) a thrombotic clot structure with impaired fibrinolysis is evident during hyperthyroidism, 2) partial normalization of clot structure/lysis is achieved by restoring euthyroidism, 3) short-term exogenous hyperthyroidism is not associated with significant change in clot structure/lysis despite shifts in some coagulation factor plasma levels, and 4) modulation of clot structure/lysis during hyperthyroidism is related not only to elevated plasma levels of procoagulant and antifibrinolytic factors but also to the inflammatory environment, specifically complement C3 levels.

Compared with healthy controls, subjects with hyperthyroidism had denser ex vivo fibrin networks with more resistance to fibrinolysis. In addition to more compact clots, an increase in fibrin fiber thickness was found. These changes in clot structure and lysis may be related to higher fibrinogen and PAI-1 levels during hyperthyroidism. However, these are not the only factors because fibrinogen levels normalized after rendering patients euthyroid, whereas
Clot structure remained more compact than in controls. This suggests that plasma factors other than fibrinogen determine clot structure in subjects with endogenous hyperthyroidism. Clot lysis was significantly prolonged when comparing hyperthyroid with control samples, but this fully normalized once euthyroidism was achieved. In the control group, PAI-1 levels were associated with LT, an expected finding, but this association was lost in the hyperthyroid group where clot lysis was related to plasma C3 levels. C3 is incorporated into the fibrin clot,18 which can determine fibrinolytic potential, particularly in pathological states.19 In contrast to CRP, C3 also correlated with clot density and LA, further supporting a role for this protein in determining thrombosis potential in hyperthyroidism. Although studies have investigated the complement system in thyroid autoimmunity,31 relatively little is known about C3 plasma levels in thyroid dysfunction. Plasma C3 levels in hypothyroidism are either elevated or unaltered,32,33 whereas work on hyperthyroid subjects suggest reduced protein levels.32 This discrepancy between our findings and the latter study may be related to the two diverse populations studied (European and Chinese) or as a result of using different methodologies to measure C3 levels. The partial normalization of C3 plasma levels after rendering patients euthyroid coupled with the positive correlation between thyroid hormone and C3 levels strongly suggests a relationship between hyperthyroidism and complement C3 protein. In support of this, others have documented positive correlations between thyroid hormone and complement protein levels in both thyroid autoimmunity and in nonthyroidal autoimmune conditions.34-36 Overall our data demonstrate the presence of an inflammatory environment in hyperthyroidism, manifested as increased levels of CRP, C3, and fibrinogen, in turn consistent with previously reported increased hepatic output of inflammatory/coagulation proteins in this condition.37,39 Also, it appears that the contribution of PAI-1 to the hypofibrinolytic state in hyperthyroidism is diminished, and as such, PAI-1 studies assessing thrombosis potential in thyroid dysfunction should be interpreted with caution. This is further supported by the failure to demonstrate changes in clot LT in volunteers taking levothyroxine, despite doubling of PAI-1 plasma levels. Using a different assay, a small difference in LT was previously documented with high-dose levothyroxine,20 and therefore small changes in clot lysis, below the detection limit of the current assay, cannot be ruled out. However, it is clear that endogenous and short-term exogenous hyperthyroidism differ in their effects on clot structure/lysis. Therefore, these results indicate that short-term exogenous hyperthyroidism is associated with little, if any, fibrin-related thrombosis risk despite increased levels of PAI-1 and other procoagulant proteins.20 Previous work has shown increased D-dimer levels in overt and subclinical hyperthyroidism, suggesting activation of the fibrinolytic pathway.30,40 In our study, D-dimer levels showed a significant fall upon restoration of euthyroidism, indicating fibrinolysis can be modulated by controlling endogenous hyperthyroidism. In contrast, exogenous hyperthyroidism has not
been associated with changes in D-dimer levels. The difference between exogenous and endogenous hyperthyroidism in relation to clot structure and fibrinolysis may be attributed to differences in time exposure as endogenous hyperthyroidism was present for at least 2 months before treatment was initiated, whereas exogenous hyperthyroidism was induced for 2 wk. Alternatively, the autoimmune process itself may have contributed to this difference, and future work is needed to clarify whether longer-term exogenous hyperthyroidism, such as the therapeutically induced mild hyperthyroidism in thyroid cancer patients, is associated with thrombotic changes in clot structure/function. Differences between endogenous and exogenous hyperthyroidism are not unprecedented because SHBG levels, usually elevated in endogenous hyperthyroidism, do not necessarily increase after levothyroxine-induced thyrotoxicosis.

A possible effect of carbimazole treatment on the fibrin clot is ruled out by the in vitro studies, which demonstrated no effect of methimazole, the active metabolite of carbimazole, on clot structure or lysis. However, carbimazole has also been shown to have immunomodulatory properties, which may contribute systematically to reduce inflammation, potentially indirectly modulating the fibrin clot.

**CONCLUSION**

We offer, for the first time, convincing evidence that demonstrates altered clot structure and fibrinolysis in endogenous hyperthyroidism, a derangement that largely normalizes upon restoration of euthyroidism. The mechanisms for altered clot structure in hyperthyroidism are partly related to changes in coagulation factor plasma levels but the inflammatory pathway, more specifically complement protein C3, is also implicated. We demonstrate that PAI-1 levels, the classical fibrinolytic inhibitor, do not necessarily modulate fibrin-related thrombosis risk in hyperthyroidism, and future studies should bear this in mind when investigating thrombotic tendencies in thyroid dysfunction. In contrast to endogenous hyperthyroidism, short-term exogenous thyrotoxicosis, induced by levothyroxine administration, is not associated with significant changes in fibrin clot structure/lysis. Future work is required to assess the clinical implications of our findings and to demonstrate any association between altered clot structure and clinical thrombotic events in individuals with endogenous hyperthyroidism or in those on long-term therapy with TSH-suppressive doses of levothyroxine.

**ACKNOWLEDGMENTS**

We thank the British Thyroid Foundation for funding this work. We also thank Dr. J. Barth for his help arranging TBII assays.
21. Carter AM, Cymbalista CM, Spector TD, Grant PJ. Heritability of clot formation, morphology, and lysis: the EuroCLOT project.
Hyperthyroidism and fibrin clot structure


