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A Pilot Study: Subclinical Hypothyroidism and Free Thyroid Hormone Measurement by Immunoassay and Mass Spectrometry

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Abstract

Background—The diagnosis of subclinical hypothyroidism is defined as the presence of an elevated thyroid stimulating hormone (TSH) with a normal free thyroxine (FT4) level. The commonly used direct analogue immunoassays for the measurement of FT4 have been shown to have poor performance at the upper and lower limits of the FT4 reference interval.

Purpose—The purpose of this pilot study was to investigate the percentage of individuals classified as having subclinical hypothyroidism with a standard immunoassay, that actually have low free thyroid hormone levels by mass spectrometry measurements.

Design—Outpatient samples with elevated TSH values and normal FT4 concentrations as per standard immunoassay methods were collected. FT4 and free triiodothyronine (FT3) analyses were performed on these samples using liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Results—Sixty five percent (n=26) of patients (n=40) had (LC-MS/MS) FT4 or FT3 or both FT4 and FT3 values below mass spectrometry reference limits.

Conclusions—Our findings indicate that the direct analogue immunoassay method for FT4 measurement results in a significant proportion of patients being misclassified as having subclinical hypothyroidism.

Keywords

Mass spectrometry; Free thyroid hormone; subclinical hypothyroidism

1. Background

Subclinical hypothyroidism is defined as increased serum thyroid stimulating hormone (TSH) levels with normal serum free thyroxine (FT4) concentrations [1]. The prevalence of subclinical hypothyroidism in the population without known thyroid disease has been reported to be 4% to 10% [2,3]. Debate still exists with regards to the clinical importance of and therapy for elevation of serum TSH (in particular elevated levels <10 mIU/l) and the

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exact upper limit of normal for the serum TSH level that also varies with age [1]. Most clinical laboratories perform TSH and FT4 measurements on immunoassay (IA) platforms [4]. Whilst TSH analyses on immunoassay platforms are considered quite reliable, the validity of FT4 analysis by direct analogue immunoassay has been questioned for many years [5,6]. Significant limitations of the currently used FT4 immunoassays that have been described are the influence of changes in binding protein concentrations and a weak inverse linear log relationship to TSH in hypo- and hyperthyroid individuals [7–11]. These assays appear to perform best in euthyroid individuals but poorly in individuals with thyroid disease [12]. FT4 immunoassays' poor performance at low concentrations may lead to misclassification of patients as having subclinical hypothyroidism, when in fact they have FT4 levels lower than the reference interval when measured by tandem mass spectrometry. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) following ultrafiltration of the sample has been shown to perform better in such circumstances, and in the case of FT4, agrees better with the gold standard equilibrium dialysis assay [10]. Numerous pharmacological agents affect thyroid hormone measurements and may further confuse interpretation of results beyond the problems encountered with immunoassay measurements. Drugs may affect thyroxine binding globulin levels (for example estrogens), thyroid hormone binding (for example carbamazepine), TSH levels (for example glucocorticoids), conversion of T4 to T3 (for example amiodarone) and may even induce thyroid disease (for example lithium) [13–16].

2. Materials and Methods

2.1 Sample collection

Outpatient serum samples received at the NIH Clinical Center (NIH-CC) for 4 months in 2013 with elevated TSH values and FT4 concentrations within the laboratory reference interval were selected for inclusion in the study. All samples were collected between 8–11am. Samples were stored at –70 degrees Celsius until MS analysis. Patients' samples were excluded if they had known thyroid disease, were receiving thyroid hormone replacement therapy or other medications that are known to affect FT4 values or cause elevation in TSH values. Samples from patients older than sixty years and those that were positive for anti thyroid peroxidase antibodies were also excluded. The study was approved by the Institutional Review Board of the NIH (Clinical Protocol number 93-CC-0094).

2.2 Immunoassay measurements

Samples were analyzed at NIH-CC Department of Laboratory Medicine. TSH and FT4 samples were processed according to usual laboratory procedures. FT4, reference interval 9.8–19.4 pmol/l (0.76– 1.50 ng/dl), was measured by a direct (analogue) immunoassay method on a Dimension Vista (Siemens Healthcare Diagnostics). TSH (reference interval, RI: 0.40–4.00 mIU/l) and free triiodothyronine (FT3, RI: 2.8– 6.5 pmol/l or 1.8– 4.20 pg/ml) were also measured on the Vista. Anti-thyroid peroxidase antibody (ATPOA) (RI: < 35 IU/ml) testing was performed on Immulite XPI 2000 (Siemens Healthcare Diagnostics). Two or three levels of commercially available internal quality control material were analysed at the start of each run

2.3 LC-MS/MS measurement

The FT4, reference interval used was 17.4 –30.9 pmol/l (1.35—2.40 ng/dl) and for FT3, reference interval 2.3–9.5 pmol/l (1.5–6.1 pg/ml). Samples were analysed in batches. Analyses were performed as per methods previously published [8,10,17]. Briefly four hundred microliters of human plasma/serum was filtered through a Centrifree YM-30 ultrafiltration device by centrifugation, and 450 µl of deuterium labeled internal standard, T4-*d*₅ in methanol was then added to 150 µl of ultrafiltrate for deproteinization. After

vortexing and centrifugation, 500 µl of supernatant was diluted with 400 µl of distilled de-ionized water and a 650 µl aliquot was injected onto a C-18 column. After washing, the switching valve was activated and the analytes were eluted from the column with a water/methanol gradient into the MS/MS system. Quantification by multiple reaction-monitoring (MRM) analysis was performed in the negative mode (ESI⁻). Three levels of internal quality control were analysed at the beginning and end of each run.

2.4 Statistical analysis

Non-normally distributed data (MS FT4 values) was normalised by log-transformation before analysis and back-transformed for data presentation. We used the Kolomogorov-Smirnov test to test for normality, and we used Pearson's correlation coefficient, Bland-Altman difference plots, and Passing Bablock regression analysis to evaluate the methods. Statistical analysis was performed on Medcalc Version 12 (MedCalc Software). A *pf* <0.05 was considered statistically significant.

3. Results

Fifty seven samples with increased TSH and immunoassay FT4 within normal reference limits were collected. Following exclusion of patients > 60 y and those positive for ATPOA, a total of 40 samples from patients aged 6 – 59 y were included in the study. TSH values ranged between 4.3 and 8.2 mIU/l. Analysis of variance (ANOVA) revealed no statistically significant difference for values between males and females. See Table 1 for summary of results.

The CVs for the immunoassay methods used were as follows: CV of TSH 3–6% at a concentration of 5.8 mIU/l was 3–6%; FT4 at 12.4 pmol/l (0.96 ng/dl) was 4–6%; FT3 at 19.2 pmol/l (1.49 pg/ml) was 6.9–7.2%. The CVs for the LC-MS/MS assays were: FT4–4.1–6.6% at concentrations of 8.5 pmol/l (0.66 ng/dl) and 33.8 pmol/l (2.62 ng/dl); FT3–9% at concentrations between 0.23 pmol/l (0.15 pg/ml) and 3.44 pmol/l (2.22 pg/ml).

Sixty-five percent (n=26/40) of patients had mass spectrometry FT4 or FT3 or both FT4 and FT3 values below mass spectrometry reference limits. (Fig. 1 summarises findings). Sixteen of the 26 patients (62%) had only low MS FT4 results. Three of the 26 patients (12%) had only low MS FT3 results and 7 patients (27%) had both low MS FT4 and MS FT3 results. Fifty eight percent (n=23/40) of patients that would be classified as subclinical hypothyroidism as per immunoassay FT4 measurements had LC-MS/MS FT4 values that were below the reference interval. Patients with LC-MS/MS FT4 results below the reference interval had on average values 16% below the lower limit of the LC- MS/MS specific reference interval. The majority had values that were greater than 10% below the lower limit. Thirteen patients (n=13/23) had MS FT4 results that were >10% below the low reference limit. Nine patient (9 of 23) had MS FT4 values >15% below the low reference limit.

Pearson's correlation coefficient (n=34) between IA FT4 and MS FT4 was 0.55 with a 95% confidence interval (CI) of 0.28–0.73 and between IA and MS for FT3 it was 0.30 (95% CI 0.14–0.45). Regression analysis in the population studied (Fig. 2 and 3) for mass spectrometry versus immunoassay showed poor correlations between the two methods for both FT4: Slope 2.85 (95% CI 1.80 to 6.29), intercept –1.40 (95% CI –4.56 to –0.40) and FT3: Slope 1.29 (95% CI 0.66 to 2.09), intercept –2.16 (95% CI –4.72 to –0.12)

There was significant bias between MS and immunoassay values, with FT4 immunoassay values being on average 48 % lower than MS values and FT3 immunoassay values on average 36 % higher than MS results. Bland Altman percentage difference plots show poor

agreement between IA and MS data (Figs. 4 and 5); the 95% limits of agreement for IA FT4 and MS FT4 are between 13.4% and -82.3%. The 95% limits of agreement for IA FT3 and MS FT3 are between -7.3% to 102%.

4. Discussion

The findings of this preliminary study reveal that the direct analogue immunoassay method for FT4 measurement results in a significant proportion of patients being misclassified as having subclinical hypothyroidism. This is important as these individuals already have biochemically overt hypothyroidism by MS measurement. They should be followed up closely, in conjunction with clinical findings and symptoms, with a lower threshold for deciding on replacement therapy.

Mass spectrometric analysis has become the reference/ gold standard method for the measurement of various analytes. This method offers elevated analytical specificity and sensitivity [18]. Analysis of FT4 using LC-MS/MS with deuterated internal standard following sample ultrafiltration has been shown to correlate better with log TSH and with the gold standard equilibrium dialysis method in comparison to FT4 measured by direct immunoassay [8,10]. On this basis the authors believe that the “correct” FT4 (i.e., measurement by LC-MS/MS) is the one that correlates best with log TSH. Also FT4 measurement by MS is more accurate (particularly at extremes of range) than immunoassay measurement. Hence the significant difference in free thyroid hormone results and their interpretation observed between the two assays in this study is not merely a function of the different reference intervals but of the performance of the assays. In addition for this study we utilised, previously established method specific reference intervals for FT4 and FT3 tests on both immunoassay and LC-MS/MS to interpret patient values [19–21]. The LC-MS/MS reference intervals were derived from testing on over 1400 healthy children, aged 1–18 y [19] and was also confirmed in a healthy adult population (20–60 y; n=300) (unpublished data). The immunoassay FT4 and FT3 reference intervals provided by the manufacturer for the Siemens Dimension Vista (n=199) [20] were confirmed by the laboratory using healthy volunteers (n=20). The quoted reference intervals for FT4 (0.76–1.50 ng/dl) used in this study also concur with other reference interval studies by Lepoutre et al .10.3 –16.8 pmol/l (0.8–1.3 ng/dl) performed using the same immunoassay platform (n=129) [21].

We used outpatient samples and excluded patients on medications known to affect thyroid function tests. As TSH increases with age we also limited the age range from 6–59 years. It is of interest to note that the two patients excluded from final analysis due to positive ATPOA were defined as having subclinical hypothyroidism using immunoassay FT4 values but analysis with LC-MS/MS also revealed that both individuals had FT4 levels below the reference interval for the mass spectrometric method.

An important limitation of this study is the possible bias that may have been introduced by selecting those samples with FT4 values within the IA reference interval (and increased TSH), that were first analysed by immunoassay. We intend to perform a follow-up study where FT4 testing is performed first by LC-MS/MS and then selected samples run by immunoassay to confirm the findings of the current study. We intend to confirm the results of this study using a larger cohort whose only apparent abnormality is that they have been classified as having subclinical hypothyroidism.

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Highlights

1. MS analysis of outpatient samples with elevated TSH and normal immunoassay FT4 values was performed.
2. This study revealed that 65% of these patients had low FT4 values by LC-MS/MS measurement.
3. MS analysis of free thyroid hormones is recommended where subclinical hypothyroidism is suspected.
4. Consider MS analysis of FT4 & FT3 when immunoassay results are discrepant to TSH/clinical findings.

Figure 1.

Figure 2.

Correlation between Immunoassay and LC-MS/MS for FT4

Abbreviations: IA: Immunoassay; MS: Mass spectroscopy; FT4: Free thyroxine

Figure 3.
Correlation of Immunoassay and LC-MS/MS FT3
Abbreviations: IA: Immunoassay; MS: Mass spectroscopy; FT3 Free triiodothyronine

Figure 4.

Bland-Altman Percentage Plot for IA FT4 versus MS FT4 (ng/dL)

Abbreviations: IA: Immunoassay; MS: Mass spectroscopy; FT4: Free thyroxine

Figure 5.
Bland-Altman Percentage Plot for IA FT3 versus MS FT3 (pg/mL)
Abbreviations: IA: Immunoassay; MS: Mass spectroscopy; FT3: Free triiodothyronine

Summary of results

Table 1

Test	All patients (n=40)	Female (n=24)	Male (n=16)	ANOVA P value
Age (years)	Mean : 39.7 (24.5–44.9) SD: 16.2	Mean: 39.3 (32.3–46.4) SD: 16.6	Mean:40.3 (31.7–48.8) SD:16.1	P=0.32
TSH (mIU/L)	Mean : 5.8 (95% CI, 5.3–6.3) SD: 1.5	Mean: 6.0 (95% CI, 5.3–6.7) SD:1.7	Mean: 5.5 (95% CI, 4.8 – 6.2) SD:1.2	P=0.36
FT4 (IA) (ng/dL)	Mean: 1.0 (95% CI, 0.9–1.1) SD:0.2	Mean: 1.1 (95% CI,0.9–1.2) SD:0.2	Mean : 0.9 (95% CI,0.9–1.0) SD:0.1	P=0.07
FT3 (IA) (pg/mL)	Mean: 3.0 (95% CI, 2.9–3.2) SD:0.4	Mean: 3.0 (95% CI, 2.8–3.2) SD: 0.4	Mean: 3.0 (95% CI, 2.8–3.3) SD: 0.4	P=0.73
FT4 (MS) (ng/dL)	Mean: 1.4 (95% CI, 1.3–1.6) SD: 0.6	Mean: 1.5 (95% CI, 1.3–1.7) SD: 0.7	Mean: 1.3 (95% CI, 1.2–1.5) SD: 0.3	P=0.39
FT3 (MS) (pg/mL)	Mean: 1.9 (95% CI, 1.7–2.1) SD:0.6	Mean: 2.0 (95% CI, 1.8–2.2) SD:0.6	Mean: 1.8 (95% CI, 1.4–2.1) SD:0.5	P=0.26

^a Abbreviations: SD (standard deviation); TSH (Thyroid stimulating hormone); FT4 (free thyroxine); FT3 (free tri-iodothyronine); IA (immunoassay) ; MS (mass spectrometry)