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Serum Lipidomic Analysis Reveals Biomarkers and Metabolic Pathways of Thyroid Dysfunction

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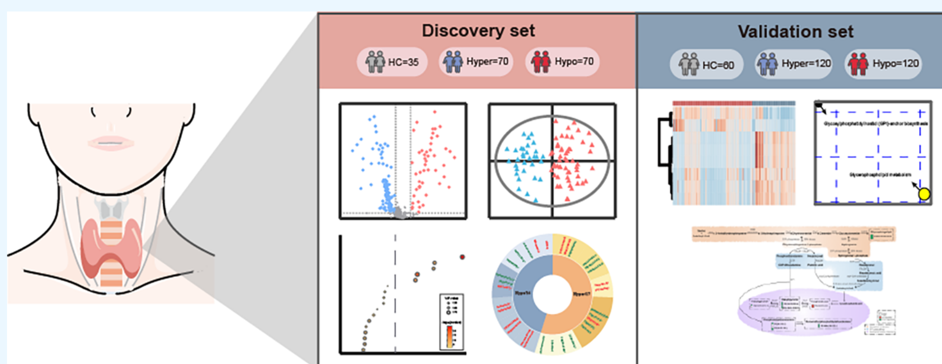

ABSTRACT: Abnormal thyroid hormone secretion is the most important feature of hypothyroidism and plays an important role in lipid metabolism. However, their connection has not been clearly established. This study aimed to identify the serum biomarkers and metabolic pathways associated with hyperthyroidism and hypothyroidism. The study enrolled discovery and validation sets of 175 and 300 participants, respectively, to identify and validate the serum biomarkers of hyperthyroidism and hypothyroidism via ultra-high performance liquid chromatography−quadrupole time-of-flight mass spectrometry lipidomics through univariate and multivariate analyses. Eight and six biomarkers were identified for hyperthyroidism and hypothyroidism, respectively. Spearman correlation analysis was used to assess the correlation between the biomarkers and thyroid dysfunction indicators; subsequently, metabolic pathway and network analyses were performed for these biomarkers. Most biomarkers exhibited significant correlation with thyroid dysfunction indicators, mainly being enriched in the glycerophospholipid (GPL) metabolism. The diagnostic accuracies of the biomarkers and biomarker panels were assessed via receiver operating characteristic curve analysis. All the biomarkers demonstrated good diagnostic performance, and the hyperthyroidism and hypothyroidism biomarker panels reached an area under the curve value of 1.000. The results were validated using the validation set. Therefore, our findings revealed that thyroid dysfunction primarily affects the human metabolism via the GPL metabolism, thus providing a theoretical basis for the clinical prevention and control of thyroid dysfunction.

1. INTRODUCTION

The thyroid gland constitutes the largest endocrine gland in the human body. Its chief function is to synthesize thyroid hormones to regulate body metabolism.[1,2] Thyroid hormones are key regulators of metabolism, development, and growth, and adequate levels of circulating thyroid hormones are essential for the proper functioning of nearly all tissues and organs in the body.[3,4] Owing to lifestyle changes, the incidence of thyroid diseases due to endocrine disorders is increasing every year, becoming the endocrine disease second only to diabetes in prevalence.[5,6] Thyroid diseases can disbalance the circulating thyroid hormones in the body, subsequently causing thyroid dysfunction and seriously affecting the human health and threatening the human life.[7,8] Hyperthyroidism and hypothyroidism are common thyroid diseases with potentially devastating health consequences that affect the global population.[3,9]

The clinical presentation of thyroid diseases is highly variable and often nonspecific.[3] Clinically, thyroid dysfunction diagnosis is primarily based on biochemical indicators, such as serum thyroid stimulating hormone (TSH) and serum free thyroxine (FT4). The diagnostic criteria for hyperthyroidism include serum TSH levels below the lower reference limit and

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FT4 levels within or above the reference range.[10] The diagnostic criteria for hypothyroidism include serum TSH levels above the upper reference limit and FT4 levels within or below the reference range.[11] According to current statistics, the prevalence of hyperthyroidism and hypothyroidism in the general population ranges from 0.2 to 1.3 and 0.2 to 5.3%, respectively, with the incidence of thyroid dysfunction increasing annually.[10,12−15] Therefore, characterizing the metabolic features of patients with hyperthyroidism and hypothyroidism and clarifying the biomarkers and metabolic pathways associated with thyroid dysfunction can help broaden the ideas and measures of clinicians for diagnosing and treating thyroid diseases and provide metabolic references for diagnosing and treating hyperthyroidism and hypothyroidism.

Thyroid hormones play an important role in lipid metabolism.[16] Thyroid dysfunction may alter lipid synthesis and degradation and the functions of various key enzymes involved in the lipid metabolic pathways.[17] Lipidomics research has been rapidly emerging as a technique for exploring the role of lipids in body metabolism.[18] Lipidomics studies the interaction of lipids in vivo and their interactions with lipid-associated proteins to delineate the mechanism of lipid metabolism regulation.[19] It builds a comprehensive map of the lipidomes of biological samples.[20] As lipidomics enables the comprehensive, large-scale analysis of lipids in biological systems, it has been increasingly applied in recent years to discover and identify disease biomarkers and to elucidate disease development mechanisms.[21] Mass spectrometry (MS) constitutes a common analysis platform for lipidomics. Using its positive and negative mode ionization methods and versatile mass analyzers, intact molecular ions of metabolites can be produced, and highly resolved MS/MS spectra can be acquired. Thus, MS exhibits high sensitivity and selectivity as well as the potential for metabolite identification.[22]

Currently, some progress has been made in the field of thyroid diseases using lipidomics; however, in practical clinical research, there have been few studies involving lipidomics related to hyperthyroidism and hypothyroidism using human serum as biological samples. Herein, we analyzed serum samples from patients with hyperthyroidism and hypothyroidism and healthy control (HC) via lipidomics using ultra-high-performance liquid chromatography−quadrupole time-of-flight mass spectrometry (UPLC−Q-TOF/MS) to investigate changes in serum metabolites in patients with hyperthyroidism and hypothyroidism and to identify potential biomarkers and metabolic pathways to provide a theoretical basis for the clinical prevention and control of hyperthyroidism and hypothyroidism.

2. RESULTS

2.1. Demographics of the Study Population. The workflow of this study is shown in Figure 1. The demographic information of the participants is presented as mean ± standard deviation, and differences were considered statistically significant at $p < 0.05$. The gender and age distribution between the groups were matched as closely as possible, and the details are listed in Table 1. The serum levels of the thyroid dysfunction indicators, including TSH, free triiodothyronine (FT3), and FT4, in the thyroid dysfunction groups significantly differed from those in the HC group ($p < 0.05$).

2.2. Metabolic Profiles of the Serum Samples of the HC and Patients with Hyperthyroidism and Hypothyroidism. The serum samples of the participants were subjected to lipidomic analysis using UPLC−Q-TOF/MS in the positive electrospray ionization (ESI) detection mode for 20 min. Base peak chromatograms of the quality control (QC) samples are shown in Figure S1A. The results of methodology validation (instrument precision, method repeatability, and

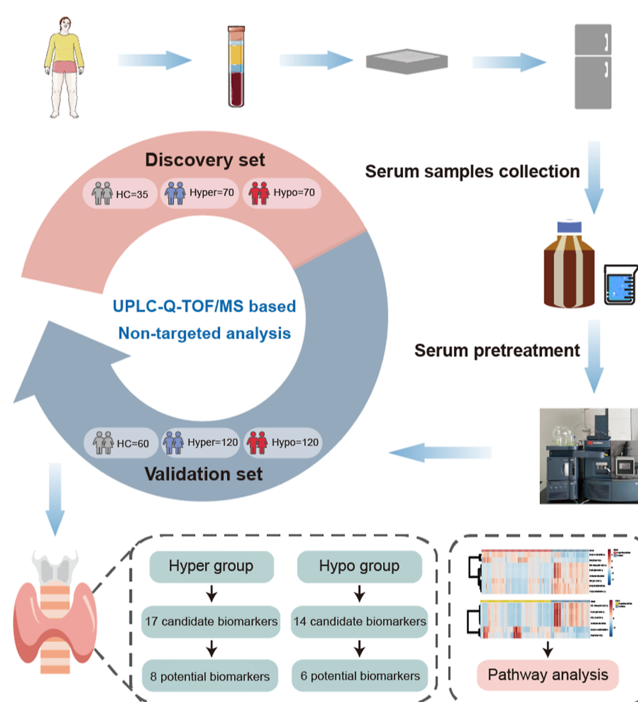

Figure 1. Design of the study. HC, healthy control group; hyper, hyperthyroidism group; and hypo, hypothyroidism group.

Table 1. Each Participant's Demographic and Clinical Information[a]

characteristics	HC	hypo	hyper
	Discovery Set		
number	35	70	70
gender (male/female)	8/27	12/58	15/55
age (years)	47.86 ± 7.94	50.59 ± 10.71	48.19 ± 7.04
age range (minimum--to-maximum)	25−68	24−68	35−63
TSH (uIU/mL)	2.14 ± 0.97	23.71 ± 25.90*	0.05 ± 0.10#
FT3 (pmol/L)	4.79 ± 0.48	3.72 ± 0.91*	9.48 ± 5.40#
FT4 (pmol/L)	15.15 ± 3.09	12.98 ± 4.48*	27.44 ± 12.86#
	Validation Set		
number	60	120	120
gender (male/female)	13/47	16/104	22/98
age (years)	50.90 ± 7.86	51.01 ± 14.08	49.84 ± 12.10
age range (minimum--to-maximum)	32−76	22−84	27−79
TSH (uIU/mL)	2.21 ± 0.99	27.03 ± 29.54*	0.07 ± 0.12#
FT3 (pmol/L)	4.81 ± 0.43	3.69 ± 0.95*	8.53 ± 5.67#
FT4 (pmol/L)	16.16 ± 2.20	12.38 ± 4.32*	23.84 ± 12.58#

[a]Note: *$p < 0.05$, **$p < 0.01$, hypothyroidism group compared to the healthy control group; #$p < 0.05$, ##$p < 0.01$, hyperthyroidism group compared to the healthy control group; abbreviations: HC, healthy control group; hypo, hypothyroidism group; hyper, hyperthyroidism group; TSH, thyroid stimulating hormone; FT3, free triiodothyronine; and FT4, free thyroxine.

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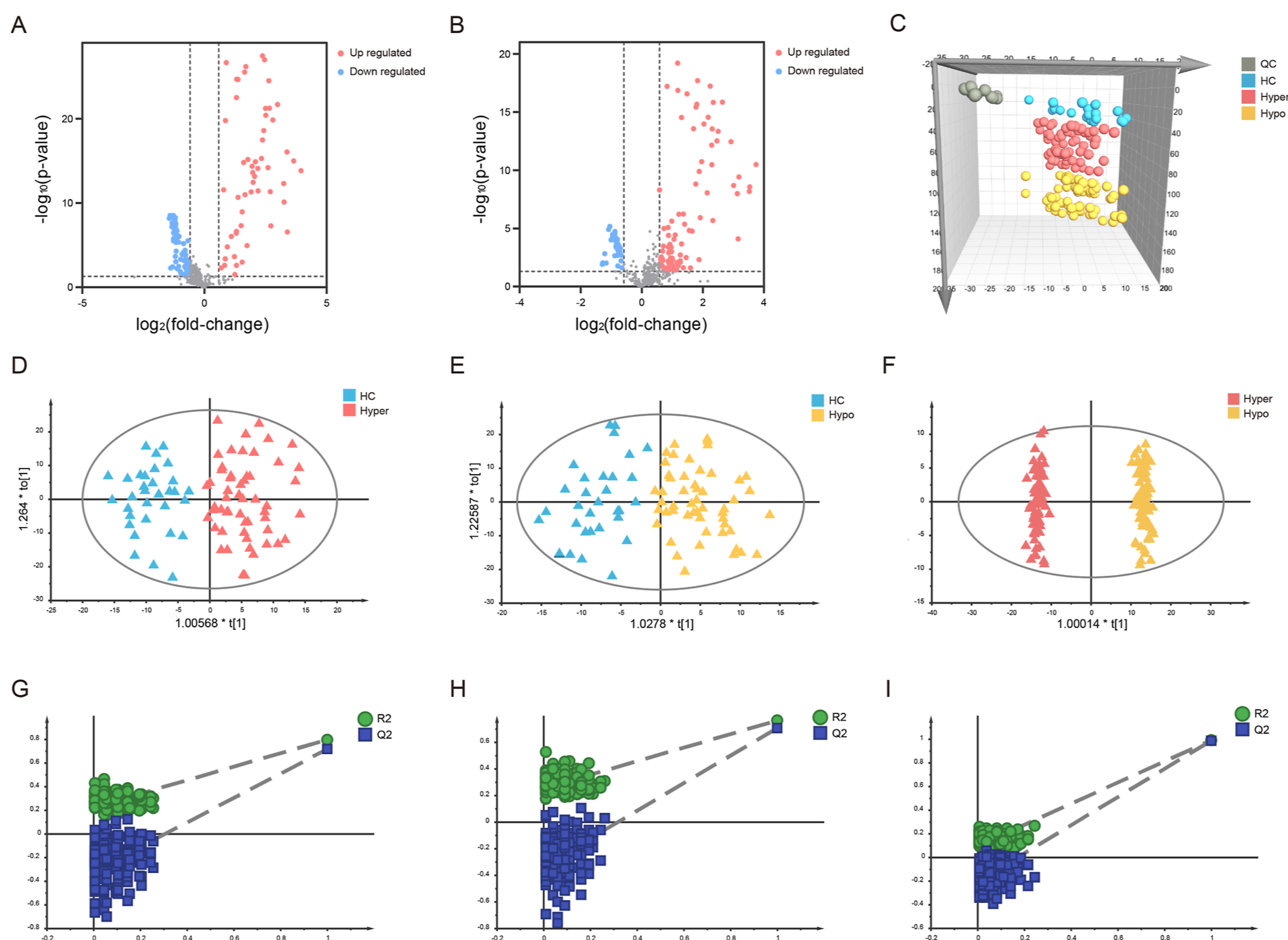

Figure 2. Univariate and multivariate statistical analysis of participants. (A) Volcano plot of the features of the serum metabolic ions in patients with hyper compared with HC. (B) Volcano plot of the features of serum metabolic ions in patients with hypo compared with HC. C, QC, HC, hyper, and hypo group PCA plot. (D) OPLS-DA plot of the hyper and HC groups. (E) OPLS-DA plot of the hypo and HC groups. (F) OPLS-DA plot of the hyper and hypo groups. (G) Permutation test between the hyper and HC groups. (H) Permutation test between the hypo and HC groups. (I) Permutation test of the hyper and hypo groups. QC, quality control; HC, healthy control group; hyper, hyperthyroidism group; and hypo, hypothyroidism group.

sample stability) of the discovery set are shown in Table S1. After an 80% reduction in the raw data and filling of missing values, 1555 features were selected for the subsequent statistical analysis. To provide a more detailed picture of the overall metabolic profile of the participants and better characterize the significantly difference metabolite changes, we performed volcano plot global analysis (Figure 2A,B). Compared with the HC group, the vast majority of the metabolic characteristics of the hyperthyroidism and hypothyroidism groups exhibited an increasing trend, with a portion of the metabolic characteristics showing a decreasing trend.

Principal component analysis (PCA) was performed to characterize the metabolic profile of the lipidomic data of the serum samples of each group. The QC samples shown as gray scatter are centrally clustered, indicating good instrument reproducibility and stability throughout the lipidomics study (Figure 2C). However, the metabolic characteristics were not distinctly separated between the HC and the individual thyroid dysfunction groups. Thus, we further performed supervised orthogonal partial least squares discriminant analysis (OPLS-DA) to determine variations in the metabolic characteristics among the different groups and evaluate the predictive ability of the model. Furthermore, 200 random permutation tests were performed to evaluate the reliability of the model.

The OPLS-DA models were constructed for the HC versus hyperthyroidism, HC versus hypothyroidism, and hyperthyroidism versus hypothyroidism groups (Table S2). The metabolic characteristics between the thyroid dysfunction and HC groups and the two thyroid dysfunction groups were distinct and all the models demonstrated desirable classification and prediction ability (Figure 2D−F). In addition, the results of the permutation test revealed no over-fitting of the models between the groups, and the goodness of fit obtained relatively reliable prediction results (Figure 2G−I and Table S2).

The metabolite profiles with variable importance in projection (VIP) values of >1.0 and false discovery rate (FDR) values of <0.05 were of variable importance and considered potential between-group differential metabolites. A screening based on the results derived from the OPLS-DA model in the discovery set revealed that compared with the HC group, 17 and 14 candidate biomarkers were identified in the hyperthyroidism and hypothyroidism groups, respectively (Figure S2). The specific change trends of biomarkers in the hyperthyroidism and hypothyroidism groups are shown in

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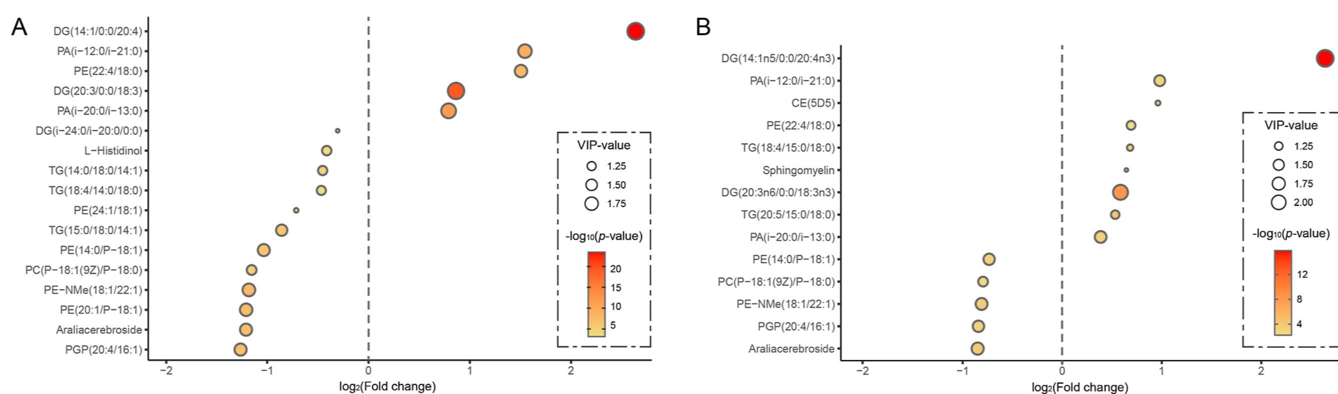

Figure 3. Change trend chart of the candidate biomarkers. (A) Change trend chart of the hyperthyroidism biomarkers. (B) Change trend chart of the hypothyroidism biomarkers.

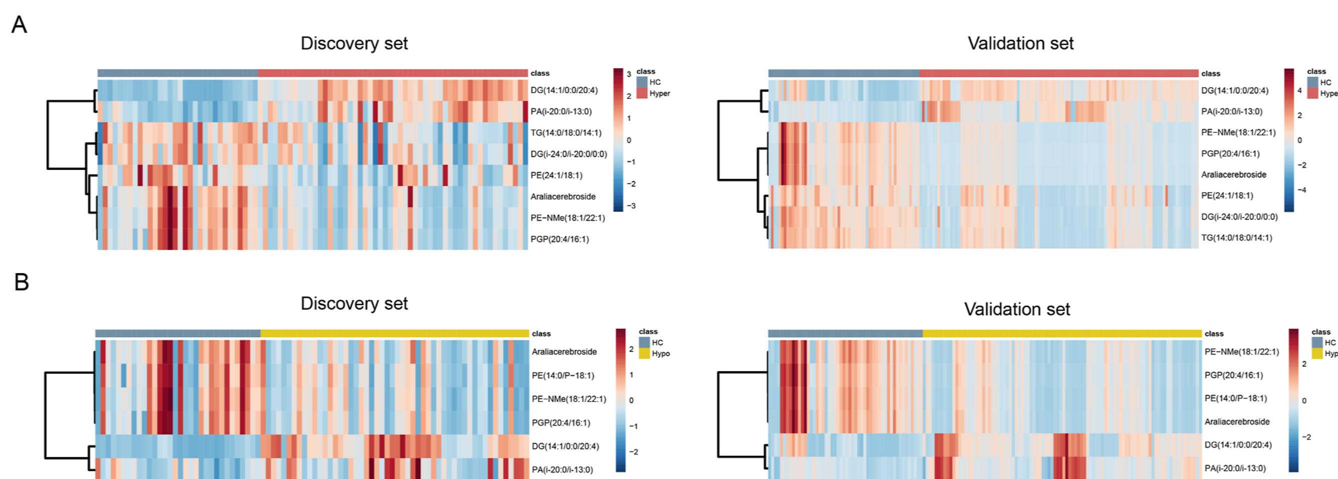

Figure 4. Hierarchical clustering analysis of the thyroid dysfunction and HC groups in the discovery and validation sets. (A) Heatmaps of the HC and hyperthyroidism groups. (B) Heatmaps of the HC and hypothyroidism groups. HC, healthy control; hyper, hyperthyroidism group; and hypo, hypothyroidism group.

Figure 3A,B. Among them, the biomarker levels in the hyperthyroidism group primarily exhibited a decreasing trend, and only phosphatidic acid (PA) metabolites, DG (14:1/0:0/20:4), DG (20:3/0:0/18:3), and PE (22:4/18:0) depicted an increasing trend. The biomarker levels in the hypothyroidism group primarily exhibited an increasing trend, and only phosphatidylglycerophosphate, phosphatidylcholine, monomethylphosphatidylethanolamine, glycosphingolipid metabolites, and PE (14:0/P-18:1) depicted a decreasing trend. Notably, the hyperthyroidism and hypothyroidism groups had eight identical biomarkers, all showing the same trend of change.

2.3. Validation of Candidate Differential Biomarkers. To verify the reliability of the findings in the discovery set, we used power analysis based on the results of the discovery set to evaluate the minimum sample size required to identify statistically significantly different metabolites between the thyroid dysfunction (hyperthyroidism or hypothyroidism) and HC groups. The results revealed that when the sample size reached 30 participants in each group, the power of detecting the difference between the hyperthyroidism group and HC group was more than 80% (Figure S3A). When the sample size reached 60 participants in each group, the power of the difference between hypothyroidism group and HC group can be detected >80% (Figure S3B). Therefore, in the validation set, the serum samples of 300 participants, including 60 HC, 120 patients with hyperthyroidism, and 120 patients with hypothyroidism, were included in the UPLC−Q-TOF/MS lipidomic analysis to obtain accurate results with true statistical differences to validate the metabolites identified in the discovery set.

The base peak chromatograms of the QC samples from the validation set are shown in Figure S1B. The results of the methodology validation (instrument precision, method repeatability, and sample stability) of the QC samples from the validation set are shown in Table S1. Eight and six specific biomarkers could distinguish HC from hyperthyroidism and hypothyroidism, respectively (Tables S3 and S4). The mass spectrum data of the hyperthyroidism and hypothyroidism biomarkers were identified, verified, and compared with the MS/MS spectra of these biomarkers obtained from the Human Metabolome Database (HMDB) (Figure S4 and Table S5). The heatmaps revealed that compared with the HC group, both the thyroid dysfunction groups exhibited significantly different metabolic profiles in the discovery and validation sets (Figure 4A,B).

2.4. Correlation Analysis of the Thyroid Biomarkers. Spearman correlation analysis was performed to determine the correlation between the biomarker levels and thyroid serum indicators of the patients with hyperthyroidism and hypothyroidism (Figure S5). Except PE (24:1/18:1) among the eight biomarkers in the hyperthyroidism group, the levels of

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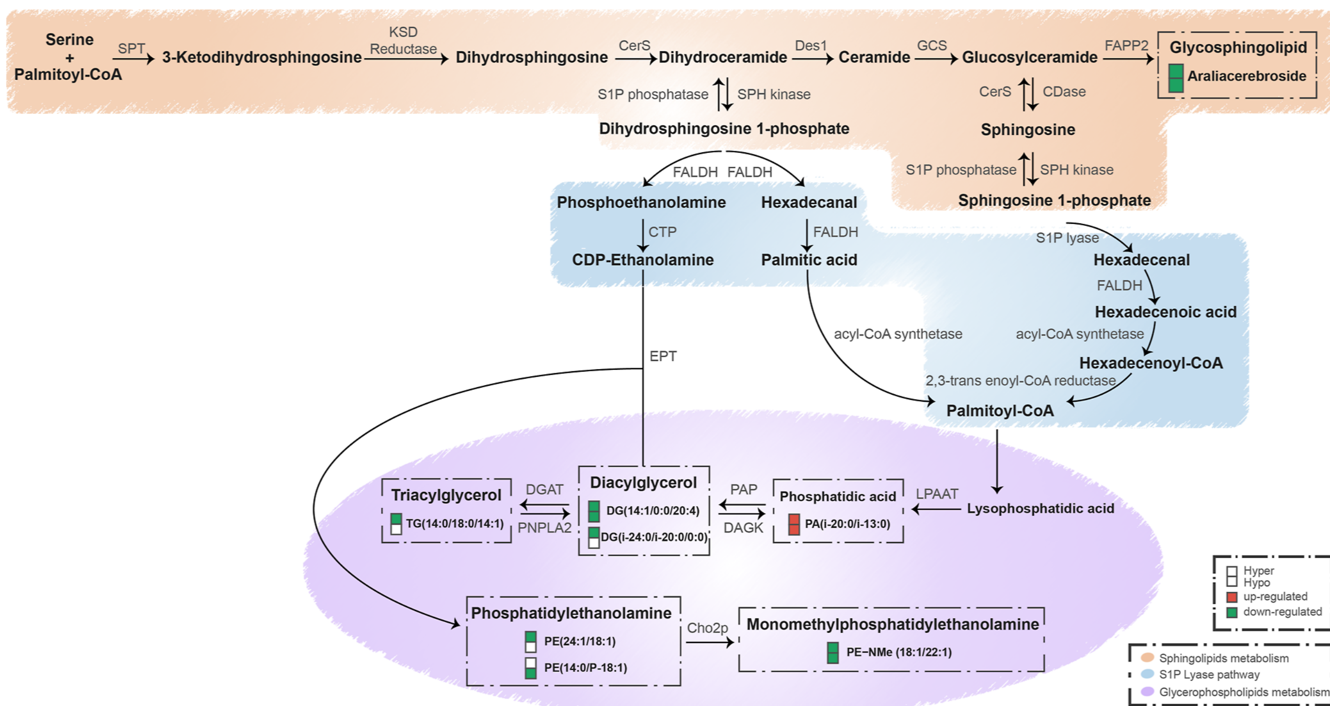

Figure 5. Biomarker interaction network diagram. Hyper, hyperthyroidism group; hypo, hypothyroidism group.

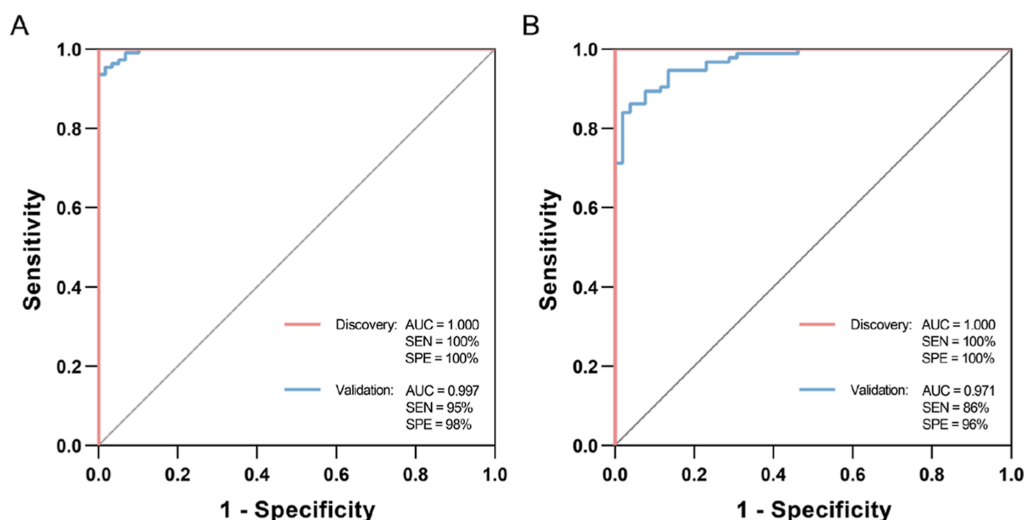

Figure 6. ROC curve analysis of biomarker panels. (A) ROC curve analysis of the combination of the hyperthyroidism biomarkers in the discovery and validation sets. (B) ROC curve analysis of the combination of the hypothyroidism biomarkers in the discovery and validation sets.

the remaining biomarkers were significantly correlated with those of TSH, FT3, and FT4 (Table S6). Except PA (20:0/13:0), which was not associated with FT4, among the six biomarkers in the hypothyroidism group, the levels of the other biomarkers were significantly correlated with those of TSH, FT3, and FT4 (Table S7). Notably, the hyperthyroidism biomarkers and thyroid dysfunction indicators exhibited negative correlation and hypothyroidism biomarkers and thyroid dysfunction indicators exhibited positive correlation.

2.5. Pathway and Network Analysis of the Thyroid Biomarkers. The differential biomarkers were mapped to their biochemical pathways via metabolic pathway analysis. The results revealed that the significantly altered metabolic pathways chiefly included glycerophospholipid (GPL) metabolism and glycosylphosphatidylinositol-anchored biosynthesis ($p < 0.05$; Figure S6). Of these, disorders of GPL metabolism were ranked first among the most severely affected pathways (impact = 0.104 > 0.1). In addition, metabolite network analysis revealed that eight hyperthyroidism biomarkers and six hypothyroidism biomarkers were primarily associated with GPL metabolism and sphingolipid metabolism, and the metabolites in both the metabolic pathways were linked via the sphingosine 1-phosphate (S1P)-mediated S1P cleavage enzyme pathway (Figure 5).

2.6. Evaluation of the Diagnostic Accuracy of the Hyperthyroidism and Hypothyroidism Biomarkers. To further explore the reliability of the identified hyperthyroidism and hypothyroidism biomarkers, we used receiver operating characteristic (ROC) curve analysis to verify their diagnostic accuracy. The results showed that the area under the curve

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(AUC) values of all the hypothyroidism and hyperthyroidism biomarkers except DG (i-24:0/i-20:0/0:0) were >0.7, indicating good diagnostic performance (Figures S7 and S8; Tables S8 and S9). The relative levels of these hyperthyroidism and hypothyroidism biomarkers between the different groups in the discovery and validation sets were compared using scatterplots (Figures S9 and S10).

In addition, we constructed binary logistic regression classification models for the hyperthyroidism and hypothyroidism biomarkers and used the predicted probability value to establish an ROC curve to evaluate the combined diagnostic ability of the two biomarker panels. The results revealed that the algorithm combining the eight hyperthyroidism biomarkers accurately distinguished patients with hyperthyroidism from the healthy population with an AUC of 1.000 (95% confidence intervals [CI] = 1.000−1.000), sensitivity of 100% (95% CI = 0.9336−1.0000), specificity of 100% (95% CI = 0.8928−1.0000), and prediction accuracy of 100%; this prediction result was also confirmed in the validation set (Figure 6A and Table S10). Similarly, the biomarker panel comprising the six hypothyroidism biomarkers also exhibited good classification ability to distinguish patients with hypothyroidism from the healthy population with an AUC of 1.000 (95% CI = 1.000−1.000), sensitivity of 100% (95% CI = 0.9312−1.0000), specificity of 100% (95% CI = 0.8928−1.0000), and predictive accuracy of 100%; these results were further validated in the validation set (Figure 6B and Table S10).

3. DISCUSSION

The main clinical feature of hyperthyroidism and hypothyroidism is abnormal serum TSH levels. Therefore, measuring serum TSH levels is the primary screening method for abnormal thyroid function.[3] However, despite the widespread availability of this screening tool, a few of its drawbacks and shortcomings have gradually emerged. TSH levels vary diurnally, fluctuating up to 40−50% of the mean value within the normal range.[23,24] Furthermore, serum FT4 levels affect TSH secretion, which is hypersensitive to small increases or decreases in FT4 levels.[25] In addition, abnormal serum TSH levels have been observed in various nonthyroid disease states that is dependent on different geographical regions and patient age.[26−28] These findings suggest that serum TSH levels are somewhat variable, and thus, do not always reflect the true state of the thyroid. Therefore, although assessing serum TSH levels for thyroid dysfunction screening is simple and widely used, establishing an objective and accurate serum diagnostic biomarker for hyperthyroidism and hypothyroidism can complement serum TSH testing to more accurately diagnose thyroid dysfunction, which is also essential for the early and effective treatment of patients.

Abnormal TSH secretion is often accompanied by biochemical alterations in lipid metabolism. To comprehensively investigate thyroid dysfunction-associated lipid metabolite changes in living organisms, nontargeted UPLC−Q-TOF/MS lipidomics techniques have been increasingly applied owing to their high sensitivity, high specificity, and high peak resolution.[29] Herein, we performed a comprehensive untargeted UPLC−Q-TOF/MS lipidomic analysis of serum samples collected from participants in the discovery and validation sets of two centers to characterize abnormal lipid metabolism in the serum of patients with hyperthyroidism and hypothyroidism compared with the HC population. We employed multivariate statistical analysis to identify and validate the eight and six differential biomarkers of hyperthyroidism and hypothyroidism, respectively, which can be used to differentiate hyperthyroidism and hypothyroidism individually from HC. Correlation analysis revealed that the levels of most of the hyperthyroidism and hypothyroidism biomarkers were significantly correlated with those of TSH, FT3, and FT4 and that the correlation between the biomarker levels and thyroid dysfunction indicators of the two diseases exhibited opposite trends. The metabolic pathway analysis of these potential biomarkers revealed that GPL metabolism was the most significantly altered metabolic pathway in patients with hyperthyroidism and those with hypothyroidism. ROC curve analysis revealed that most hyperthyroidism and hypothyroidism biomarkers had AUC values of >0.7, indicating desirable diagnostic value, and the AUC values of the biomarker panel of hyperthyroidism and hypothyroidism constructed using binary logistic regression analysis were all 1.000, with sensitivity, specificity, and predictive accuracy all reaching 100%, suggesting the potential diagnostic value of the biomarker panel for thyroid dysfunction. In addition, the diagnostic performance of individual biomarkers and the biomarker panel for hyperthyroidism and hypothyroidism was validated in the validation set.

Recently, significant changes in numerous lipid species have been observed in patients with hyperthyroidism and hypothyroidism, suggesting these altered lipid metabolites to play a central role in hyperthyroidism and hypothyroidism pathogeneses.[29,30] For example, triglyceride (TG) and diacylglycerol (DG) are neutral lipids that have been implicated in various diseases.[31] Clinically significant hyperthyroidism has been associated with low TG levels, which may reduce plasma TG concentrations in patients with hyperthyroidism by altering very low-density lipoprotein−TG metabolism.[32,33] In addition, some studies have reported that TSH increased DG levels in thyroid cells, which may be associated with the development of hypothyroidism.[34] The elevated or decreased expression of DG lipase alpha, a metabolic enzyme downstream of DG, is accompanied by changes in circulating FT4 levels, potentially associated with hyperthyroidism development.[35] Consistent with the results of this study, we found that the serum levels of TG metabolites were lower in patients with hyperthyroidism compared with the HC group, and changes in the DG metabolite levels were also closely related to thyroid dysfunction.

PA constitutes a key intermediate in synthesizing cell membranes and storing lipids and plays an important role in mediating various cellular and physiological processes in eukaryotes.[36] Reportedly, TSH can increase PA accumulation, thereby affecting thyroid metabolism.[37] Phosphatidylethanolamine (PE), the most abundant phospholipid class in mammalian cells, exhibits significant changes during the progression of numerous diseases.[32,38] The plasma of pregnant women with subclinical hypothyroidism demonstrates several downregulated PE-like metabolites.[39] Consistent with the findings of previous studies, patients with thyroid dysfunction exhibited a decreasing trend of DG and PE metabolites and an increasing trend of PA metabolites in the serum; however, the exact action mechanism remains to be explored in depth. In addition, our findings reveal that the abovementioned lipid metabolites are closely associated with the GPL metabolism. Some studies have reported a close association of the GPL metabolism with other thyroid disorders, such as thyroid

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cancer, in addition to severely affecting hyperthyroidism and hypothyroidism.[40]

This study has some limitations. First, a detailed classification of the thyroid dysfunction could not be performed as diagnosing hyperthyroidism and hypothyroidism using their clinical indicators is not precise. Second, our clinical sample size was limited. A larger sample cohort needs to be investigated in the future to validate the results of the biomarkers using targeted lipidomics.

4. CONCLUSIONS

This study used the serum samples of two independent cohorts of participants from two centers and performed UPLC−Q-TOF/MS lipidomic analyses to identify the potential biomarkers of hyperthyroidism and hypothyroidism, explore metabolic changes in the patients with thyroid dysfunction, and assess the diagnostic accuracy of the identified biomarkers. In addition, the important influence of the GPL metabolism in the development of hyperthyroidism and hypothyroidism was identified via pathway analysis. Thus, our findings provide insights into hyperthyroidism and hypothyroidism pathogeneses.

5. MATERIALS AND METHODS

5.1. Instruments and Reagents. This study used the following equipment and reagents: a desktop high-speed refrigerated centrifuge TGL-20M (Hunan Hexi Instrument Equipment Co., Ltd.), a visible nitrogen air purge concentrator (Beijing Spot Technology Co., Ltd.), an IKA MS3 vortexer (Guangheng Technology Co., Ltd.), distilled water (Guangzhou Watsons Food & Beverage Co., Ltd.), chromatographically pure isopropanol (EMD Millipore, USA), methanol (Tedia, USA), dichloromethane (DIKMA, USA), acetonitrile (EMD Millipore, USA), a Waters ACQUITY UPLC liquid chromatograph (Waters, USA), a Waters Xevo G2-XS QTOF time-of-flight mass spectrometer (Waters, USA), and an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) (Waters, America).

5.2. Serum Sample Collection and Participants. Overall, 475 human participants (175 and 300 participants in the discovery and validation sets, respectively) were recruited from the Second Hospital of Tianjin Medical University and Tianjin Peking University Medical Offshore Oil Hospital (Tianjin, China). All the participants were recruited on the basis of symptoms and indicators for diagnosis and classification. After collecting the serum samples, they were immediately frozen and stored at −80 °C for subsequent analysis. Detailed information regarding the diagnosis, inclusion, and exclusion criteria of the participants is presented in the Supporting Information (Method S1). The Institutional Review Committee of the Second Hospital of Tianjin Medical University and Peking University Medical Offshore Oil Hospital approved this study. This study followed the Declaration of Helsinki and has been registered with the China Clinical Trials Registry with the registration number ChiCTR1900026717 and the ethics batch number KL2019K007.

5.3. Sample Preparation for Lipidomics. The serum samples were thawed at 4 °C. Serum (80 μL) was taken in a 1.5 mL centrifuge tube, to which 320 μL solvent mixture (CH_2CL_2/methanol 3:1, v/v) was added and mixed via vortexing for 60 s. Then, the mixture was centrifuged at 13,000 rpm for 10 min, and 90 μL of organic phase layer (lower CH_2CL_2 layer) was collected and dried using nitrogen at room temperature. Subsequently, 180 μL of solvent mixture (acetonitrile/isopropanol 1:1, v/v) was added to the centrifuge tube and dissolved, vortexed for 60 s, centrifuged at 13,000 rpm for 5 min, and 150 μL supernatant was used for lipidomic analysis. To ensure data quality in the lipidomic analysis, we analyzed the samples randomly and prepared mixed QC samples by mixing 10 μL of supernatant from each sample. The QC samples were inserted uniformly into each set of analytical run sequences for the methodology validation of the large-scale metabolomics analysis method.[41] We investigated the precision of the instrument by performing six consecutive injections of the same QC sample. To determine the method repeatability, seven QC samples were parallelly prepared and analyzed consecutively. Sample stability was examined by running the same QC sample every 15 samples.

5.4. UPLC−Q-TOF/MS Analysis. This study was performed using a Waters ACQUITY UPLC liquid chromatograph (Waters, USA) and a Waters Xevo G2-XS QTOF time-of-flight mass spectrometer (Waters, USA). An ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) (Waters, USA) was used in the positive ESI mode. The detailed experimental conditions for UPLC separation and MS detection are as follows.

5.4.1. Chromatographic Analysis Conditions. Column temperature, 50 °C; flow rate, 0.25 mL/min; and injection volume, 2 μL. Mobile phase composition: phase A, water/methanol/acetonitrile (3:1:1 with 5 mM ammonium formate) and phase B, isopropanol (with 10 mM ammonium formate). The elution gradient was 0−0.5 min, 20% B; 0.5−5 min, 20%−40% B; 5−15 min, 40%−98% B; 15−17 min, 98% B; and 17−17.1 min, 20% B.

5.4.2. MS Analysis Conditions. Auxiliary spray ionization and desolvation gas, high-purity N_2; desolvation gas flow rate, 800 L/h; cone hole voltage, 40 V; cone hole blowback nitrogen flow rate, 50 L/h; desolvation gas temperature, 400 °C; ionization source temperature, 120 °C; capillary ionization voltage, 3 kV; and quadrupole scan range, m/z 50−1000.

5.5. Data Processing. The demographic information of this study was statistically analyzed using SPSS 26.0 software to compare whether there was a significant difference in each indicator between the HC and thyroid dysfunction groups. If the continuous variables conformed to a normal distribution, independent sample t-test was used to assess the differences between the groups. If the data conformed to a non-normal distribution, Kruskal−Wallis test (nonparametric test) was used to assess the differences between groups. The data preprocessing procedure was as follows: MassLynx software (Version 4.1, Waters, USA) was used to collect the original data of UPLC−Q-TOF/MS nontargeted lipidomics, including retention time, m/z value, and peak intensity. The data were exported after chromatographic peak matching, peak alignment, and normalization. Subsequently, 80% reduction was performed, and the missing values were filled using the K-nearest neighbor method (the number of neighbors was five). Multivariate statistical analysis was performed using SIMCA 14.1 (Umetrics AB, Umea, Sweden). PCA was performed to view clustering trends in multidimensional data and to monitor the stability of the study. Next, OPLS-DA was performed to further investigate the metabolic changes, screen potential metabolites, and identify significant contributors to the classification using their VIP variable.

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In addition to multivariate statistical methods, the p value of each variable was obtained using t-test, and multiple hypothesis testing using Benjamini−Hochberg (BH) correction was performed to calculate multiple testing adjusted p value (FDR value) to evaluate the statistical significance of the results. We set FDR < 0.05 as the significant difference level. Features with a VIP value of >1 and p value of <0.05 (after BH FDR correction) were selected for metabolite identification as potential disease biomarkers. Ion chromatograms were used for metabolite annotation. The m/z values of the metabolites were used to search the HMDB (http://www.hmdb.ca/) for potential metabolites, and the MS/MS spectra obtained from the HMDB were compared with the MS/MS spectra of the metabolites found in this study to identify them. Spearman correlation analysis was used to determine the correlation between biomarker levels and thyroid serum indicators. The potential biomarkers were subsequently subjected to hierarchical cluster analysis using MetaboAnalyst 5.0 and mapped to their biochemical pathways. The AUC, sensitivity, and specificity of individual hyperthyroidism and hypothyroidism biomarkers at 95% CIs were calculated using ROC curves, and binary logistic regression models were developed to assess the combined diagnostic value of the biomarker panel.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c08048.

> Details of participants' diagnosis, inclusion and exclusion criteria, results of the methodology validation of the discovery and validation sets, results of the OPLS-DA model and permutation test for each group, statistical analysis of differential metabolites to distinguish the HC and hyperthyroidism groups, statistical analysis of differential metabolites to distinguish the HC and hypothyroidism groups, MS/MS spectra of the hyperthyroidism and hypothyroidism biomarkers obtained from the HMDB, statistics of correlation coefficients between biomarkers of hyperthyroidism and thyroid indicators, statistics of correlation coefficients between biomarkers of hypothyroidism and thyroid indicators, ROC analysis of the biomarkers to distinguish the hyperthyroidism and HC groups, ROC analysis of the biomarkers to distinguish the hypothyroidism and HC groups, evaluation results of the combined diagnostic model of biomarkers, base peak chromatogram under the positive ion mode, candidate biomarker information of the hyperthyroidism and hypothyroidism groups, sample size estimation using power analysis to ensure the detection of true differences, mass spectral data of nine biomarkers of hyperthyroidism and hypothyroidism, correlation analysis of the biomarkers and thyroid indicators, pathway analysis of the hyperthyroidism and hypothyroidism biomarkers, ROC curve analysis of the hyperthyroidism biomarkers, ROC curve analysis of the hypothyroidism biomarkers, relative levels of the hyperthyroidism biomarkers in the hyperthyroidism and HC groups, and relative levels of hypothyroidism biomarkers in the hypothyroidism and HC groups (PDF)

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Notes

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ABBREVIATIONS

AUC, area under the curve; CI, confidence interval; DG, diacylglycerol; ESI, electrospray ionization; FDR, false discovery rate; FT3, free triiodothyronine; FT4, free thyroxine; GPL, glycerophospholipid; HC, healthy control; HMDB, Human Metabolome Database; MS, mass spectrometry; OPLS-DA, orthogonal partial least-squares discriminant analysis; PA, phosphatidic acid; PCA, principal component

analysis; PE, phosphatidylethanolamine; QC, quality control; ROC, receiver operating characteristic; S1P, sphingosine 1-phosphate; TG, triglyceride; TSH, thyroid stimulating hormone; UPLC-Q-TOF/MS, ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry; VIP, variable importance in projection

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