Original article

Metabolomic profile of overweight patients on LT4 treatment for hypothyroidism

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Short title: LT4 Treatment and hypothyroidism metabolomics

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Abstract

**Background:** Hypothyroidism is clinically characterized by decrease in levels of the circulating thyroid hormones namely thyroxine and triiodothyronine. The main treatment for hypothyroidism is thyroid hormone replacement using levothyroxine to normalize serum thyroid hormone levels.

**Objectives:** In this study, we explored the metabolic changes in the plasma of patients with hypothyroidism after reaching a euthyroid state with levothyroxine treatment.

**Methods:** Plasma samples from 18 patients diagnosed as overt hypothyroidism were collected before and after levothyroxine treatment upon reaching a euthyroid state and were analyzed by high-resolution mass spectrometry-based metabolomics. Multivariate and univariate analyses evaluated data to highlight potential metabolic biomarkers.

**Results:** LC-MS-based metabolomics revealed significant decrease in the levels of ceramide, phosphatidylcholine, triglycerides, acylcarnitine, and peptides after levothyroxine treatment; this could indicate a change in the fatty acid transportation system and an enhanced β oxidation, compared with a hypothyroid state. At the same time, the decrease in the peptides suggested a shift in protein synthesis. In addition, there was a considerable rise in glycocholic acid following therapy, suggesting the involvement of thyroid hormones in stimulating bile acid production and secretion.

**Conclusions:** A metabolomic analysis of patients with hypothyroidism revealed significant changes in several metabolites and lipids after treatment. This study showed the value of the metabolomics technique in providing a complementary understanding of the pathophysiology of hypothyroidism and as a crucial tool for examining the molecular impact of LT4 treatment on hypothyroidism. It was an important tool for investigating the therapeutic effects of levothyroxine on hypothyroidism at the molecular level.
1. Introduction

The thyroid hormone affects almost every organ system in the body, including the cardiovascular, central nervous, skeletal, and gastrointestinal systems and metabolism. Tetraiodothyronine or thyroxine (T4) and triiodothyronine (T3) are the main hormones released by the thyroid gland (1). The hypothalamus and anterior pituitary gland produce and release several hormones that work synchronously to maintain the proper feedback mechanisms and homeostasis of T4 and T3 in the bloodstream (2).

Numerous in vitro and in vivo previously conducted studies on animals and humans have examined the effect of thyroid hormones on metabolic balance (3). The research findings prompted the development of different diagnostic tests and therapeutic agents now implemented in the clinical setting. To assess thyroid gland function, several blood tests can be used to measure the levels of thyroid hormones and other related hormones. These tests include those for thyroid-stimulating hormone (TSH), total T4 and T3, free T4 and T3. The other tests for thyroid antibodies and thyroglobulin identify autoimmune thyroid conditions and thyroiditis, respectively (4).

Hypothyroidism, or an underactive thyroid, is a medical condition characterized by a decrease in the thyroid hormones released into the bloodstream (5, 6). It is clinically distinguished by an indirect but broad range of signs, depending on the severity of the hormone deficiency that reflects a hypometabolic state. This metabolic condition is marked by decreased basal metabolic rate, heat production, and increased resting energy expenditure, and is associated with increased cholesterol levels, decreased lipolysis, and decreased gluconeogenesis (7).

Hypothyroidism is divided into primary, secondary, and tertiary stages. In primary hypothyroidism, diminished thyroid gland production of hormones causes a compensatory increase in TSH. On the other hand, secondary hypothyroidism is characterized by decreased TSH and T3/T4 levels and is usually caused by pituitary disorders (8). Tertiary hypothyroidism is caused by hypothalamic disorders and results in decreased levels of thyrotropin-releasing hormone released from the hypothalamus, TSH, and T3/T4 (8).

Thyroid hormone replacement is currently the primary treatment method for hypothyroidism (6). According to patient feedback, levothyroxine (LT4) is a synthetic replacement for thyroid tests. The recommended dose for adults is 1.6 mcg/kg, adjusted every eight weeks, depending on the response, and thyroid test results.
Although patients may have partial symptom alleviation with medicine intake, they typically experience over
replacement, which makes them more vulnerable to osteoporosis, liver damage, and cardiovascular diseases.
Therefore, novel approaches to managing hypothyroidism are urgently needed.
Metabolomics is a well-known and widely utilized approach to understanding perturbation in an organ system
under stress. This approach can provide novel insights into the end product of various biochemical processes
within the body, which could reveal the physiological status of that system and identify new therapeutic
targets (9-15). In hypothyroidism, metabolomics has been applied to understand the molecular mechanism in
the urine of rats (16), detect metabolic changes associated with the transition from hypothyroidism to
euthyroidism using NMR-based metabolomics (17), identify biomarkers to distinguish between hypothyroid
and euthyroid individuals (9), and evaluate the effect of herbs with hot properties on hypothyroidism rats
(18). The current study used a metabolomic technique based on liquid chromatography-mass spectrometry
(LC-MS) to evaluate the metabolic changes connected with hypothyroidism therapy.

2. Materials and methods

2.1 Ethics approval, patient recruitment, and treatment

The study was approved by the King Khalid University Hospital (KUUH), College of Medicine, Riyadh,
Saudi Arabia (registration no. E-10-172). The recruited patients, referred to the KUUH Obesity Research
Center, were asked to sign a written informed consent form before enrolment. Eighteen overweight patients
(10 women, 8 men) with BMI ranging 25-27 kg/m² who were diagnosed as overt hypothyroidism were
included in this study. TSH values >10 mIU/L and FT4 levels <12 pmol/L were considered to indicate overt
hypothyroidism. All patients were in good health and had no other pathological disorders, such as type 2
diabetes, hypertension, and inflammatory or autoimmune diseases.

The patients were treated with an appropriate dose of LT4. Samples for the hypothyroid group were taken
before initiation of treatment and the posttreatment samples were collected after the TSH levels were
normalized after giving the recommended dose. The TSH levels normalized in fifteen patients after 6 weeks
of therapy while two patients took 8 weeks and one patient took 10 weeks to reach euthyroid levels.
2.2 Biochemical analysis

Standard clinical tests were performed at the first visit to the hospital after diagnosis and repeated when a euthyroid state was achieved. The following parameters were evaluated using blood samples that were obtained after overnight fasting: FT4, TSH, fasting blood glucose, urea, creatinine, sodium, potassium, aspartate transaminase, alanine transaminase, alkaline phosphatase, total cholesterol, low-density lipoprotein cholesterol (LDL), triglyceride (TG), and high-density lipoprotein cholesterol. An integrated clinical chemistry autoanalyzer (Dimension® Xpand Plus; Siemens Healthcare Diagnostics, Deerfield, IL, USA) was used to determine all parameters for biochemical and hormone studies listed in Table 1.

2.3 Sample preparation and liquid chromatography-mass spectrometry analysis

The blood samples were placed in EDTA-coated tubes (BD Vacutainer, USA) and centrifuged at 3000 g for 15 minutes; after that, plasma samples were kept for analysis at −80°C (19). For plasma metabolite extraction, proteins were precipitated using a mixture of 50% acetonitrile in methanol. The mixtures were processed in a thermomixer (Eppendorf, Germany) at 600 g and 4°C for one hour, followed by centrifugation at 16000 g for 10 minutes at 4°C. The supernatant was transferred into a 1.5-mL tube (Eppendorf) and then evaporated completely in a chamber (SpeedVac; Christ, Germany). The dried samples were suspended using 50% mobile phase A: 50% mobile phase B.

Metabolic profiling was performed using a Waters Acquity UPLC system coupled with a Xevo G2-S QTOF mass spectrometer. Metabolites were first separated in a column (100 × 2.1 mm, 2.5 μm) (XSelect; Waters Ltd., Elstree, UK) using ACQUITY UPLC and a binary mobile phase that comprised 0.1% formic acid in dH2O as solvent A and 0.1% formic acid in 50% acetonitrile: methanol as solvent B running at a flow rate of 300 μL/min. The gradient elution was as follows: 0–16 minutes 95- 5% A, 16–19 minutes 5% A, 19–20 minutes 5% to 95% A, and 20–22 minutes 95% A. The MS data for the separated metabolites were acquired using positive and negative ionization under the following conditions: source temperature, 150 °C; desolvation temperature, 500 °C (ESI+) or 140 (ESI−); capillary voltage, 3.20 kV (ESI+) or 3 kV (ESI−); cone voltage, 40 V; desolvation gas flow, 800.0 L/h; and cone gas flow 50 L/h. The collision energy values of the low and high functions were set at off and 10–50 V, respectively, in MSE mode. Quality control samples were prepared by pooling aliquots from all samples and were injected every 10 samples to check the
reproducibility of the LC-MS system. All data were collected with Masslynx™ V4.1 workstation (Waters Inc., Milford, Massachusetts, USA) in continuum mode.

2.4 Data processing and statistical analysis

Peak picking and alignment of the detected ion (m/z, Rt) were processed using Progenesis QI v.3.0 software (Waters Technologies, Milford, MA., USA). Thereafter, the processed data were statistically evaluated using several approaches. After data treatment, including log-transformation, mean centering, and Pareto scaling, MetaboAnalyst v5.0 (McGill University, Montreal, QC, Canada) was used to highlight the metabolic changes associated with LT4 treatment (18). In addition, in order to maximize the covariance between the measured data (peak intensities) and the response (class assignment) within the groups, the supervised classification method orthogonal partial least squares discriminant analysis (OPLS-DA) was utilized. This test showed how two variables differed and allowed enhanced interpretation of the metabolic variations between the pre and posttreatment groups by removing information that had no impact on the discrimination and features. A variable importance in the projection (VIP) value of >1 was considered significant. Thereafter, Mass Profiler Professional software was used for univariate analysis. A Volcano plot was used to identify the metabolites that significantly differed between the pre and posttreatment groups, based on a p value of <0.05 and a fold change (FC) cutoff of 2. Significantly altered features were chosen based on the univariate and multivariate analyses.

Next, the metabolites were putatively annotated based on the exact mass, isotopic distribution, and fragmentation pattern in different databases, including the Human Metabolome Database ((www.hmdb.ca) and METLIN ((http://metlin.scripps.edu), within a mass difference of 5 ppm. Exogenous metabolites, such as those from drugs and environmental exposures, were excluded, and the remaining identified metabolites were retained for further pathway analyses. The receiver operating characteristic (ROC) curve was used to assess the diagnostic ability of the potential biomarkers to discriminate between a hypothyroid and a euthyroid state during LT4 treatment. Sensitivity, specificity, and area under the ROC curve (AUC) were determined using the MetaboAnalyst program (https://www.metaboanalyst.ca/).

3. Results
3.1 Biochemical analysis

The measurements at baseline and after treatment in a euthyroid state are listed in Table 1.

3.2. Mass ion detection and significantly altered metabolites

A total of 20,406 mass ion features were detected (13,010 and 7,396 in positive and negative 18 modes, respectively) (Table S1). After applying a frequency threshold (cutoff, 80% of all samples) and excluding 19% of missing values during the peak detection and alignment steps, 16,565 features were retained for statistical analysis. Afterward, these features were normalized by total signal median and then log-transformed and Pareto-scaled before univariate and multivariate analyses.

Based on the multivariate analysis, the OPLS-DA model (Figure 1) clearly showed separation between the two groups; this result highlighted that the metabolic profile of patients with hypothyroidism significantly differed before and after treatment. Using an extended dataset (n = 100), a permutation test to validate the OPLS-DA model showed $R^2_Y = 0.991$ and $Q^2 = 0.404$. The discriminant mass ions with a VIP of >1 were responsible for the proposed separation and were subjected to univariate analysis to determine their statistical significance.

On univariate analysis and volcano plot, 334 features were significantly dysregulated by LT4 treatment (Figure 2A). Of these features, 14 were upregulated, and 320 were downregulated after treatment (Table S2).

Table S3 shows a summary of 210 of the 334 compounds identified. The top 94 metabolites were identified as endogenous and retained for further pathway and biomarkers analyses (Table S4).

The folate biosynthesis; glycerolipid, glycerophospholipid, phosphatidylinositol signaling systems; and primary bile acid biosynthesis were the most affected pathways between the two groups (Figure 2B). Compared with the pretreatment group, the posttreatment group had a metabolic plasma profile of significantly higher levels of glycocholic acid, lysophosphatidylinositol (LysoPI) (16:0/0:0), monoacylglyceride (MG) (0:0/24:1(15Z)/0:0), 1,2-diglyceride (DG) (8:0/13:0/0:0), and tracylglycerol (TG) (14:0/14:0/18:1(11Z)) and significantly lower levels of diacylglycerol (DG) (24:0/20:2), phosphatidylcholine (PC) (18:1(12Z)-O(9S,10R)/2:0), Asparagine (Asn), Aspartic acid (Asp), Metionine (Met), tetracosatetraenoyl carnitine, 3-hydroxyhexanoylcarnitine, 2-octanoic, and
DG (16:1n7/0:0/18:2n6). These metabolites might be considered promising biomarkers for transitioning from hypothyroid to euthyroid following LT4 treatment.

Figure 3A shows the specificity and sensitivity of the potential biomarkers. Three features in the ROC curve had an AUC value of 0.924. Feature ranking in OPLS-DA (Figure 3B) revealed that LysoPI(16:0/0:0), MG(0:0/24:1(15Z)/0:0), DG(8:0/13:0/0:0), TG(14:0/14:0/18:1(11Z)), and N-Acetylasparagine were the most discriminative metabolites.

4. Discussion

Thyroid hormones affect nearly every cell in the body when they cross the plasma membrane and bind to receptors on the mitochondria that control metabolism, including energy production and glucose oxidation (20, 21). In 1930, hypothyroidism was first reported to have a huge impact on blood lipid components (22). Since then, several studies that examined the association and impact of thyroid hormones on metabolism have shed light on the pathophysiology of hypothyroidism and the mechanism of action of LT4.

In this study, we demonstrated distinct sample clustering and group separation between the hypothyroid and euthyroid groups as a result of LT4 treatment, as shown in the OPLS-DA model, which had acceptable goodness of fit and prediction (23). Moreover, LysoPI (16:0/0:0), MG(0:0/24:1(15Z)/0:0), DG (8:0/13:0/0:0) and TG(14:0/14:0/18:1(11Z)) were the highest ranking metabolites that could discriminate between hypothyroidism and euthyroidism before and after treatment, respectively. Therefore, these metabolites may be further evaluated to monitor treatment with LT4. The significant increase in the T4 level, which is expected to significantly decrease the TSH level, in the euthyroid state from the T4 level in the hypothyroid state reflected a positive therapeutic effect of LT4 on hypothyroidism.

Inadequate levels of circulating thyroid hormones secondary to hypothyroidism decrease hepatic cholesterol synthesis, probably by inhibition of hydroxy methylglutaryl coenzyme (HMG-CoA) reductase. Furthermore, thyroid hormones are known to stimulate transcription of the LDL receptor and HMG-CoA reductase genes. HMG-CoA reductase is expressed in the liver and stimulates the production of cholesterol. However, decrease in hepatic cholesterol synthesis is outweighed by the action of thyroid hormones on LDL cholesterol receptor expression, resulting in net accumulation of serum LDL cholesterol. Therefore, compared with healthy subjects, patients with hypothyroidism usually present with increased TC and LDL concentrations. Moreover,
they manifest elevated TG levels secondary to a decrease in the activity of lipoprotein lipase, which is an enzyme that degrades circulating TG in the bloodstream (24-27). LT4 can be extremely effective for the treatment of hyperlipidemia associated with hypothyroidism (26). However, our results showed no notable changes in the serum concentrations of TC, LDL, and TG after LT4 treatment. This implied that a longer time may be needed to observe for changes in the lipid profile, even if a euthyroid state has been achieved. Another reason for the absence of significant changes in the lipid profile after LT4 treatment may be the fact that the recruited patients had lipid profiles within the accepted range before treatment.

In addition to dyslipidemia, which is consistently seen in hypothyroidism, considerably greater plasma levels of glycerophospholipids in individuals with clinical and subclinical hypothyroidism than in euthyroid individuals were previously reported (9). Moreover, several animal studies reported that thyroid hormones could greatly affect sphingolipid metabolism, de novo ceramide synthesis, glycerophospholipid metabolism, and fatty acid β oxidation in the liver (28, 29). In other studies, the level of ceramides increased upon LT4 administration to hypothyroid tissue and was even higher in hyperthyroid hepatocytes, liver tissue, and heart muscle (29, 30). LT4 is believed to increase the activity of ceramide phosphocholine transferase, which is an enzyme that transfers the phosphocholine group from phosphatidyl cholines (PC) to ceramide to generate sphingomyelin (SM), and diglycerides (DG) (31). In a recent plasma metabolomics profiling study Feifei Shao et al, the level of ceramide was higher in individuals with clinical and subclinical hypothyroidism than in the control group (9). Consistent with previous reports, this present study found low plasma levels of ceramides, trihexosyl ceramide (d18:1/22:0), and trihexosyl ceramide (d18:1/24:0) upon LT4 treatment. In addition, the downregulation of 10 metabolites identified as PC following LT4 treatment could reflect that more PCs were redirected to the liver and consumed during SM synthesis, while the liver lipoprotein-associated PC efflux decreased. This provided evidence on the key role of thyroid hormones in lipid synthesis and metabolism, particularly sphingolipid metabolism in the liver. Moreover, monitoring plasma and/ or liver ceramides and probably SM could serve as gold standard regulators of hypothyroid treatment and may be used to monitor LT4 overdose.

Elevated level of DG is a common finding in hypothyroidism. DG accumulation had been attributed to multiple routes, including lipid efflux, and ceramide, polyphosphoinositide, and PC hydrolysis, in addition to
reduced hepatic lipogenesis, all of which resulted in reduced levels of PC and TG (32). Sustained elevation of DG level is believed to be associated with decreased protein kinase C (PKC) activity (33). PKC comprises a family of closely related enzymes that are activated by the second messenger calcium (Ca²⁺) and DG, depending on the duration, and magnitude of these signals. Inappropriate DG buildup promotes different cellular transformations, including hepatocarcinogenesis, hyperglycemia, hypertriglyceridermia, other cardiovascular complications of diabetes, and hypothyroidism (34-38). In addition, the work of Bansode R et al on mice liver and skeletal muscle showed reductions in adipose tissue depots and TG content upon deletion of the PKCβ isoform (37, 38). Therefore, the level of intracellular DG must be closely regulated. Upon LT4 administration, PKC is reactivated, the amount of DG synthesized de novo will be decreased, and the level of TG is expected to increase (33, 39, 40). In the current study, the levels of three of four identified DGs were downregulated and the level of one of two identified TGs was upregulated after treatment. Although this result coincides with that of previous studies, not all DGs, and TGs follow the same trend, thereby, suggesting that a complete metabolic change was not achieved after reaching a euthyroid state with treatment, because T3, and T4 levels do not reflect all the changes reversed in hypothyroidism. Consistent with our findings, the results of C. Piras et al showed that upon LT4 administration to 18 patients with overt primary hypothyroidism, the metabolic changes persisted despite normalization of serum TSH and thyroid hormone concentrations (17). Therefore, the metabolomics technique may help integrate traditional hormone assays and more effectively assess the accomplishment of a euthyroid state.

Thyroid hormones are known to increase the transcription of fibroblast growth factor 21 (FGF21), which is a coded protein that serves as an endocrine factor and a key metabolic regulator of glucose and lipids in adipose tissue (41). Reduced level of FGF21 was reported in hypothyroidism, whereas elevated FGF21 level was observed in hyperthyroidism (42, 43). FGF21 inhibits the synthesis of fatty acids, cholesterol, and TG and stimulates fatty acid uptake and β oxidation, which enhance thermogenesis and increase energy expenditure (44, 45). β oxidation is a multistep process that takes place in the mitochondria, where fatty acids are broken down by different tissues to produce acetyl-CoA units that can be employed in the tricarboxylic acid (TCA) cycle to produce ATP. Therefore, accumulation of plasma long-chain acylcarnitine in patients with hypothyroidism supports the decrease in the activity of fatty acid oxidation, which may indicate dysfunction
in the long-chain fatty acid transport protein (FATP) 1 (28) or incomplete β oxidation relative to the tricarboxylic acid cycle flux (46). In this study, a low level of long-chain acylcarnitine was detected upon treatment; this reflected enhanced β oxidation in euthyroid patients than in hypothyroid patients. Thyroid hormones activate Ca\(^{2+}\)/calmodulin-dependent protein kinase-β, which, in turn, mediates phosphorylation of AMP-activated protein kinase (AMPK) and increases fatty acid transport from the periphery into the cells by translocation of CD36 through activation of Ras-related protein Rab-8A (Rab8a) or Fatty Acid Transport Protein (FATP) on the plasma membrane. In addition, AMPK activates acetyl-CoA carboxylase (ACC), which is an enzyme that catalyzes the conversion of acetyl-CoA to malonyl-CoA, which promotes fatty acid synthesis (47). However, when activated by AMPK, ACC activity is inhibited, thereby, decreasing the amount of malonyl-CoA released and activating carnitine palmitoyl transferase 1, which is an outer mitochondrial membrane protein that converts acyl-CoA to acyl carnitine, accompanied by an increase in fatty acid trafficking into mitochondria (47). In addition, thyroid hormones increase the production of several mitochondrial enzymes required for β oxidation, including mitochondrial uncoupling protein (48), pyruvate dehydrogenase kinase (49), and medium-chain acyl-CoA dehydrogenase (50).

Furthermore, glycocholic acid was increased upon restoration of a euthyroid state. Thyroid hormone facilitates the conversion of cholesterol to bile acid by stimulating the rate-limiting enzyme cholesterol 7α-hydroxylase through hepatic TH β receptors and stimulates bile acid secretion in the liver and intestine (51-53). Therefore, increase in the glycocholic acid concentration was expected in the posttreatment samples.

Compared with a hypothyroid state, a euthyroid state led to downregulation of small peptides upon achieving normal plasma free T4; this suggested a change in protein metabolism. Since 1956, whole body protein breakdown and decreased synthesis had been documented upon disruption in thyroid hormone levels (either hypothyroidism or hyperthyroidism). However, reversal of these changes following treatment indicated the importance of sustaining normal thyroid hormone levels for maintaining anabolic effects (54, 55).

The findings from the study have to be taken into the consideration keeping in mind the small sample size and lack of gender base analysis and the time duration to reach euthyroid levels. Another point that needs to be considered is the fact that although our study shows significant improvement in the metabolomic profile after thyroxine treatment it is not known at which FT4 levels there is a complete normalization of the metabolomic profile, which is the real euthyroidism at cellular level, and not only the FT4 within the normal range for that laboratory. Further investigations are necessary to confirm the results obtained in this study.
Conclusions

A metabolomic analysis of patients with hypothyroidism revealed significant changes in the level of several metabolites and lipids after treatment. This study showed the value of the metabolomics technique in providing a complementary understanding of the pathophysiology of hypothyroidism and as a crucial tool for examining the molecular impact of LT4 treatment on hypothyroidism. However, it is important to note that metabolic changes might persist upon reaching a euthyroid state after treatment.

Statements

Acknowledgments

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Statement of Ethics and Informed Consent Statement

The study was conducted in accordance with the Declaration of Helsinki, and all procedures and protocols, including the clinical samples, were reviewed, and approved by the ethics committee of the College of Medicine, King Saud University, Riyadh, Saudi Arabia (registration no. E-10-172). Informed consent was obtained from all participants involved in the study.

Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as potential conflict of interest.

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Author Contributions

, Anas M. Abdel Rahman., and Assim A. Alfadda wrote the manuscript. All authors have read and approved the final manuscript.

**Data Availability Statement**

Metabolomics data were deposited to the EMBL-EBI MetaboLights database with the identifier MTBLS7600. The complete dataset can be accessed at https://www.ebi.ac.uk/metabolights/MTBLS7600 (30 March 2023).

**Supplementary Materials**

The following are available online.

**References**


22. Rizos CV, Elisas MS, Liberooulos EN. Effects of thyroid dysfunction on lipid profile. The open cardiovascular medicine journal. 2011;5:76-84. Epub 2011/06/11.


Figure 1:
The orthogonal partial least squares discriminant analysis (OPLS-DA) model

There is evident group separation between the post and pretreatment groups. The robustness of the created models is evaluated by the fitness of the model ($R^2_Y = 0.991$) and the predictive ability ($Q^2 = 0.404$) values on larger data ($n = 100$). (green dots represent the pre-treatment group, and red dots represent the post-treatment group)

Figure 2:
(A) This is a volcano plot between the post and pretreatment groups after applying an 80% filter on all data, (paired t-test, no correction, $p \leq 0.05$, FC 2). Of 334 dysregulated metabolites, 14 (red) are upregulated, and 320 (blue) are downregulated between the two groups, respectively. Orange and light blue squares refer to metabolites that failed to pass fold change cutoffs and were up- and downregulated, respectively. Gray square metabolites failed to pass both cutoffs. (B) Pathway analysis of the significantly dysregulated metabolites of patients with hypothyroidism before and after treatment. Colors (varying from yellow to red) mean the metabolites are in the data with different significance levels ($p$-value).

Figure 3:
Receiver operating characteristics (ROC) curve was generated by the orthogonal partial least squares discriminant analysis (OPLS-DA) model, with Area Under the Curve (AUC) values calculated from the combination of 5, 10, 15, 25, 50, and 100 metabolites and (B) loading for significantly altered metabolites before and after hypothyroidism treatment are shown. (C) TG (14:0/14:0/18:1) (AUC, 0.901) and (D) N-acetyl asparagine (AUC, 0.951) as examples of upregulated after hypothyroidism treatment
Table 1: Biochemical parameters of the hypothyroid patients at baseline and after levothyroxine therapy.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Hypothyroid</th>
<th>Euthyroid</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients number</td>
<td>18</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>39 ± 11.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Female/ male</td>
<td>10/8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FT4 (pmol/L)</td>
<td>7.9 ± 5.8</td>
<td>17.9 ± 3.5</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>TSH (mIU/L) median (IQR)</td>
<td>23.79 (36.39)</td>
<td>1.20 (1.08)</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>5.2 ± 0.5</td>
<td>5.1 ± 0.3</td>
<td>0.067</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>4.4 ± 0.2</td>
<td>4.8 ± 0.8</td>
<td>0.318</td>
</tr>
<tr>
<td>Creatinine (umol/L)</td>
<td>74.5 ± 11.1</td>
<td>75.7 ± 20.4</td>
<td>0.450</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>140.1 ± 1.7</td>
<td>139.5 ± 0.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.6 ± 0.2</td>
<td>4.6 ± 0.1</td>
<td>1.20</td>
</tr>
<tr>
<td>Aspartate transaminase (IU/L)</td>
<td>35.3 ± 7.1</td>
<td>36.8 ± 7.5</td>
<td>0.450</td>
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<tr>
<td>Alanine transaminase (IU/L)</td>
<td>18.8 ± 6.2</td>
<td>17.9 ± 3.5</td>
<td>0.21</td>
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<td>Alkaline phosphatase (IU/L)</td>
<td>95.8 ± 19.9</td>
<td>97.2 ± 21.1</td>
<td>0.86</td>
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<td>Total cholesterol (mmol/L)</td>
<td>4.5 ± 0.4</td>
<td>4.9 ± 0.8</td>
<td>0.66</td>
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<tr>
<td>Low-density lipoprotein cholesterol (mmol/L)</td>
<td>1.4 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>0.25</td>
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<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.5 ± 0.2</td>
<td>1.4 ± 0.4</td>
<td>0.35</td>
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<tr>
<td>High-density lipoprotein cholesterol (mmol/L)</td>
<td>2.3 ± 0.7</td>
<td>2.9 ± 0.5</td>
<td>0.32</td>
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</tbody>
</table>

FT4, free thyroxine; TSH, thyroid stimulating hormone. Values are expressed as means ± SD, median (IQR) and * indicate significant p-value.
LysoPI(16:0/0:0)
N-Acetyl asparagine
DG(8:0/13:0/0:0)
MG(0:0/24:1/0:0)
TG(14:0/14:0/18:1)
Tryptophyl-Phenylalanine
Glycocholic acid
N-Acetyl leucine
Asn Asp Met Met
Salicyluric beta-D-glucuronide
15-Acetyl-4-deoxynivalenol
2-Hydroxyundec-3-enoylcarnitine
7-Acetoxy-6-methoxycoumarin
Phenylalanylarginine
4-Hydroxynonenal

A

B

C

D

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