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Rising stars in thyroid endocrinology 2023

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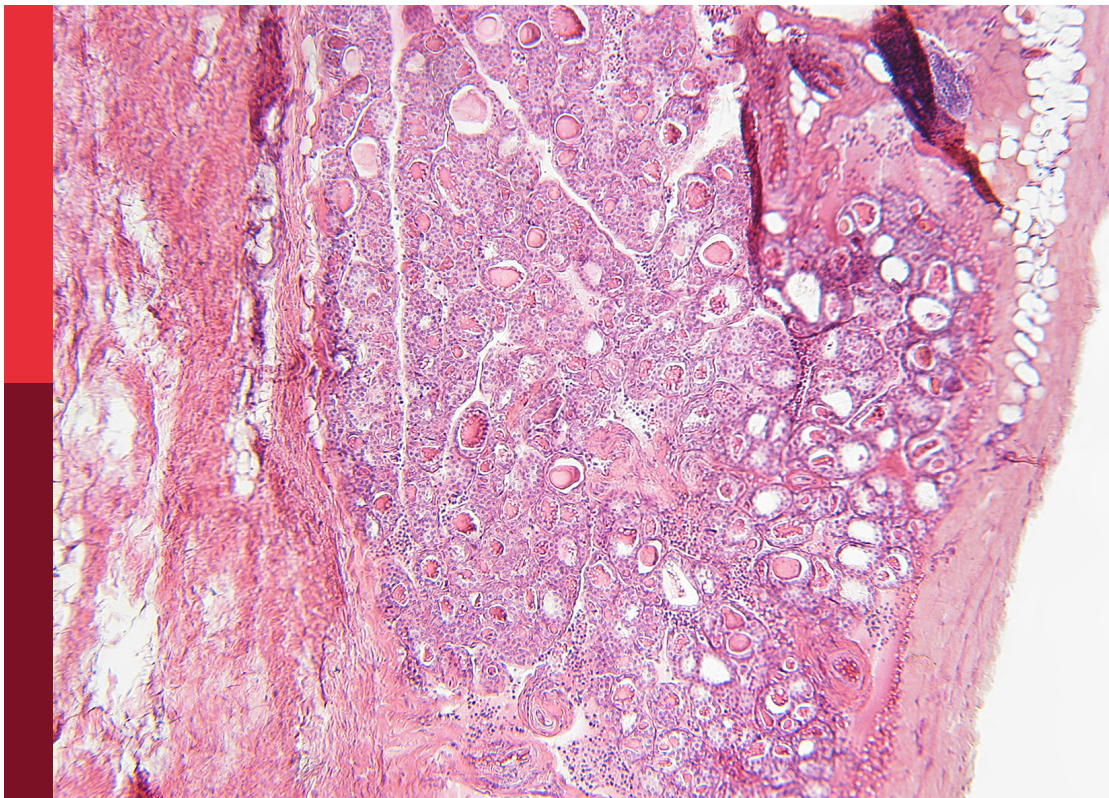

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Rising stars in thyroid endocrinology: 2023

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Effect of ^{131}I with and without artificial liver support system in patients with Graves' disease and severe liver dysfunction: A retrospective study

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Objective: Treatment decision-making in Graves' disease (GD) with severe liver dysfunction (LD) is a clinical challenge. This research was carried out to evaluate the effect of radioiodine (^{131}I) with or without an artificial liver support system (ALSS) in GD patients with severe LD.

Methods: In total, 45 patients diagnosed with GD and severe LD were enrolled and allocated to two groups: patients treated with ^{131}I alone (n=30) (Group A)and patients by a combination of ^{131}I and ALSS (n=15)(Group B). Liver function, thyroid hormone concentrations, therapeutic efficacy, and the cost of treatment were compared between the two groups.

Results: Thyroid hormone concentrations were lower 2 weeks after ^{131}I treatment, but no deterioration in liver function was identified. There was no statistically significant difference in the treatment efficacy between the two groups. The hospital stay, total cost, and daily cost were lower in patients treated with ^{131}I alone than in those treated with ^{131}I and an ALSS (p<0.05).

Conclusion: The key point of treating GD patients with severe LD is to control the GD.^{131}I is recommended as an effective and safe and should be applied as soon as possible once the diagnosis is clarified; however, when used in combination with an ALSS, there was no substantial improvement in therapeutic efficacy.

KEYWORDS

thyroid, severe liver dysfunction, artificial liver support system, radioiodine, therapeutics

Introduction

Graves' disease (GD) is an autoimmune thyroid disorder characterized by overproduction and over-release of thyroid hormone (TH) secondary to stimulation of the thyroid-stimulating hormone (TSH) receptor caused by TSH receptor antibody (TRAb) (1). The physiological relationship between the thyroid and the liver is widely recognized and may contribute to the etiology of liver dysfunction (LD) in patients with GD. Previous studies have shown that the mechanism of LD in patients with hyperthyroidism involves elevated oxygen consumption caused by increased metabolic rate; resulting in relative hypoxia in the perivenous zones of liver lobules, which leads to apoptosis and oxidative stress (2). GD may impair hepatic function, resulting in hyperbilirubinemia, liver failure, and even death (3, 4). LD in patients with GD may be caused by the effects of TH excess, drug-related hepatic injury, and the existence of accessory liver disease (5). Moreover, severe LD is a rare but potentially lethal complication of GD, and its treatment is clinically important. Only a few cases of GD with severe LD have been reported to date (6, 7), and there is no clear guidance or expert consensus regarding the management of this clinical condition.

Thyroidectomy, anti-thyroid drugs (ATDs), and radioiodine (^{131}I) therapy are the most common treatments for GD. Thyroid resection generally requires treatment with ATDs to reach a euthyroid state prior to surgery. ATDs are not suitable for use in patients who have been diagnosed with GD plus severe liver insufficiency or failure (8–12). For these patients,^{131}I therapy is therefore the only option. However, according to the European Association of Nuclear Medicine guidelines (13) and Shen and Liu (3), severe hyperthyroidism with jaundice is a contraindication for ^{131}I therapy. Previous study has shown that the TH concentration could increase after ^{131}I therapy (14), because of the induction of acute radiation thyroiditis, which is associated with a large release of stored TH into the circulation, resulting in worsening of the symptoms of hyperthyroidism or even a thyroid storm (9, 15).

As a mature method for treating severe LD, artificial liver support system (ALSS) is a device used to help recovering multiple reason-induced LD by *in vitro* mechanical, physical, chemical and biological reaction. The mechanisms of ALSS include molecular adsorbent recirculating system, plasma exchange(PE), or PE in combination with hemofiltration. ALSS can filter and remove kinds of toxins produced by LD, supplementing proteins that are synthesized/metabolized by liver, promoting the hemeostasis, which instantly replaces the basic function of liver and creates an advantageous physiological environment for hepatic repair (16, 17), thus helping with hepatocyte regeneration and liver function recovery (18). A previous study demonstrated that an ALSS improved the safety of ^{131}I treatment for patients with GD and LD (19). After the use of the ALSS, the decreases in the mean free triiodothyronine (FT3) and free thyroxine (FT4) levels were 57% and 73% (20), respectively. However, another study showed no significant decreases in the FT3 and FT4 levels (21). Notably, ALSS is high in cost and has certain associated risks (22). Very few studies have focused on how an ALSS affects the recovery of liver function in patients with GD combined with severe LD.

To the best of our knowledge, there has been no comparison of the value of using ^{131}I alone or in combination with ALSS for the treatment of patients with GD and severe LD. Therefore, we performed a retrospective study to determine the safety of ^{131}I therapy and to compare the efficacy of these two methods of treatment, aiming to provide evidence and guidance for the management of patients with GD and severe LD.

Abbreviations: GD, Graves' disease; ALSS, artificial liver support system; LD, liver dysfunction; ATD, anti-thyroid drug; ALT, alanine aminotransferase; PTA, prothrombin time activity; AST, aspartate aminotransferase; TBil, total bilirubin; FT4, free thyroxine; DB, direct bilirubin; TSH, thyroid-stimulating hormone; TBA, total bile acids; PT, prothrombin time; FT3, free triiodothyronine; TH, thyroid hormone; TRAb, thyroid-stimulating hormone receptor antibody; MELD, Model for End-Stage Liver Disease; PE, plasma exchange; TCM, traditional Chinese medicine.

Materials and methods

Patients and recruitment criteria

In this retrospective study, 45 patients diagnosed with GD and severe LD were selected from 368 patients who received ^{131}I therapy in the Second Affiliated Hospital of Chongqing Medical University from January 2011 to January 2021. The study was approved by the Ethics Committee of the Hospital's (approval no. 2021E115). The diagnosis of GD was based on typical manifestations including diffuse goiter, thyrotoxicosis, high ^{131}I uptake, and positivity for TRAb (23). Severe LD was defined as LD with a prothrombin time activity (PTA) of <60% and/or a total bilirubin (TBil) concentration of >85.5 μmol/L (24, 25). Patients who simultaneously met the diagnostic criteria for both diseases were enrolled. The exclusion criteria was set as: obstructive jaundice, liver cancer, failure of another organ, severe infection, and defective coagulation due to the presence of another disease. The enrolled patients were allocated to two groups: Group A (n=30), in which the patients were treated using ^{131}I alone, and Group B (n=15), in which the patients were treated by ^{131}I plus ALSS.

Comprehensive inpatient program

After admission, the patients stopped taking ATDs or other drugs that might cause hepatic failure for quite a long time, including traditional Chinese medicine (TCM) and anti-

tuberculosis drugs. Beta-blockers and digoxin were used to control the tachycardia caused by GD since ATDs were withdrawn.

^{131}I treatment

Patients in both groups accepted ^{131}I therapy after commencing a low-iodine diet. Thyroid ultrasonography, ^{131}I thyroid scintigraphy, and ^{131}I uptake were used to measure the thyroid mass, length and the effective half-life of the iodine. After consultation with more than two nuclear medicine specialists, personalized ^{131}I doses were calculated by following formula: therapeutic radioactivity (MBq) = (thyroid mass [g] × ^{131}I activity [MBq] per g thyroid tissue)/24-h ^{131}I uptake. The patients in Group B underwent ALSS therapy at least once during the ^{131}I therapy.

ALSS

After admission, the Model for End-Stage Liver Disease (MELD) score was compared between the two groups of patients. The MELD score is calculated according to the following formula: MELD = 6.43 + 11.2 × ln [international normalized ratio] + 9.57 × ln (serum creatinine [mg/dL]) + 3.78 × ln (serum bilirubin [mg/dL]) (26).

The patients in Group B underwent ALSS therapy at least once during the ^{131}I therapy. For the part, extracorporeal circulation was established by placing a single-needle dual-lumen catheter in the anterior right inguinal vein of each patient,then blood was remained in an anticoagulation state by heparin I.V.The device type was Kawasumi KM-9000, the parameters of ALSS were set as: blood flow velocity was 80-130mL/min; circulating albumin was 80-130ml/min, the dialysate velocity was 500mL/min. Each time of treatment lasted for 4-6 hours. The treatment was repeated every 3-7 days according to the condition of the patients.

Treatment assessment and follow-up

Laboratory results (including liver function and thyroid function) were obtained 1 to 3 days, 4 to 7 days, 2 weeks,2 and 6 months after treatment, and the hospitalization stay/expenses were collected. The participants were followed for 6 months after discharge. The results of treatment were classified into three levels, including cured, improved, and no response. Cured was defined as both liver function and thyroid function returned to normal or hypothyroidism was present. Improved was defined as liver function and/or thyroid function has improvement. Ne response was defined as neither liver function nor thyroid function has improvement and death. Effective rate equals to the rate of the cured and the rate of the improved.

Statistical analysis

SPSS 23.0 statistical software (IBM, Inc., Armonk, NY, USA) was used for data analysis. The baseline characteristics of the two groups were analyzed using the independent-samples *t*-test, and the therapeutic efficacy of the two treatments was analyzed using the paired *t*-test. Non-normally distributed data were analyzed using the rank sum test. Odds ratios for the differences between the two groups were calculated using the χ^2 test, and Fisher's exact test was used when the expected values were <5. P value <0.05 was considered statistically significant.

Results

Patients' baseline characteristics

As shown in Table 1,there were no significant differences in the sex distribution, duration of GD, FT3, FT4, TSH, ALT, AST, TBil, PTA, or ^{131}I dose between the two groups. However, there were significant differences in age (p=0.026), international normalized ratio (p=0.049), and prothrombin time (p=0.046). The mean MELD score in Groups A and B was 13.0 ± 4.51 and 15.7 ± 7.38, respectively (p=0.07). The patients in Group B tended to have worse liver indices than those in Group A. The three main causes of severe LD were TH excess, drug-related hepatic injury, and viral hepatitis. The detail of the distribution of causes was shown in Table 1.

Treatment efficacy

The data regarding treatment efficacy at different time points were shown in Table 2. At all the time points, no statistically significant difference in the efficacy of the treatments was found between the two groups. One patient died after discharge in Group A, and three in Group B. The death of the patient in Group A, and three Group B. TH replacement was given to six patients who developed hypothyroidism. Six months later, two patients accepted ATD treatment because of recurrence of GD.

Changes in liver and thyroid indices by ^{131}I therapy in Group A

As shown in Figure 1. The FT3 and FT4 concentrations significantly decreased after treatment (p<0.05):FT3 decreased from

TABLE 1 Baseline characteristics of patients with Graves' disease and severe liver dysfunction.

	Group A	Group B	P value
Sex(male/female)	17/13	9/6	0.831
Age,year	44.3 ± 13.86	32.7 ± 13.11	0.01
Duration of GD(month)	33.7 ± 67.11	50.7 ± 88.61	0.99
Cause of liver dysfunction,n[a]			0.731
GD induce	16	7	
GD+virus	9	4	
GD+(ATD/other drugs)	(1/4)	(1/3)	
FT3(pmol/L)	18.6 ± 11.93	20.4 ± 9.9	0.367
FT4(pmol/L)	75.3 ± 38.84	82.2 ± 19.9	0.296
TSH(μIU/mL)	0 ± 0.01	0 ± 0	0.798
Alb(g/L)	31.5 ± 5.46	31.2 ± 5.7	0.857
ALT(U/L)	452 ± 482.56	307 ± 432.65	0.413
AST(U/L)	436.9 ± 542.96	339 ± 419.21	0.933
TBil(μmol/L)	295.7 ± 159.82	362.4 ± 141.14	0.178
DB(μmol/L)	215.2 ± 108.15	251.4 ± 119.07	0.312
TBA(μmol/L)	213.7 ± 130.45	269.8 ± 175.57	0.233
INR	1.4 ± 0.37	2 ± 0.94	0.049
PTA(%)	70.9 ± 24.69	55.3 ± 32.54	0.081
PT(s)	16.9 ± 3.64	22 ± 8.49	0.046
MELD	13.0 ± 4.51	15.7 ± 7.38	0.07
Iodine dose(MBq)	262.7 ± 79.18	344.1 ± 138.75	0.077

Data are presented as number of patients or mean ± standard deviation.
INR, international normalized ratio; ALT, alanine aminotransferase; PTA, prothrombin time activity; AST, aspartate aminotransferase; TBil, total bilirubin; FT4, free thyroxine; DB, direct bilirubin; TSH, thyroid-stimulating hormone; TBA, total bile acids; PT, prothrombin time; FT3, free triiodothyronine; MELD, Model for End-Stage Liver Disease; Alb, albumin; GD, Graves' disease.
[a]For causes of liver dysfunction.
Reference ranges were as follows: FT3, 2.9–9.1 pmol/L; FT4, 9.1–25.5 pmol/L; TSH, 0.4–5.1 mIU/mL; Alb, 40–55 g/L; TBil, 1.7–17.1 μmol/L; DB, <6.8 μmol/L; TBA, 0–10 μmol/L; INR, 0.7–1.3; PTA, 70%–120%; PT, 9.8–12.1 s; and ALT and AST, both ≤40 U/L.

TABLE 2 Comparison of therapeutic efficacy of the two treatments.

	Time	Cured	Improved	No response	Effective rate
Group A(n=30)	discharge	–	23	7	76.67%
	Follow-up in two months	–	27	3	90.00%
	Follow-up in six months	8	20	2	93.33%
Group B(n=15)	discharge	–	11	4	73.33%
	Follow-up in two months	1	11	3	80.00%
	Follow-up in six months	3	8	4	73.33%

The efficacy of the two treatments did not significantly differ (χ^2 = 0.000, 0.216, and 1.947; P=1.000, 0.642, and 0.163, respectively).

baseline(18.6 ± 11.93 pmol/L) to 14.3 ± 10.92 pmol/L 1 week post-treatment and further down to 7.3 ± 5.89 pmol/L at 2 weeks post-treatment, while FT4 decreased from baseline(75.3 ± 38.84 pmol/L) to 57.6 ± 27.47 pmol/L 1 week after treatment, then down to 35.5 ± 25.42 pmol/L 2 weeks after treatment. In Figure 2, the liver indices gradually improved after treatment: the ALT (p=0.001) and AST (p=0.004) activities in Group A significantly decreased within 3 days after ^{131}I therapy. The prothrombin time was not significantly lowered until 2 weeks after treatment (p=0.014).

Changes in liver and thyroid indices by combination treatment in Group B

As shown in Figure 1. The TH concentration significantly decreased 1 week after the combination treatment (p<0.05). FT3 decreased from baseline(20.4 ± 9.9 pmol/L) to 6.4 ± 2.45 pmol/L 1 week after treatment, and 4.14 ± 1.57 pmol/L 2 weeks after treatment, and FT4 decreased from baseline(82.2 ± 19.9 pmol/L) to 36.7 ± 13.26 pmol/L 1 week after treatment and 26.1 ± 14.01

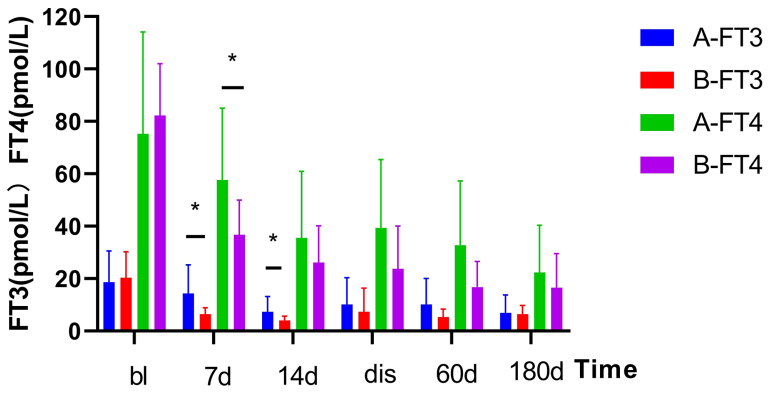

FIGURE 1
Changes in serum thyroid hormone levels before and after treatment in two Groups. After treatment, the serum thyroid hormone decreased significantly. Results are presented as the mean value ± standard deviation. *Statistically significant difference between the two groups (p<0.05). bl, baseline; dis, discharge; FT3, triiodothyronine; FT4, free thyroxine.

pmol/L 2 weeks after treatment. In Figure 2, PTA also significantly changed after 1 week (p=0.008). The AST activity significantly decreased 3 days after treatment (p=0.037), and the ALT activity (p=0.03) and TBil concentration (p=0.046) significantly decreased 2 weeks after treatment.

Comparisons of post-treatment changes in thyroid and liver indices between the two groups

As shown in Figure 2, the thyroid and liver indices of both groups significantly improved after treatment. The decrease of FT3 at 1 and 2 weeks after treatment was statistically significant between two groups, and decrease of FT4 was statistically significant at 1 week after treatment. However, no differences in FT3 and FT4 detected at discharge or at follow-up was found to be statistically significant between two groups. In Figure 2, there was no significant difference in liver function between the two groups (e.g., TBil, PTA, ALT, AST) after treatment, at discharge, or at follow-up.

Costs and recovery time

The treatment cost and hospital stay were recorded and compared between the two groups. As shown in Supplementary Table SA. The total cost of treatment, mean daily cost, and duration of hospital stay were much lower in Group A than those in Group B, and the difference were statistically significant (p<0.05). The mean length of time required for the patients recovering from severe LD to mild LD was 91.3 ± 56.56 days in Group A and 106.8 ± 69.07 days in Group B (p=0.661).

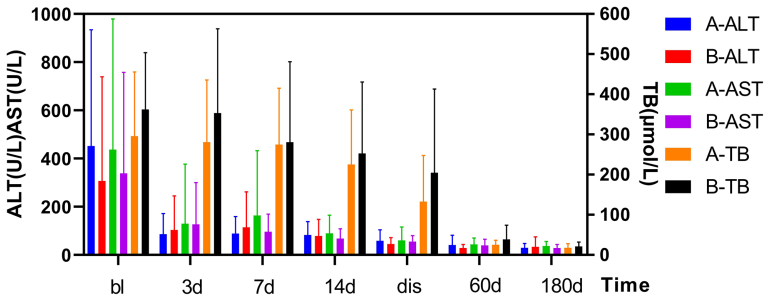

FIGURE 2
Changes in TBil and ALT/AST levels before and after treatment in two Groups. After treatment, the TBil, and ALT/AST levels decreased significantly. Results are presented as the mean value ± standard deviation. There was not a statistically significant in the TBil, and ALT/AST levels between the two groups. bl, baseline; dis, discharge; TBil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Changes in TRAb

The changes in TRAb in some patients were shown in Supplementary Table SB. After treatment, the TRAb level decreased in both groups, and the decrease in Group B was more obvious, and the difference between two groups was statistically significant.

Discussion

This study was the first retrospective analysis focusing on the evaluation of ^{131}I therapy treating GD with severe LD and the addition of an ALSS. Abnormal liver function is often found in patients newly diagnosed with thyrotoxicosis/hyperthyroidism (prevalence of 15%–76%) (27). A previous study has reported that the incidence rate of severe LD was 6.6% (28). In our research, 12.22% (45 of 368) of GD patients were diagnosed of severe LD. The difference in incidence rate may be explained by admission bias. According to the 2018 edition of the Chinese Guidelines for the Diagnosis and Treatment of Liver Failure, patients who currently have or are at risk of developing liver failure should be managed with ALSS therapy as early as possible (29, 30). In our study, both groups of patients had indications for ALSS therapy, but only patients in Group B received ALSS treatment according to their willingness and their financial status.

The etiology of liver injury in GD patients enrolled in our research varied, and more than one cause were identified. Previously, it has been recommended to recover the functions of both vital organs using multidisciplinary treatment. In this study, the main cause of LD was GD, followed by viral hepatitis and drug-related hepatitis. For a patient with GD-associated LD, the control of LD relies mainly on treating GD. We have observed that LD in 7 patients was caused by TCM, which was possibly due to the unclear chemical composition of TCMs (31).

As shown in Figures 1, 2, after the corresponding treatment, the TH concentration decreased within 2 weeks in both groups, and descended to normal levels during the follow-up for most patients, which was consistent with previous studies that ^{131}I therapy is effective for GD patients with LD (32). Previous studies (3, 14) have also reported that the mean TH concentration in patients with GD treated by ^{131}I without ATDs pretreatment decreased rapidly. However, results of some studies showed that TH concentration increased after ^{131}I treatment (33) or on the day after ALSS (34). There was another study (19) which has reported that the serum concentrations of TBil and FT4 increased 1 week after ^{131}I and ALSS. The function of thyroid cells and their TH storage and ATDs all have impacts on serum TH level. We thus hypothesized that the consumption of TH was promoted in GD patients without ATD treatment, whereas the storage of TH was lowered. Thus, after ^{131}I treatment, the release of TH were decreased. Conversely, TH level increased rapidly in patients treated by ATDs, This could explain for the different in the TH level after ^{131}I treatment as mentioned above. Different from the routine use of ATDs In Western countries, patients in our study did not receive ATDs or stopped receiving ATDs for quite a long time before treatment because of LD.

The key point of treating GD patients with severe LD is the rapid control of the GD, as previously (19). In our study, the liver function was gradually promoted after GD was treated and thyroid function was improved in both groups, and no statistically significant difference in the degree of the improvement in liver function/treatment efficacy/recover time was found between the two groups. At this point, ALSS didn't show an obvious advantage in the treatment of GD patient with GD patients with LD in our study. However, considering the limitations in sample size and sample bias, further study is required to assess the value of ALSS in the treatment.

The TH concentration in Group B decreased more than that in Group A 1 week after treatment, and the difference was statistically significant. After ALSS treatment in Group B, the level of TRAb decreased by an average of 48% one week after treatment, and a small increase (11%) was detected in 6th months during follow-up. However, the changes of TRAb in Group A were less apparent, showing only a slight decrease (17.4%) during the 6-month follow-up. The data indeed showed a significant ALSS-reduce of TH level, which may be due to the remove of a part of TH by ALSS.

Despite of the uncertainty of ALSS in the improvement of treatment, some of its disadvantages needed to be clarified. First of all, the cost of applying ALSS is significantly higher due to ALSS itself and the prolonged hospital stay. Secondly, ALSS has certain adverse effects, including transfusion reactions, citrate-related nausea and vomiting, hypocalcemia, vasovagal or hypotensive reactions, respiratory distress, catheter dysfunction, bleeding, and tetany or seizure (30, 35). Death is a potential but rare complication. In our study, one patient in Group A was dead due to infectious shock, whereas three patients were dead in Group B due to severe coagulation defect, hepatic encephalopathy and cerebral edema with infection, and heart failure caused by myocardial infarction, respectively. Two of the three patients died in Group B had complications such as hypotension and secondary infection during ALSS treatment. The mortality was 8.89% in this study, where the mortality rate of 2.9% in Group A and 20.0% in Group B.

In conclusion, the key point in the treatment of GD patients with severe LD is the control of GD. ^{131}I is recommended as an effective and safe therapy and should be applied as soon as possible once the diagnosis is clarified. The auxiliary treatment by ALSS didn't show apparent advantage in our study, however, as a preliminary study that requires further confirmation in a larger cohort, we can not negate the function of ALSS in the treatment of GD with severe LD.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (approval no. 2021E115). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization: MR and SL. Data acquisition and analysis: YW and YW. Writing - original draft: YW and MR. Writing - review and editing: MR, SL, CZ, QY, and YC. Acquistion of funding: JR, MR, and SL. Supervision: GY. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1034374/full#supplementary-material

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Correlation of thyroid-related hormones with vascular complications in type 2 diabetes patients with euthyroid

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Background: This study aimed to evaluate the relationship between thyroid-related hormones and vascular complications in type 2 diabetes mellitus (T2DM) patients with euthyroidism.

Methods: We enrolled 849 patients with T2DM after screening out the ineligible. Multivariate logistic regression was used to analyze the relationship between fT3, fT4, the fT3/fT4 ratio, thyroid-stimulating hormone, and diabetic vascular complications. Spearman correlation analysis was used to determine the correlation between thyroid-related hormones and vascular complications.

Results: In this cross-sectional study of T2DM, 538 patients with carotid atherosclerosis (CA) and 299 patients with diabetic peripheral neuropathy (DPN). The prevalence of DPN was negatively correlated with fT3 and the fT3/fT4 ratio but positively correlated with fT4 (all P<0.01). At the same time, the odds ratio for DPN decreased with increasing fT3 (T1: reference; T2: OR: 0.689, 95%CI: 0.477, 0.993; T3: OR: 0.426, 95% CI: 0.286, 0.633, all P<0.05) and fT3/fT4 ratio (T1: reference; T2: OR: 0.528, 95% CI: 0.365, 0.763; T3: OR: 0.413, 95% CI: 0.278, 0.613, all P<0.001). In terms of sensitivity and specificity, fT4 was found to be 39.5% and 71.4% accurate, respectively, with a 95% CI of 0.531-0.611.

Conclusions: We found a negative correlation between fT3 and fT3/fT4 ratio and the number of individuals with DPN, and a positive correlation between fT4 and the prevalence of DPN.

KEYWORDS

free triiodothyronine, free thyroxine, thyroid-stimulating hormone, type 2 diabetes mellitus, diabetic peripheral neuropathy, carotid atherosclerosis

Introduction

One of the most frequent endocrine illnesses is diabetes mellitus (1). According to a recent report from the International Diabetes Federation (version 2019), the global prevalence of diabetes is expected to increase from 9.3% in 2019 to 10.9% in 2045 (2). More than 90% of people with diabetes have type 2 diabetes mellitus (T2DM) (3–5). The worldwide pandemic of type 2 diabetes indicates that more people are suffering from the psychological and physical burden of diabetes (6–8). T2DM can cause many vascular complications, such as coronary heart disease, renal disease, retinopathy, and diabetic peripheral neuropathy (DPN) (9, 10). Cardiovascular problems are the primary cause of death in T2DM patients (4). Therefore, other modifiable risk factors need to be investigation to prevent cardiovascular complications.

Like T2DM, thyroid diseases are also common endocrine system disorders (11). A growing number of studies suggest that abnormal thyroid function may impact glucose metabolism, insulin sensitivity, and the development of chronic complications of diabetes (12, 13). This indicates that T2DM and its long-term consequences may be associated with abnormal thyroid function (14, 15). Recent studies have shown that free triiodothyronine (fT3), free thyroxine (fT4) and thyroid-stimulating hormone (TSH) levels have been linked to insulin resistance and chronic complications of diabetes such as carotid atherosclerosis (CA) and DPN (16–19). However, patients with T2DM who have normal thyroid function have rarely been found to have a link between thyroid-related hormones and vascular problems like DPN and CA.

Therefore, the current study aimed to evaluate the association between fT3, fT4, fT3/fT4, TSH and DPN, CA in T2DM patients with euthyroidism.

Methods

Study subjects

A retrospective cross-sectional study was performed by the Department of Endocrinology and Metabolism at the Affiliated Hospital of Southwest Medical University on T2DM patients hospitalized in the department from 2018 to 2020. Inclusion criteria: (1) age > 18 years old; (2) T2DM diagnosis according to the American Diabetes Association "Standards of Medical Care in Diabetes" diagnostic criteria (2019 version (20).; (3) Normal thyroid function was defined as: fT3: 1.80–3.80 pg/mL, fT4: 0.78–1.86 ng/dL, TSH: 0.38–5.57 mIU/L. Exclusion criteria: (1) abnormal thyroid function test index; (2) a history of thyroid disease or thyroid surgery; (3) use of thyroxine preparations, amiodarone, and other drugs affecting thyroid function. Information for the study was collected by health professionals using a traditional questionnaire and validated equipment. Each participant provided demographic information and medical record information. We divided smokers into current smokers and nonsmokers. Similarly, alcohol drinkers were divided into current drinkers and non-drinkers. Mean arterial blood pressure was derived from three right arm arterial blood pressure measurements taken continuously for at least 30 min during a physical examination.

The study complied with the ethical standards of the Declaration of Helsinki (2013) and was approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University (ethical approval code: 2018017) (21). All enrolled participants signed the informed consent form.

FT3/fT4 ratio assessment

FT3/fT4 ratio was determined as follows: fT3 (pg/mL)/fT4 (ng/dL). For further analysis, the fT3/fT4 ratio was transformed into categorical variables: T1: <1.88, T2: 1.88–2.24, T3: >2.24.

Diagnosis of diabetic vascular complications

All subjects were scanned bilaterally in the carotid arteries by the same color Doppler ultrasound diagnostic instrument under the guidance of an ultrasound operator. During data collection, measurements were performed by a single operator. The criteria for atherosclerotic plaque formation were local carotid intima-media thickness (CIMT) ≥1.5 mm or local CIMT greater than 50% of the peripheral area. The diagnosis of carotid atherosclerosis was defined as an increase in CIMT ≥1.0 mm and/or the presence of carotid plaque (22–25). At least one symptom and/or one nerve conduction study neuropathy and aberrant signs are needed to diagnose DPN, as defined by the Toronto International Consensus for DPN (26).

Statistics analysis

Descriptive statistics were used to compare baseline clinical characteristics of patients according to sex. For comparison between groups, one-way analysis of variance (ANOVA) (normally distributed continuous variables), Kruskal-Wallis H test (non-normally distributed continuous variables), and χ^2 test (categorical variables) were used. Logistic regression analysis models were used to detect variables affecting diabetic complications. The relationship between thyroid-related hormones and vascular complications was identified by Spearman correlation analysis. We examined the anticipated

accuracy of thyroid-related hormones using receiver operating characteristic (ROC) curves. All hypotheses were tested bilaterally at an α level of 0.05. The weighting of each risk factor was according to the values of odds ratio (OR). GraphPad Prism (version 9.0) was used to generate forest plots. SPSS (version 26.0) was used for all data analyses.

Results

In this study, 869 patients with T2DM participated. There were 468 men and 401 women in the group. The mean age was 55.23 ± 11.14 years in men and 59.30 ± 10.23 years in women. and an average of 88.83 ± 73.43 months of diabetes on record. There were 538 patients with CA and 299 patients with DPN. The detailed demographic information and biochemical indicators of the participants are shown in Table 1. Compared with women, men had higher values of height, weight, body mass index (BMI), waist circumference (WC), diastolic blood pressure (DBP), alanine aminotransferase (ALT), FT3, FT3/FT4, visceral fat area (VFA), current smoking rates, current alcohol consumption, and use of antihypertensive medication (all P<0.05). Conversely, women had higher values for mean age, systolic blood pressure (SBP), high-density lipoprotein cholesterol (HDL), TSH, subcutaneous fat area (SFA), use of hypoglycemic drugs, and duration of diabetes (all P<0.05).

Table 2 demonstrates the distribution of CA and DPN in the fT3, fT4, fT3/fT4 ratio, and TSH tertiles. The prevalence of CA in the fT3/fT4 tertiles was 66.2%, 56.0%, and 63.5%, respectively. Moreover, the prevalence of DPN negatively correlated with fT3 (42.7%, 33.6%, 26.8%, P<0.05) and fT3/fT4 ratio (46.9%, 30.9%, 25.3%, P<0.001), but positively correlated with fT4 (29.4%, 32.3%, 42.0%). Importantly, the prevalence of CA and DPN in all tertiles of TSH were virtually identical.

Multivariate regression models were used to calculate the odds ratios for DPN (Table 3). A higher fT3/fT4 ratio was associated

TABLE 1 Clinical characteristics according to gender.

Variables	Men (n=468)	Women (n=401)	P
Age, years old	55.23 ± 11.14	59.30 ± 10.23	<0.001*
Height, cm	166.09 ± 5.97	153.50 ± 6.01	<0.001*
Weight, kg	69.72 ± 10.36	58.27 ± 10.26	<0.001*
BMI, kg/m^2	25.24 ± 3.22	24.68 ± 3.77	<0.018*
WC, cm	89.55 ± 9.74	84.95 ± 10.81	<0.001*
SBP, mmHg	134.47 ± 20.00	140.94 ± 21.06	<0.001*
DBP, mmHg	81.03 ± 11.26	78.74 ± 11.13	0.003*
TC, mmol/L	4.87 ± 3.58	4.95 ± 1.35	0.655
TG, mmol/L	2.63 ± 2.75	2.34 ± 2.24	0.087
HDL, mmol/L	1.09 ± 0.32	1.27 ± 0.40	<0.001*
LDL, mmol/L	2.78 ± 1.08	2.90 ± 1.09	0.097
FBG, mmol/L	9.22 ± 3.03	9.07 ± 3.28	0.487
HbA1c, %	9.78 ± 2.52	9.47 ± 2.31	0.056
ALT, mmol/L	31.73 ± 31.17	25.68 ± 23.44	0.001*
AST, mmol/L	25.37 ± 32.48	22.76 ± 15.93	0.143
FT3, pg/mL	2.59 ± 0.35	2.43 ± 0.32	<0.001*
FT4, ng/dL	1.25 ± 0.21	1.23 ± 0.20	0.165
FT3/fT4	2.12 ± 0.41	2.02 ± 0.40	<0.001*
TSH, mU/L	2.04 ± 1.08	2.43 ± 1.17	<0.001*
VFA	92.04 ± 44.26	86.24 ± 41.77	0.049*
SFA	159.74 ± 52.66	168.19 ± 64.75	0.034*
Duration of diabetes, months	82.41 ± 68.18	96.32 ± 78.55	0.005*
Current smoking (No/Yes)	225/243	394/7	<0.001*
Current drinking (No/Yes)	202/266	381/20	<0.001*
Antihypertensive drugs (No/Yes)	379/89	284/117	<0.001*
Hypoglycemic drugs (No/Yes)	190/278	136/265	0.043*
Insulin (No/Yes)	302/166	275/126	0.208

The values were expressed as the mean ± SD, n. BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triacylglycerol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; ALT, alanine aminotransferase; AST, aspartate aminotransferase; fT3, free triiodothyronine1; fT4, free thyroxine; TSH, thyroid stimulating hormone; VFA, visceral fat area; SFA, subcutaneous fat area. *P<0.05.

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TABLE 2 Prevalence of CA and DPN in different thyroid-related hormones tertiles.

Events	Carotid atherosclerosis	P-value	Diabetic peripheral neuropathy	P-value
FT3				
T1 (1.80-2.36)	191 (65.2%)	0.343	125 (42.7%)	<0.001*
T2 (2.37-2.63)	178 (61.0%)	0.289	98 (33.6%)	0.024*
T3 (2.64-3.78)	169 (59.5%)	0.159	76 (26.8%)	<0.001*
FT4				
T1 (0.78-1.15)	191 (62.4%)	0.836	90 (29.4%)	0.004*
T2 (1.16-1.32)	177 (62.8%)	0.931	91 (32.3%)	0.453
T3 (1.33-1.85)	170 (60.5%)	0.633	118 (42.0%)	0.002*
FT3/fT4				
T1 (<1.88)	192 (66.2%)	0.032*	136 (46.9%)	<0.001*
T2 (1.88-2.24)	163 (56.0%)	0.012*	90 (30.9%)	<0.001*
T3 (>2.24)	183 (63.5%)	0.502	73 (25.3%)	<0.001*
TSH				
T1 (0.40-1.55)	186 (64.1%)	0.304	109 (37.6%)	0.239
T2 (1.56-2.48)	184 (63.2%)	0.820	90 (30.9%)	0.091
T3 (2.49-5.52)	168 (58.3%)	0.152	100 (34.7%)	0.474

The values were expressed as n (%). *P<0.05.

with a lower odds ratio for DPN after adjustment for age and sex in model 2 (T1: reference; T2: OR: 0.482, 95% CI: 0.342, 0.680; T3: OR: 0.369, 95% CI: 0.258, 0.528; all P<0.001). This trend remained unchanged after adjustment for the remaining confounders in model 3 (T1: reference; T2: OR: 0.518, 95% CI: 0.352, 0.760; T3: OR: 0.356, 95% CI: 0.234, 0.542; all P<0.001). FT3 and the fT3/fT4 ratio were negatively related to DPN risk, whereas fT4 had the opposite effect. Unfortunately, we did not find a correlation between TSH and DPN prevalence.

As shown in Table 4, the association between thyroid-related hormones and vascular complications in T2DM patients with euthyroidism was determined by Spearman correlation analysis. The results revealed a negative correlation between fT3 (rs=-0.144, P<0.001) and fT3/fT4 (rs=-0.193, P<0.001) and DPN, and a positive correlation between fT4 (rs=0.117, P=0.001) and DPN. However, we did not find any relationship between thyroid-related hormones and CA, and the relationship between TSH and DPN.

TABLE 3 Adjusted OR and 95% CI in tertiles of thyroid-related hormones in the DPN group.

Events	Model 1	P-value	Model 2	P-value	Model 3	P-value
FT3						
T1	1	<0.001*	1	<0.001*	1	<0.001*
T2	0.679 (0.485,0.950)	0.024*	0.641 (0.455,0.903)	0.011*	0.689 (0.477,0.993)	0.046*
T3	0.491 (0.346,0.697)	<0.001*	0.433 (0.301,0.623)	<0.001*	0.426 (0.286,0.633)	<0.001*
FT4						
T1	1	0.004*	1	0.007*	1	0.092
T2	1.143 (0.805,1.623)	0.453	1.134 (0.797,1.612)	0.484	1.068 (0.737,1.550)	0.727
T3	1.773 (1.235,2.445)	0.002*	1.689 (1.198,2.382)	0.003*	1.467 (1.014,2.124)	0.042*
FT3/fT4						
T1	1	<0.001*	1	<0.001*	1	<0.001*
T2	0.507 (0.361,0.712)	<0.001*	0.482 (0.342,0.680)	<0.001*	0.528 (0.365,0.763)	0.001*
T3	0.384 (0.270,0.547)	<0.001*	0.369 (0.258,0.528)	<0.001*	0.413 (0.278,0.613)	<0.001*
TSH						
T1	1	0.239	1	0.293	1	0.443
T2	0.744 (0.527,1.049)	0.091	0.769 (0.543,1.087)	0.137	0.798 (0.554,1.150)	0.226
T3	0.883 (0.629,1.240)	0.474	0.949 (0.670,1.343)	0.766	0.954 (0.657,1.386)	0.807

Model 1: unadjusted; Model 2: adjusted for sex, age; Model 3: adjusted for sex, age, FBG, HbA1c, BMI, duration of diabetes, current smoking, current drinking, antihypertensive drugs, hypoglycemic drugs, insulin. Abbreviations: OR, odds ratio; CI, confidence interval. *P<0.05.

TABLE 4 Association among thyroid-stimulating hormone and vascular complications.

Events	CA, rs	P	DPN, rs	P
FT3	-0.061	0.073	-0.144	<0.001*
FT4	-0.018	0.594	0.117	0.001*
FT3/fT4	-0.021	0.541	-0.193	<0.001*
TSH	-0.064	0.060	-0.025	0.455

fT3, free triiodothyronine1; fT4, free thyroxine; TSH, thyroid stimulating hormone; CA, carotid atherosclerosis; DPN, diabetic peripheral neuropathy; rs, Spearman's correlation coefficient. *P<0.05.

Furthermore, we used fT3 tertiles to separate the DPN groups by, sex and age (Figure 1). The results showed that the overall prevalence was higher in men than in women. Meanwhile, all DPN subgroups, except those aged > 65 years, showed a declining prevalence with increasing fT3 (P<0.05).

As illustrated in Figure 2, we performed multivariate regression analysis for variables independently associated with diabetic CA or DPN. CA risk factors were age (OR: 1.073, 95% CI: 1.054, 1.091) and current smoking (OR: 1.893, 95% CI: 1.244, 2.878) (all P<0.01). And the forest plot revealed that HbA1c (OR: 1.104, 95% CI: 1.027, 1.187), DBP (OR: 1.032, 95%CI: 1.017,1.047), low-density lipoprotein cholesterol (LDL) (OR: 1.182, 95% CI: 1.022, 1.367), fT4 (OR: 3.736, 95%CI: 1.691,8.256), duration of diabetes (OR: 1.004, 95% CI: 1.002, 1.007), and insulin use (OR: 2.021, 95% CI: 1.380, 2.959) were risk factors for DPN (all P <0.05). Moreover, age (OR: 0.977, 95% CI: 0.963, 0.993) and antihypertensive drugs use (OR: 0.537, 95% CI: 0.354, 0.814) were protected factors for DPN (all P <0.01).

Finally, we assessed the diagnostic value of thyroid-related hormones for DPN using a ROC curve (Figure 3). With an under the curve (AUC) of 0.571 (95% CI: 0.531, 0.611, P<0.001), fT4 was found to be the most accurate, followed by TSH (AUC: 0.485, 95%CI: 0.444, 0.525, P=0.455), fT3 (AUC: 0.412, 95%CI: 0.373, 0.452, P<0.01), fT3/fT4 ratio (AUC: 0.383, 95%CI: 0.343, 0.422, P<0.001). By calculating the Jorden index, the optimum cutoff value of fT4 was 1.325. The sensitivity of fT4 was 39.5%, and the specificity was 71.4%.

Discussion

This cross-sectional study included 869 T2DM patients. We explored the correlation between the prevalence of DPN and CA and fT3, fT4, fT3/fT4, and TSH in T2DM patients with normal thyroid function. Significantly fewer people in the higher fT3 or fT3/fT4 tertiles had DPN (P<0.001). However, the prevalence of DPN increased with increasing fT4 tertiles (P<0.01). After adjustment for confounding factors, fT3 and the fT3/fT4 ratio was negatively associated with DPN (P < 0.05). Overall, fT3 performed the best in assessing the correlation of thyroid hormones with the prevalence of DPN, followed by fT3/fT4 ratio and fT4. Interestingly, we found DPN is more associated with thyroid hormone (TH) levels than with TSH levels.

DPN is a common microvascular complication of diabetes leading to lower limb amputations, neuropathic pain, and increased mortality (27). Both T2DM and obesity exacerbate the inflammatory response, leading to increased serum

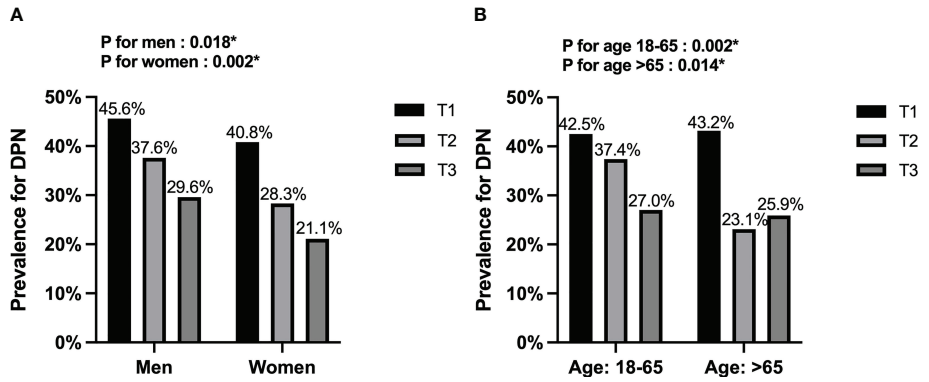

FIGURE 1
Stratification analysis of DPN among the fT3 tertiles. **(A)** Stratified by sex; **(B)** Stratified by age. DPN, diabetic peripheral neuropathy. *P<0.05.

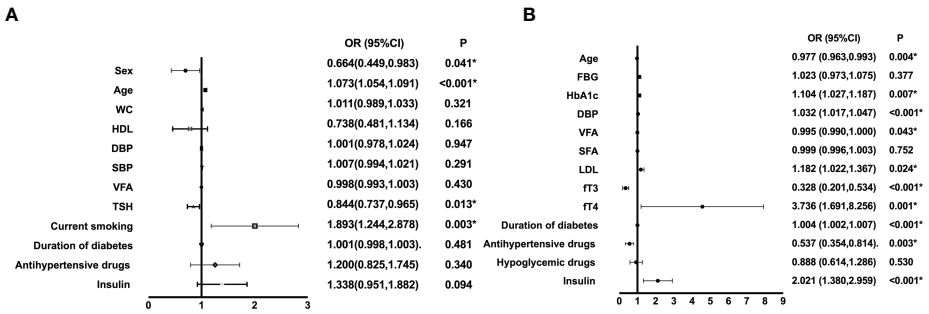

FIGURE 2
Multiple regression analysis of variables independently associated with CA **(A)** or DPN **(B)** in all participants. WC, waist circumference; SBP, systolic blood pressure; HDL, high-density lipoprotein cholesterol; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; DBP, diastolic blood pressure; VFA, visceral fat area; SFA, subcutaneous fat area; LDL, low-density lipoprotein cholesterol; fT3, free triiodothyronine1; fT4, free thyroxine; TSH, thyroid stimulating hormone; CA, carotid atherosclerosis; DPN, diabetic peripheral neuropathy. *P<0.05.

concentrations of inflammatory biomarkers such as C-reactive protein (28–30). These inflammatory biomarkers diminish the autonomic function and promote the development and progression of neuropathy (31). This may explain why LDL-c is a risk factor for DPN. Yang et al. demonstrated a linear relationship between T2DM and patients with HbA1c > 7.0% and an increased risk of DPN (32). This is because hyperglycemia and insulin resistance are essential for the development of DPN (27, 33, 34). In this study, DBP was found to be independently associated with the risk of developing DPN. The exact mechanism is unclear, and possibly due to a combination of reduced neural blood flow, slow nerve conduction, axonal atrophy, and thinning of myelinated fibers as a result of elevated blood pressure (35–37). The use of antihypertensive drugs may reverse these effects, leading to the annihilation of the risk of DPN. Several studies have shown that the prevalence of DPN increases with the duration of disease in T2DM, suggesting that we should screen for DPN early in the T2DM population (38–40). Patients with poor glycemic control may develop insulin neuritis characterized by acute severe distal limb pain, peripheral nerve fiber damage, and autonomic dysfunction (41, 42). Although rare, these patients are more likely to develop pain or autonomic neuropathy (42).

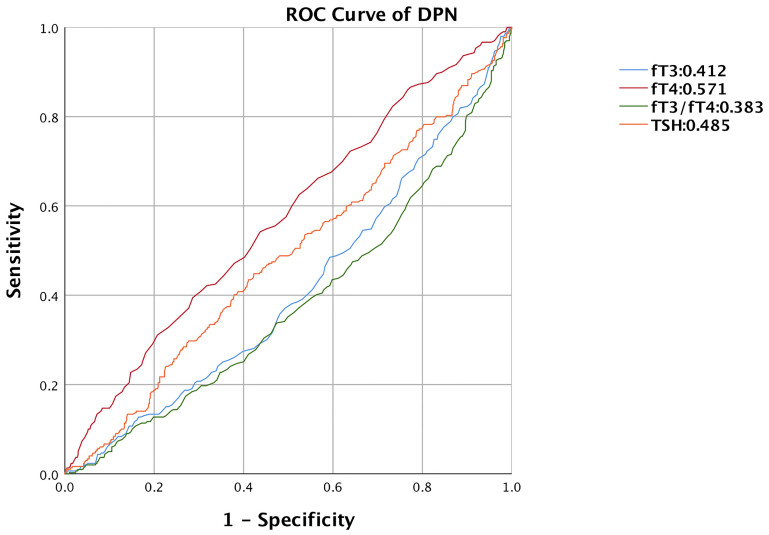

FIGURE 3
ROC curve of thyroid-related hormones predicting DPN in T2DM with euthyroid. ROC, receiver operating characteristic; DPN, diabetic peripheral neuropathy; fT3, free triiodothyronine1; fT4, free thyroxine; TSH, thyroid stimulating hormone.

CA manifests as plaque formation and stenosis of the carotid artery and is a common macrovascular complication of T2DM, the prevention and treatment of which are of critical importance because of its high lethality (43). Some studies have demonstrated that smoking promotes CA by increasing the intima-medial wall thickness of the carotid artery (44). In addition, diabetics are at high risk for arterial atherosclerosis (45) because the arterial walls become less pliable and even harden with age. Plaque composed of cholesterol, fat, calcium, and fibrous tissue accumulates, causing blood vessels to narrow and promoting atherosclerosis (46, 47). Zhou et al. identified a negative correlation between fT3 and carotid intima-media thickness (17). Our study provides evidence to confirm their findings.

The mechanisms concerning fT3 and DPN pathogenesis are unclear. Some investigators have suggested that degeneration of microvascular endothelial cells and peripheral endothelial cells is one of the characteristic changes of DPN (48–50). In addition, the decrease in NO, which can relax blood vessels, is associated with endothelial dysfunction (51, 52). Some studies have reported that T3 mediates the production of endothelial NO (53). Therefore, T3 may reduce the occurrence of DPN by protecting the endothelial cells of microvessels. In addition, the natural metabolite 3,5-diiodothyronine, generated by the deiodination pathway of T3, could reverse the process of DPN by regulating the expression of Sirtuin 1 protein (54, 55). Previous studies have shown that fT3 level was negatively associated with risk of abnormal nerve conduction (56). Meanwhile, Li et al. demonstrated a negative association of fT3 with DPN in T2DM patients with normal thyroid function (57). This is consistent with our findings.

FT4 is deiodinated by three deiodinases to form the more active fT3, which exerts potent biological effects on target organs and tissues (58). Type II deiodinase (DIO2) is the most potent in converting T3, and fT3/fT4 is an indicator of DIO2 activity (59). Intracellular activation of thyroid hormone *via* DIO2 attenuates cellular dependence on aerobic glycolysis, decreases ROS production and thus minimizes oxidative stress (60). Therefore, decreased DIO2 activity and fT3 conversion lead to increased oxidative stress, which in turn promotes an inflammatory response. Further, prolonged exposure to a hyperglycemic environment promotes oxidative stress (61). This suggests that T2DM patients have decreased DIO2 activity, increased fT4, decreased fT3 production, and enhanced oxidative stress and inflammatory responses, which promote the development of DPN (61–63). Foremost, fT4 level was an independent risk factor for DPN (OR:3.376, 95% CI: 1.691,8.256) in our study. Hu et al. showed that fT4 was negatively correlated with DPN (OR:0.800) (64). This is contrary to our findings. The possible reasons include their smaller sample size, only containing 248 patients with T2DM, and group variability, living environment may have played a role.

When subclinical hypothyroidism occurs, it can exacerbate abnormalities in glucolipid metabolism and significantly exacerbate DPN due to TSH receptor palmitoylation-induced oxidative stress and apoptosis in Schwann's cells (65). A cross-sectional study from China also demonstrated that TSH levels were independently associated with DPN in the T2DM population in patients with subclinical hypothyroidism (19). However, our study found that this correlation disappeared when the patient's thyroid function was at normal levels. Does this mean that we should control TSH levels in patients with DPN? In addition, TSH can exacerbate atherosclerosis by promoting macrophage inflammation in plaques (66). Several studies have found a positive correlation between TSH and carotid intima-media thickness in patients with normal thyroid function (67, 68). Interestingly, we did not find this correlation in patients with T2DM. As we all know, hyperglycemia, obesity, and dyslipidemia can promote inflammation and exacerbate CA. The exact mechanism is unclear, and more animal studies are needed to explore it.

As we all know, early detection, early diagnosis, and early treatment can stop and delay the development of the disease and help patients return to health as soon as possible. If the high-risk group of DPN can be detected in advance to help patients diagnose as well as treat them earlier, the development of organic diseases such as gangrene can be reduced or avoided. For people with T2DM, regular monitoring of glycosylated hemoglobin to regulate blood glucose is essential. We can have the treating physician screen the patient for glycosylated hemoglobin along with thyroid function. Reducing the incidence of DPN by controlling fT4 at low normal levels or fT3 at high normal levels after thyroid disease has been ruled out as the cause of the abnormal thyroid function.

In this study, several thyroid-related hormones (fT3, fT4, fT3/fT4, TSH) were evaluated for their association with DPN or CA. Previous research often examined only one of these hormones in relation to the thyroid. One limitation of our study was its a small sample size, which could have led to some errors in the results. Furthermore, it was a single-center cross-sectional study, so the findings cannot be directly attributed to a specific cause. Therefore, further multicenter cohort studies are urgently required to support our conclusions.

Conclusions

Individuals with a low fT3 level and fT3/fT4 ratio in T2DM with normal thyroid function and those with high fT4 levels were more likely to develop DPN. Considering that elevated fT4 is a risk factor for DPN, even in the euthyroid range, early control of fT4 levels or redefinition of its reference range (0.78-1.15 ng/dL) in the T2DM population is beneficial for the

prevention of DPN. And fT3 should be early controlled at high normal levels (2.64-3.78 pg/mL).

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of the Affiliated Hospital of Southwest Medical University (ethical approval code: 2018017). The patients/participants provided their written informed consent to participate in this study.

Author contributions

JL, XX, and YQ made equal contributions to this work, performing the statistical analysis, interpreting the results, and writing the paper. JG reviewed the data, and QW proposed the ideas. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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The clinical characteristics and gene mutations associated with thyroid hormone resistance syndrome coexisting with pituitary tumors

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Aims: Resistance to thyroid hormone (RTH) and pituitary tumors are both rare diseases, and the differential diagnosis of these two diseases is difficult in some cases. There are also patients who have both conditions, making diagnosis more difficult. To better understand this aspect, we analyzed the clinical characteristics and gene mutations of RTH coexisting with pituitary tumors.

Methods: Database retrieval was conducted in the PubMed, Cochrane Library, and SinoMed databases, and the search contents were case reports or case series of patients with RTH coexisting with pituitary tumors. The demographic, clinical manifestations, and imaging characteristics of pituitary tumors and gene mutations were summarized.

Results: Thirteen articles involving 16 patients with RTH coexistent with pituitary tumors, consisting of 13 female patients, one male patient, and two patients with unknown sex, were included. The patients were 10 to 79 years old and most patients were 41-55 years old (43.75%). The 16 patients were from seven different countries and three continents (Asia, the Americas, and Europe). All the patients showed an abnormal secretion of TSH, and five patients underwent transsphenoidal surgery. Finally, four patients were pathologically confirmed to have TSHoma. A total of 11 different mutations occurred at nine amino acid sequence sites (251, 310, 344, 347, 383, 429, 435, 438, and 453). Two different mutations occurred in both the no. 435 and no. 453 amino acid sequences. Fourteen patients provided their treatment histories, and all had undergone different treatment regimens.

Conclusions: Patients with both RTH and pituitary tumors had multiple clinical manifestations and different thyroid functions, imaging characteristics of pituitary

tumors, genetic mutations of *THRβ*, and treatments. However, due to the limited number of cases, the patients were mainly women. Further studies with more cases that focus on the mechanism are still needed.

KEYWORDS

thyroid hormone resistance syndrome, pituitary tumors, *THRβ*, clinical characteristics, gene mutation

Introduction

Resistance to thyroid hormone (RTH) and thyroid-stimulating hormone (TSH)-secreting adenoma (TSHoma) of the pituitary, as the main causes of abnormal secretion of TSH, are clinically rare diseases that are mainly characterized by unregulated thyroid hormone negative feedback, manifested as elevated thyroid hormone with normal or elevated TSH (1–3). RTH is usually caused by mutations in the thyroid hormone receptor-β (*THRβ*) gene and is characterized by a series of clinical manifestations such as tissue insensitivity to the thyroid hormone. Direct sequencing of the *THRβ* gene confirms the diagnosis of RTH in 85% of the cases (4). Since RTH is mainly inherited by autosomal dominant inheritance (it can also be autosomal recessive but is rare), it can be effectively diagnosed by measuring thyroid function in first-degree relatives. An RTH patient without mutations in the beta isoform of the thyroid hormone receptor is also occasionally seen. The diagnosis of RTH is challenging, and it is mainly differentiated from TSHoma. For pituitary tumors, TSHoma cannot be definitively diagnosed without pathological examination. Other clues to help with the diagnosis of TSHoma are visual symptoms caused by pituitary enlargement that presses on the optic chiasma or the abnormal secretion of prolactin or growth hormone from the anterior pituitary. It is interesting that a limited number of reports show that RTH can coexist with pituitary tumors (especially TSHoma) in the same patient (5–17). Sometimes, RTH and TSHoma are difficult to distinguish because some RTH patients may have signs and symptoms of thyrotoxicosis, especially tachycardia, hyperactivity, and hyperreflexes, and many patients have goiters. Conversely, a small number of TSHoma patients have mild symptoms of thyroid toxicity or even none. A correct diagnosis for RTH patients without *THRβ* gene mutations is also difficult, and they are often misdiagnosed. In addition, sporadic pituitary lesions have been reported in up to 24% of patients with RTH (18), thus increasing the complexity of differential diagnosis. Hence, this is the reason why the characteristics of patients with both diseases need to be differentiated. Therefore, we conducted this study to summarize the clinical characteristics and gene mutations of RTH coexistent with pituitary tumors.

Materials and methods

We performed a search in PubMed, Cochrane Library, and SinoMed for case reports or case series about patients with thyroid hormone resistance syndrome coexisting with pituitary tumors. We also hand-searched the reference lists of all eligible articles and related previous review articles. The literature search was restricted to published results from the earliest date to 9 December 2022 with the following search terms: ((Thyroid Hormone Resistance Syndrome[MeSH Terms]) OR (resistance to thyroid hormone) OR (RTH) OR (Thyroid hormone resistance syndrome) OR (resistance to thyroid hormone syndrome) OR (pituitary resistance to thyroid hormone) OR (PRTH)) AND ((Pituitary Neoplasms[MeSH Terms]) OR (hypophysoma) OR (pituitary adenoma) OR (pituitary tumor)). Studies that met the following criteria were included in this article: 1) published in English or Chinese language, 2) case reports or case series, 3) subjects were patients with RTH syndrome coexistent with a pituitary tumor, and 4) clinical characteristics and genetic mutations were described. Otherwise, a study was not included in the subsequent analysis. The flowchart shows the literature review inclusion process (Figure 1).

The following information was extracted from the eligible articles: country; number of patients; and characteristics of cases including age, sex, size of pituitary tumor, symptoms or diseases, thyroid function (FT3, FT4, and TSH), and molecular biology results (including gene mutations and protein). The demographic, clinical, and genetic mutation information of the cases above was described utilizing simple summary statistics.

Results

General data

This study included 13 articles (Figure 1) that were first published in 2001 and recently published in 2022. In total, the articles involved 16 patients with RTH syndrome coexisting with pituitary tumors, consisting of 13 female patients, 1 male patient, and two patients with unknown sex. The patients were 10 to 79 years old. The age group with the largest number of patients was 41-55 years old, accounting for 43.75% of the total number of patients, followed by the 26-40-year-old group (18.75%). There was no significant difference in the number of patients under 25 years old (four patients) and over 56 years old (two patients) (Figure 2). The 16 patients were from seven different countries: five from China (one from Hong Kong, China); three from America; six from Thailand, Brazil, and Italy, each with two patients; and one each from Spain and Portugal (Figure 3A). Patients were grouped according to the distribution of the continents, and Asia accounted for the largest proportion of patients (43.75%), followed by the Americas (18.75% in North America and 12.5% in South America). No cases have been reported in Oceania or Africa (Figure 3B).

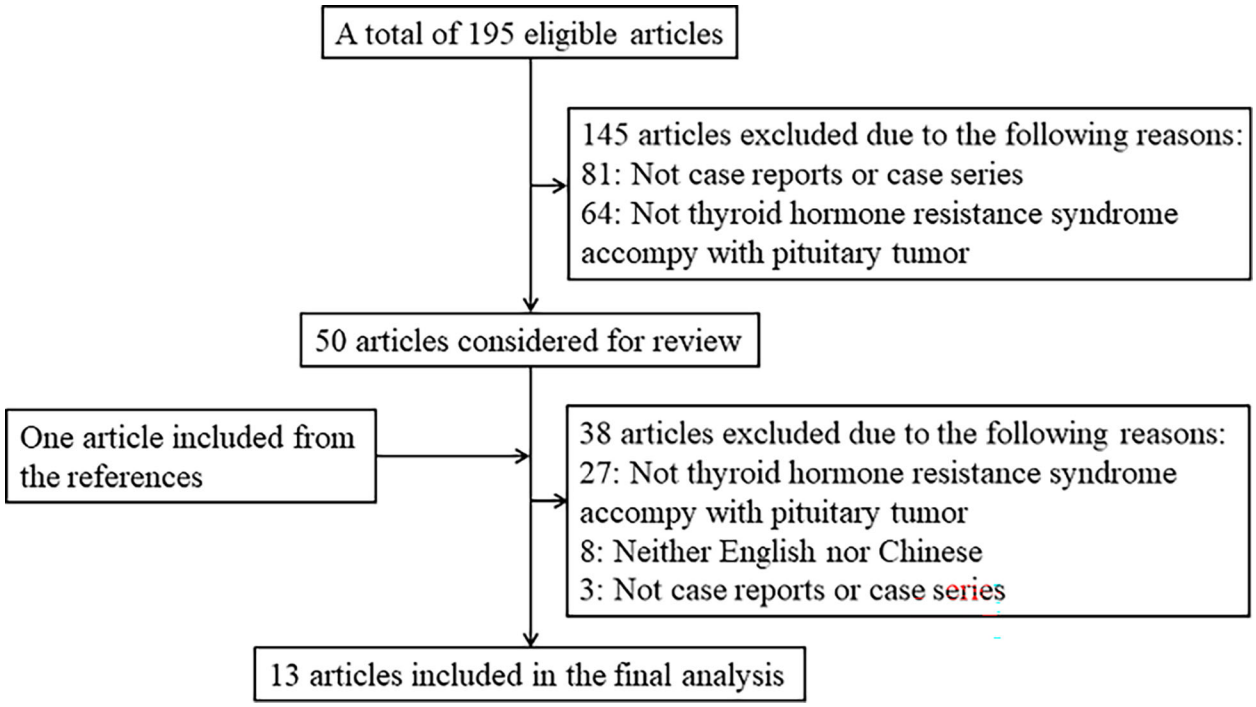

FIGURE 1
Literature review inclusion process.

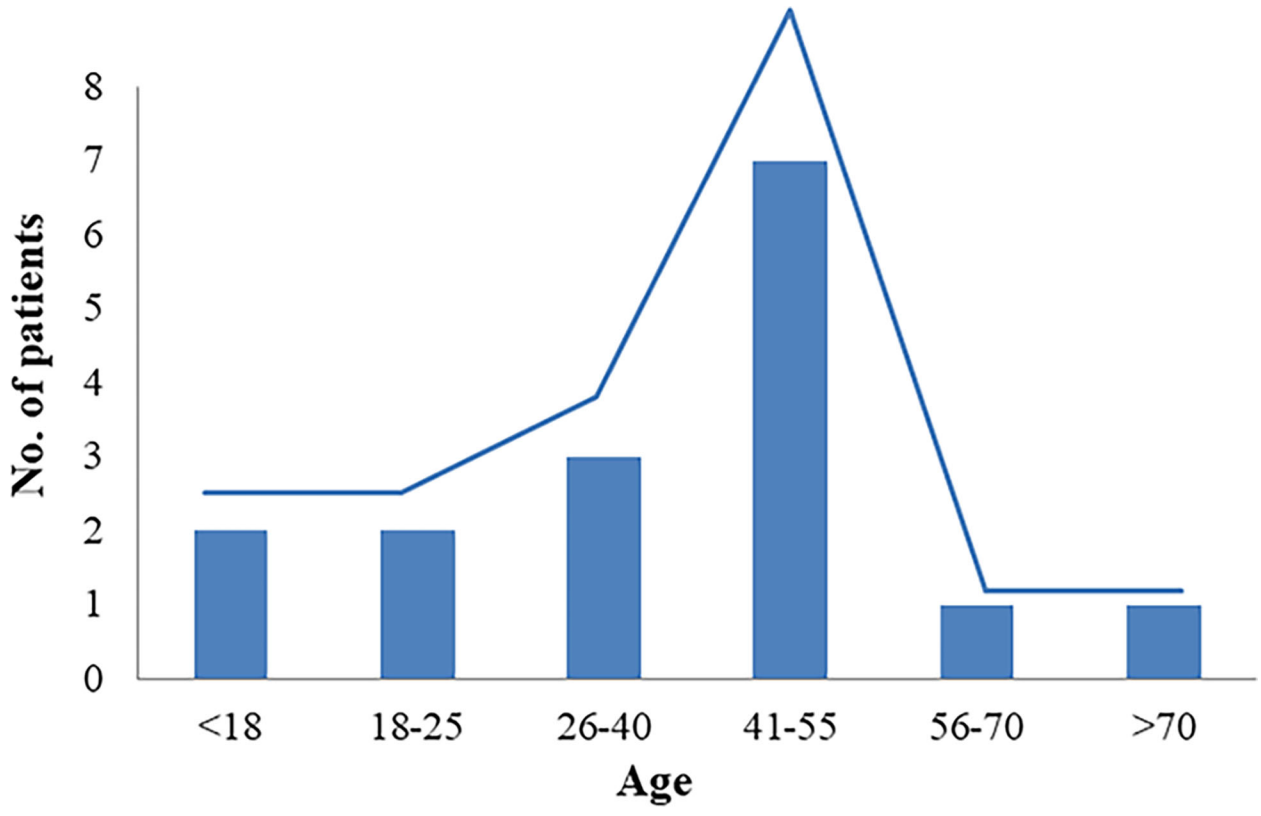

FIGURE 2
Patients' age distribution.

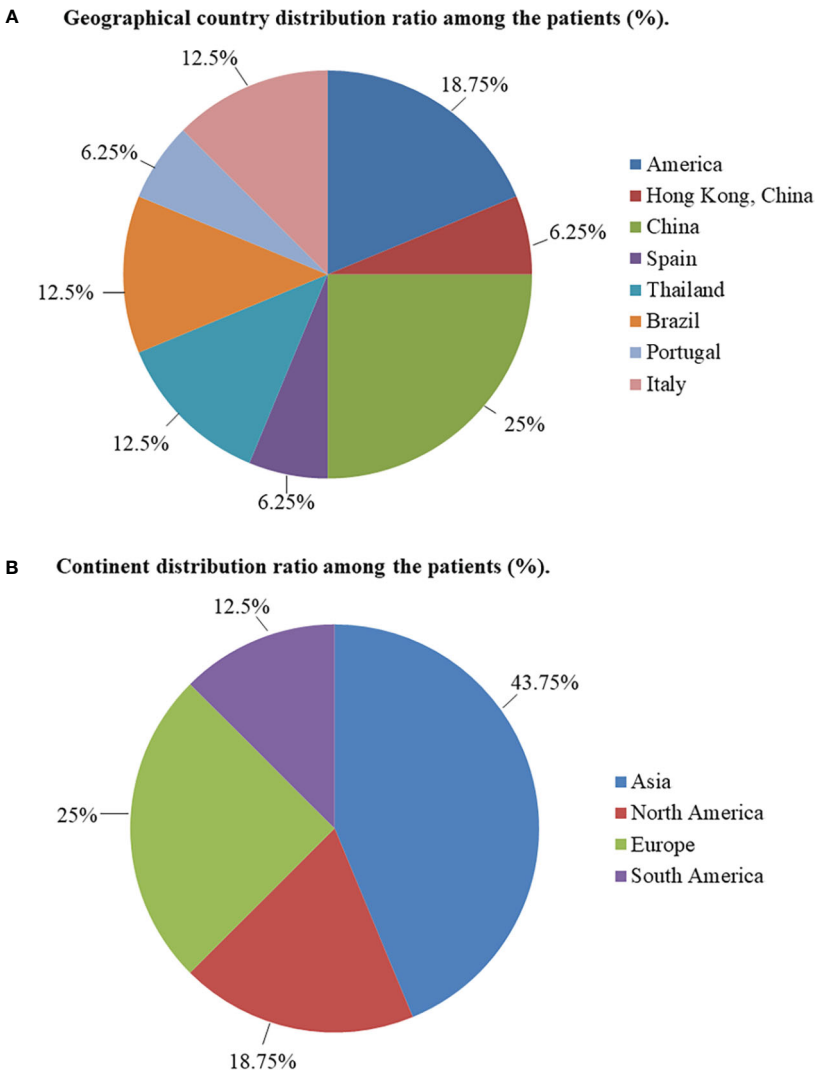

FIGURE 3
(A) Geographical country distribution ratio among the patients (%). **(B)** Continent distribution ratio among the patients (%).

Clinical manifestations

Clinical symptoms varied among the 16 patients included in the analysis. Three patients did not provide any information about symptoms (6, 17), and another three patients had no clinical symptoms (11, 12). Palpitations were reported in six patients (5, 8–10, 14, 17), two patients had cervical swelling (7, 13), and one patient was suspected to be diagnosed with hypothyroidism because she had several typical symptoms, such as fatigue, weakness, weight gain, memory loss, dry eyes, anxiety, and depression (7). Among these 16 patients, four did not have a physical examination of the thyroid (6, 16, 17). Eight patients had goiter (5, 8–10, 12–15), and three had thyroid nodule(s) (12, 13, 15). Prior to a definitive diagnosis of RTH, most of the patients (9/16) were diagnosed with hyperthyroidism or thyrotoxicosis due to clinical symptoms and elevated thyroid hormone levels (5, 6, 8, 9, 12, 14, 15, 17). All sixteen patients showed abnormal TSH secretion, such as elevated thyroid hormone, but normal or elevated TSH. The thyroid function details (FT3, FT4, and TSH) of the 16 patients are summarized in Table 1.

Imaging characteristics of the pituitary tumors

Pituitary MRI examination was abnormal in all 16 included patients. One patient did not report the size of the pituitary tumor (8), four patients were diagnosed with macroadenoma with diameters greater than 10 mm (5, 6, 10, 17), and the remaining 11 patients were diagnosed with microadenoma (7, 9, 11–16). Five patients underwent transsphenoidal surgery (5, 6, 8, 9, 16), and four patients were confirmed to have TSHoma by a pathological examination (5, 6, 9, 16). One patient was diagnosed with a pituitary cyst (16), another was diagnosed with pituitary hyperplasia (17), and the remaining 10 were all diagnosed with pituitary adenoma (7, 8, 10–15) either by a pituitary MRI scan or pathological examination.

Gene mutations

All 16 patients underwent genetic testing, and 15 of them had abnormalities in the *THRβ* and were diagnosed with RTH. RTH

TABLE 1 Summary of thyroid function.

First author, year	FT3	FT4	TSH
Ando S, 2001-1	NA	7.4 ng/dl (0.9-1.6)	10.5 μU/ml (0.43-4.60)
Ando S, 2001-2	NA	1.7 ng/dl (0.9-1.6)	121.6 μU/ml (0.43-4.60)
Safer JD, 2001	606 pg/dl (210-440)	2.6 ng/dl (0.8-2.7)	0.4 mIU/L (0.3-5.0)
Kong AP, 2005	9.65-12.3 pmol/L (3.28-8.2)	20.7 pmol/L (8.5-20.7)	16.7 mIU/L (0.3-4.0)
Teng X, 2015	14.25 pmol/L (2.63-5.7)	28.79 pmol/L (9.01-19.05)	21.11 mIU/L (0.35-4.94)
Ramos-Leví AM, 2016	7.32 pg/ml (2.5-3.9)	2.2 ng/dl (0.8-1.7)	10.5 μU/ml (0.3-5.6)
Sriphrapradang C, 2016	NA	13.9 (6-11.5)	2.8 mU/L (0.4-3.6)
Ramos LS, 2018	NA	2.8 ng/dl (0.6-1.5)	1.5 mIU/L (0.3-4.0)
	NA	2.9 ng/dl (0.6-1.5)	6.7 mIU/L (0.3-4.0)
Yu C, 2018	9.76 pg/ml (4.1-7.9)	26.78 pg/ml (12-22)	5.21 μIU/ml (0.27-4.2)
Carvalho Cunha N, 2019	NA	2.1 pg/dl (0.8-1.9)	9.6 μIU/ml (0.4-4.0)
Jiaqi L, 2019	NA	56.7-70.7 pmol/L (12.0-22.0)	4.85-10.93 mU/L (0.27-4.20)
	NA	32.34-32.09 pmol/L (12.0-22.0)	10.73-26.34 mU/L (0.27-4.20)
Campi I, 2020	8.13 pmol/L (4.2-7.5)	35.3 pmol/L (10-20)	1.3 μIU/ml (0.3-5.1)
	6.9 pmol/L (4.2-7.5)	26.8 pmol/L (10-20)	1.58 μIU/ml (0.3-5.1)
Suntornlohanakul O, 2022	3.14 pg/ml (2-4.4)	2.09 ng/dl (0.7-1.75)	7.27 mIU/L (0.25-4)

FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; NA, not available.

without mutations in the *THRβ* gene (non-TR-RTH) was considered when patients did not have mutations in the beta isoform of the THR but had other characteristics of RTH according to laboratory evaluations, such as the α-subunit, TRH stimulation test, T3 suppression test, and sexual hormone binding globulin. Finally, one patient was diagnosed with non-TR-RTH (12). Ando S et al. (5) reported aberrant alternative splicing of *THRβ2* (135 bp deletion) that caused hormonal dysregulation and hormone resistance (Figure 4B). The details of the gene mutations in the 16 patients are shown in Table 2. A total of 11 different mutations occurred at nine amino acid sequence sites (251, 310, 344, 347, 383, 429, 435, 438, and 453), and a schematic representation of *THRβ1* is shown in Figure 4A. These mutations involve the following gene loci: c.1030G>A, c.1037G>T, c.1040G>A, c.1286G>A, c.1303C>A, c.1433G>A, c.1642C>A, and c.1642C>G. Two different mutations occurred in both the no. 435 and no. 453 amino acid sequences. Three patients had the same point mutation in the no. 429 amino acid sequence causing the replacement of normal arginine with glutamine (8, 15, 16), and two patients had the same point mutation in the *THRβ* gene (c.1642C>A), leading to a missense change of proline 453 to threonine (p.Pro453Thr) (9, 10). No relationship was found between the site and types of genetic mutations and the patients' country, age, sex, thyroid function, and pituitary tumor characteristics by further analysis.

Treatment

Fourteen patients provided their treatment histories, and all 14 patients had undergone different treatment regimens. Due to TSHoma or suspected TSHoma (finally pathologically confirmed as a pituitary adenoma), five patients underwent transsphenoidal surgery (5, 6, 8, 9, 16). Two patients underwent a thyroidectomy for Graves' disease or nodular goiter and thyroid adenoma (6, 13). Three patients had received ^{131}I therapy for hyperthyroidism or goiter accompanied by increased heart rate and thyroid hormone levels (6, 10, 15). Antithyroid drugs, such as thiamazole (methimazole), were taken by five patients for suspected hyperthyroid (9, 12, 14, 15) or TSHoma (16). Levothyroxine was administered to four patients who were suspected of having hypothyroidism or secondary hypothyroidism after thyroidectomy and ^{131}I therapy (7, 10, 13, 15). Only two patients received triiodothyroacetic acid or T3 therapy for RTH (11, 12). Furthermore, most of the patients had abnormal TSH during follow-up.

Discussion

Abnormal TSH secretion with high levels of FT3 and FT4 and high or normal levels of TSH is due to either a TSHoma or RTH (1–3). Although RTH and TSHoma are both syndromes of abnormal secretion of TSH, their pathogenesis, treatment strategies, and prognosis are completely different. TSHoma, a rare functional pituitary tumor, accounts for less than 1% of all pituitary adenomas (19). Due to the clonal expansion of abnormal cells, the secretion of thyroid hormone is not affected by its negative feedback. Therefore, currently, the first-line treatment is surgical resection. However, the mechanism of TSHoma is still unclear. A hypothesis was proposed by Beck-Peccoz P et al. (1) in 1996 that the downregulation of the thyroid hormone may be one mechanism. In addition, mice with a mutation in the β-isoform of the thyroid hormone receptor spontaneously develop TSHoma (20). This suggests the role of thyroid hormone receptors in pituitary tumors or even predisposes patients to TSHoma (9).

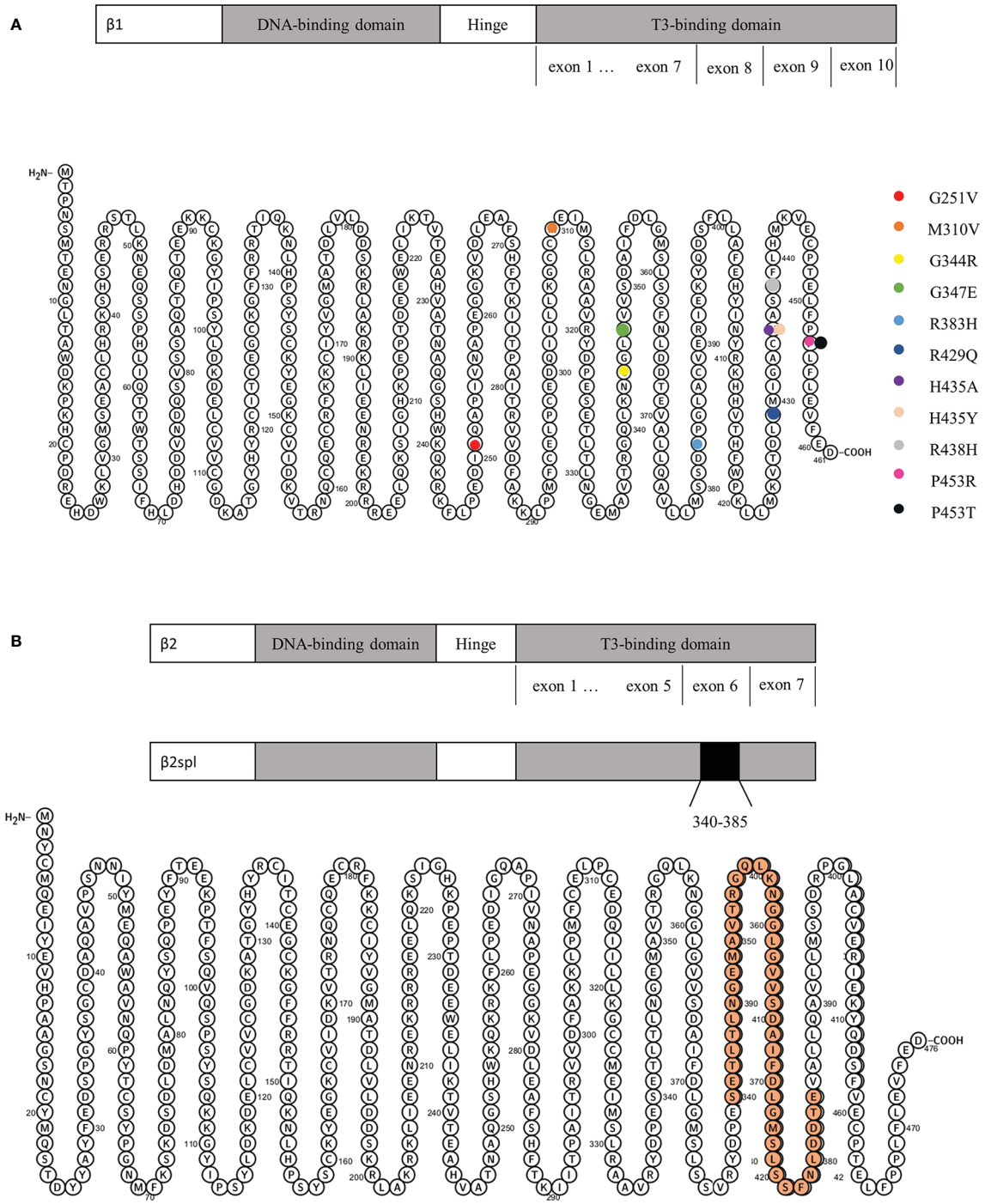

FIGURE 4
(A) Schematic representation of *THRβ1* and its gene mutations. **(B)** Schematic representation of *THRβ2* and its deletion from amino acids 340 to 385.

RTH is a disease first reported by Refetoff et al. in 1967 (21), and some of the main causes of RTH include 1) mutation of the thyroid hormone receptor, 2) binding disorder of the thyroid hormone and its receptor, and 3) abnormal post-binding action of the thyroid hormone receptors, etc., leading to the insensitivity of the tissues to the thyroid hormone, thus causing metabolic disorders, thyroid dysfunction, etc. In addition to the brain, testis, and lymph, thyroid hormone receptors are widely expressed in diverse tissues, organs, and cells. The α- and β-isoforms are two subtypes of THR, and mutation or dysfunction is an important cause of RTH. Currently, approximately 80% of RTH is caused by *THRβ* mutations, and approximately 10%–15% of cases are not caused by *THRβ* mutations (18, 22, 23). RTH, as it is often referred to, is mainly a rare autosomal dominant disease characterized by mutation in the β-isoform of the thyroid hormone receptor (2, 3). The *THRβ* gene is located at 3p24.2 and consists of 10 exons. RTH can be divided into two main categories according to the site of RTH and clinical manifestations, that is, generalized RTH and central or pituitary RTH (24). Partial resistance is commonly seen in the clinic, while true complete resistance is rare. However, various organs and tissues

TABLE 2 Summary of the *THRβ* mutations of RTH patients coexistent with pituitary tumor.

Study	Gender	Gene mutations	Protein
Ando S, 2001-1	F	THRβ2spl has a deletion from amino acids 340 to 385	
Ando S, 2001-2	F	c.1303C>T	p.His435Tyr
Safer JD,2001	F	c.1313G>A	p.Arg438His
Kong AP, 2005	M	c.1286G>A	p.Arg429Gln
Teng X, 2015	F	c.1642C>A	p.Pro453Thr
Ramos-Leví AM, 2016	F	c.1642C>G	p.Pro453Arg
Sriphrapradang C, 2016	F	c.1037G>T	p.Gly251Val
Ramos LS, 2018	F	c.1642C>A	p.Pro453Thr
	F	Without mutations	
Yu C, 2018	F	c.1303C>A	p.His435Ala
Carvalho Cunha N, 2019	F	c.1030G>A	p.Gly344Arg
Jiaqi L, 2019	F	c.1040G>A	p.Gly347Glu
	F	c.1286G>A	p.Arg429Gln
Campi I, 2020	NA	c.1286G>A	p.Arg429Gln
	NA	NA	p.Met310Val
Suntornlohanakul O, 2022	F	c.1433G>A	p.Arg383His

F, female; M, male; NA, not available.

have different degrees of resistance to the thyroid hormone, and patients have different compensatory abilities, so there are different clinical manifestations and laboratory characteristics. Clinical manifestations can include hyperthyroidism, normal thyroid function, or hypothyroidism. To date, no efficient therapy has been found to correct the defect in THR function especially that caused by gene mutation. Therefore, symptomatic treatment to relieve the symptoms of hypo- or hyperthyroidism is the main therapy for RTH. For patients with hyperthyroidism, antihyperthyroidism drugs can be used, and thyroid hormone supplementation should be considered. Although the diagnosis was the same, the clinical symptoms, thyroid function, and treatment regimen were all different in the 16 patients in this study. It is important to stress that invasive treatments such as thyroidectomy or radioactive iodine ablation are not needed. Unfortunately, in the few cases we included, some patients underwent unnecessary invasive treatment.

The symptoms and signs of RTH and TSHoma have similar characteristics, such as palpitations, goiter, and similar thyroid function in both diseases. For RTH patients with coexisting pituitary tumors, the signs and symptoms might be worse and diverse, and the abnormalities caused by the high level of prolactin or growth hormone from the anterior pituitary and the visual symptoms caused by pituitary enlargement pressing on the optic chiasma might occur at the same time. Prior to the correct diagnosis of these different diseases, some aggressive treatments may result in serious adverse outcomes, such as inappropriate thyroid ablation in patients with TSHoma or unnecessary pituitary surgery in patients with RTH. These problems lead to the need for further testing, including laboratory tests, dynamic tests, genetic tests, and pituitary imaging. To evaluate patients with abnormal TSH secretion and pituitary imaging abnormalities, combined examination is usually required to distinguish TSHoma from RTH. There have been several previous reports of RTH with non-functional or functional pituitary adenomas (5–17). Both RTH and pituitary tumors are rare diseases, but it is still possible for a patient to have both, although it is highly unlikely. Is there a connection between the two diseases? It is known that up to 15% of people without any diseases may have a small and non-functional pituitary adenoma, and patients with RTH may occasionally have abnormal imaging findings (25). As we mentioned above, thyroid hormone receptors may play a role in the development of pituitary tumors (or TSHoma). In addition, a somatic mutation of *THRβ* was found in a TSH-secreting pituitary adenoma in one of the patients included in our study (5). However, the relationship between RTH and pituitary tumors (especially TSHoma) and whether RTH can promote the occurrence of TSHoma are still unknown due to the limitations of current studies.

Although genetic testing could assist in the diagnosis of RTH, there are still up to 10%–15% of RTH patients without *THRβ* mutations (which we called non-TR-RTH), which may affect the diagnosis of such patients, especially for patients with pituitary tumors, leading to the wrong choice of surgical treatment (4, 26). It has also been described in the literature that 15% of RTH families may have non-TR-RTH patients (27). In this study, we reported a non-TR-RTH patient with pituitary microadenoma, but whether the pituitary microadenoma was a TSHoma was unknown because pituitary pathological examination was not performed.

In this paper, a total of 11 different mutations occurred at nine amino acid sequence sites, but no regular mutations or mutations associated with any particular phenomenon were found. *THRβ1* (widely expressed), *THRβ2* (mainly expressed in the brain, retina, and inner ear), and *THRβ3* (expressed in the kidney, liver, and lung) are the three main T3-binding splicing products of *THRβ* (28, 29).

The mutation of *THRβ* has both dominant and negative effects. Point mutations often manifest as autosomal dominant, and a deletion is often autosomal recessive. It was reported that exons 7-10 of *THRβ* mainly encode the T3-ligand-binding region (30), and the mutations reported in patients in this study were mainly concentrated in exons 7-10 encoding the T3-ligand-binding region.

Thyroid disease is more common in women than in men. As an autosomal disorder, the prevalence of RTH is approximately 1/40,000-19,000, and the frequency among the sexes is equal (18, 31). Daly AF et al. (32) reviewed the prevalence studies of clinically relevant pituitary adenomas in the general population that had been published before 2020 and reported that the prevalence of pituitary adenomas differed from 1/865 to 1/1,322. At the same time, it is higher in women than in men (differing from 62.2% to 77.3%). However, it is still unknown whether a sex difference exists in *THRβ* mutation patients. Santos Mata MA et al. (33) summarized a multicentrical case series (a total of 22 RTH patients) and showed that of the 12 patients who underwent THR genetic testing, eight men and three women had *THRβ* mutations and one woman had normal *THRβ* and *THRα*. Interestingly, in our study, except for two patients who did not provide gender information, only one of the other 14 patients was a male patient and the rest (92.86%) were all female patients, which means that the dataset is mostly made of female individuals. In other words, in this dataset, the proportion of female patients with RTH complicated with pituitary tumors was significantly higher than that of male patients. Due to the limitations of the dataset compared to the general incidence of the diseases, the results still need to be supported and verified with more data. At the same time, the mechanism by which sex and RTH coexist with pituitary tumors needs further study.

Summary

Both RTH and pituitary tumors are rare diseases, and patients diagnosed with both are even rarer. This study shows that patients with both RTH and pituitary tumors have multiple clinical manifestations and different thyroid functions, imaging characteristics of pituitary tumors, genetic mutations of *THRβ*, and treatments. However, due to the limited number of cases, patients were mainly women. Further studies with more cases that focus on the mechanism are still needed.

Author contributions

JZ and LX: document retrieval, data extraction, data analysis, essay writing, and paper submission. CL and FW: data extraction and data analysis. JD: article innovation. LL: article innovation and paper submission. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Development and validation of a nomogram model for predicting residue of partially cystic thyroid nodules after ultrasound-guided ethanol and thermal ablation

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Objectives: To develop and validate a nomogram model for predicting residue of partially cystic thyroid nodules (PCTNs) after ethanol and thermal ablation.

Materials and Methods: From July 2015 to August 2022, a total of 97 patients (age 40.78 ± 12.61 years) with 107 treated benign PCTNs receiving ethanol and thermal ablation were enrolled. Pre-ablative laboratory test results and the ultrasound (US) and contrast-enhanced ultrasound (CEUS) features of lesions were collected. They were categorized into non-residue group and residue group according to the CEUS examination assessment after ablation. Univariate and multivariate logistic regression analysis were adopted to build a nomogram. The nomogram was validated by internal stratified fivefold cross-validation. The calibration, discrimination and clinical utility of the nomogram were investigated to assess the performance of the model.

Results: Residue was reported in 30 out of 107nodules (28.0%). Multivariate logistic regression analysis revealed initial volume (OR=1.12, 95%CI 1.06-1.19) and presence of septum (OR=3.19, 95%CI 1.09-9.36) were predictors of residue of PCTNs. The nomogram developed by the above factors showed good calibration and discrimination. The area under the curve (AUC), sensitivity and specificity of this model were 0.832, 86.7% and 68.8%, respectively. When applied to internal validation, the model revealed good generalizability with stratified fivefold cross-validation in the cohort (mean AUC = 0.821).

Conclusions: The nomogram model has good performance for predicting the residue of PCTNs undergoing ethanol and thermal ablation. This could play a role in the decision of treatment and follow-up in clinical practice.

KEYWORDS

thermal ablation, ethanol ablation, partially cystic thyroid nodules, ultrasonography, nomogram

Introduction

Thyroid nodules are common in population, affecting up to 65% of them (1). Partially cystic thyroid nodules(PCTNs)are nodules with both solid and cystic components. With a malignancy rate less than 20%, most of PCTNs are benign and do not need intervention (2). But some of them can cause compressive symptoms or cosmetic problems and require treatment. Traditionally surgery was the standard solution of these problems but it still has some disadvantages, including scar formation, potential complications and damage of thyroid function (3). Thus, minimally invasive treatments are needed for benign PCTNs. Ultrasound(US)-guided thermal ablation, including radiofrequency ablation(RFA) and microwave ablation(MWA), was proved to be an effective substitute for surgery, which can decrease the nodule size significantly and simultaneously preserve the thyroid function and minimize complications (4–8). A meta-analysis showed that volume reduction rate (VRR) at 3, 6, and 12 months was 56.0%, 80.8% and 86.2% after RFA. And VRR after MWA was 53.9%, 74.9% and 80.0%, respectively. Significant decreased symptomatic and cosmetic scores were found after 6 and 12 months for both RFA and MWA (6). Besides, another study demonstrated that the incidence of hoarseness, hypothyroidism and postoperative pain were lower after thermal ablation compared to conventional thyroidectomy (9).

At the same time, for PCTNs, especially those with cystic components≥50%, simple aspiration of internal fluid with or without ethanol ablation (EA) was an initial recommended treatment (10). To enhance the efficacy, we adopted an EA-RFA combination therapy for these nodules in our study (11). The volume reduction rate (VRR) was commonly used as a measurement of the efficacy after ablation of thyroid nodules (12). However, just simple aspiration of the fluid and EA can achieve a great VRR but still reported a considerable rate of recurrence (13), which means VRR may not be an appropriate indicator of the prognosis of patients. The presence of solid component was considered a main cause of recurrence (14). For PCTNs that underwent RFA or MWA, the rate of recurrence was reported to be 5.6%-18.0% after ablation (15–17), which needs supplementary treatment. In the studies of Sim et al. (18) and Yan et al. (19), the incompletely treated residual vital tissue after ablation of thyroid nodules was independently related to recurrence. With the widespread use of contrast-enhanced ultrasound(CEUS), the residual vital tissue can be easily detected by CEUS after ablation and defined as enhanced area at ablative margin during both arterial phase and venous phase. Similar concept has been generally used to assess disease progression in image-guided tumor ablation (20). Therefore, the presence of residual vital tissue can be a preferable parameter of efficacy, compared to VRR. As we know, the pre-ablative prediction of the residual vital tissue is still unexplored for PCTNs, which is important for the decision-making before the management of PCTNs and follow-up strategy after ablation.

Therefore, the aim of this study was to construct a prognostic nomogram model based on pre-ablative parameters to predict the residual vital tissue after ablation of PCTNs.

Materials and methods

The protocol of this retrospective study was approved by the Institutional Review Board of the First Affiliated Hospital of Sun Yat-Sen University. Since it was a retrospective study, informed consent of the patients could not be obtained, but all patients has signed informed consent before the ablation and CEUS.

Patients

From July 2015 to August 2022, a total of 97 consecutive PCTNs patients (age 40.78 ± 12.61 years, with 107 treated nodules) were enrolled. The inclusion criteria were as follows: (1)patients with benign nodules confirmed cytologically by ultrasound-guided fine-needle aspiration (FNA); (2)nodules without suspicious echographic features of malignancy; (3)patients with partially cystic thyroid nodules (10%≤cystic components ≤ 90%); (4)patients who complained about compression or cosmetic problems, or with anxiety about malignant transformation; (5) patients who refused or ineligible for surgery; (6) patients with a post-ablative follow-up time≥1 month. The exclusion criteria were: (1) malignant nodules on US imaging; (2) cytologically confirmed malignancy; (3) patients with totally solid or cystic thyroid nodules; (4) patients allergic to ultrasonic contrast agent; (5)patients with severe cardiopulmonary disfunction or coagulation disorder; (6) patients with incomplete data.

Pre-ablation assessment

Before ablation, patients underwent US and CEUS examination, FNA and laboratory examination. All CEUS examinations were carried out by multiple senior sonographers with more than 5 years of experience in thyroid examination. US and CEUS examination were performed using a Toshiba Aplio i900 Ultrasound System (Canon, Tokyo, Japan) or a Philips iU22 Ultrasound System (Philips Healthcare, Bothell, WA) with a linear multifrequency probe. Nodule volume, location, adjacent structures, proportion of cystic component, echogenicity of cystic component, vascularity, presence and type of septum and enhancement of solid component were assessed on US and CEUS. The nodule volume was calculated using three orthogonal diameters (the largest one(a) and two diameters perpendicular to it (b and c)): $V = \pi abc/6$. The location of each nodule was classified as right lobe, left lobe and Isthmus. The adjacent structures (<2mm) were classified as trachea, esophagus, large cervical vessels (carotid artery and jugular vein), recurrent laryngeal nerve and none of the above. The proportion of cystic component was measured using software ImageJ on the largest section of the nodule. The echogenicity of cystic component was classified as echoless and mixed echo. Nodule vascularity was evaluated for the solid component according to the Alder grading system of blood flow: grade 1 (absent): no blood flow visualized; grade 2 (minimal): blood flow of one or two pixels; grade 3 (moderate): a main vessel or several small vessels; grade 4 (marked): four or more vessels (21). Septum was defined as one or more bands (thickness <

2mm, length > 2mm) in the largest cyst with both fixed ends, reported as absent or present. According to the type of septum and distribution of cystic and solid components, the nodules were further classified into 5 types: type A: one or more small cysts scattered among continuous solid component; type B: discontinuous solid components adhered to the wall separated by cystic component; type C: one septum in the largest cyst; type D: several septa in the largest cyst with a radial pattern; type E: several septa in the largest cyst with a random pattern (Figure 1). The type A and B were non-segregated nodules, and type C, D and E were segregated nodules. The process of CEUS examination consists of arterial phase (0–30s after the administration of contrast agent) and venous phase (30–120s after contrast agent injection). Enhancement of solid component in the two phases was evaluated as hypo-, iso- and hyper-enhanced compared to surrounding normal tissue. Nodule location, adjacent structures, echogenicity of cystic component, vascularity, presence and type of septum and enhancement of solid component were assessed by 2 doctors with more than 5 years of experience in thyroid examination independently. If there was difference between them, a third doctor with more than 10 years of experience in thyroid examination would re-assessed the characteristics and made the final decision.

Laboratory results collection included the level of thyroid stimulating hormone (TSH), serum free triiodothyronine (fT3), serum free thyroxine (fT4), complete blood count and coagulation tests. Additionally, all patients underwent electrocardiogram, laryngoscopy and chest X-ray or CT before ablation.

Ablation procedures

All invasive operations were performed by 2 interventional doctors with more than 5 years of experience in ablation. The operator adopted simple aspiration of fluid and ethanol ablation as a combined therapy of thermal ablation when (1): the proportion of cystic component was more than 50% and the type of septum was not type A; (2) the volume of cystic component was estimated to be more than 10ml on the largest section and its perpendicular section. For nodules with small cysts, only thermal ablation is enough to damage the structure of cyst wall. However, for nodules with large cysts, thermal ablation is not enough so simple aspiration and ethanol ablation are needed to damage the wall completely. Patients were requested to assume a supine position with the neck extended. Intravenous analgesia plus local anesthesia was used during ablation. The operator first aspirated the fluid in the capsule and then repeatedly flushed the capsule cavity with anhydrous alcohol to complete the ethanol ablation. The dosage of alcohol was approximately 50% of the aspirated fluid volume. Then if the distance between the target area and the adjacent important structures was less than 2mm, 5% glucose solution would be introduced to form hydrodissection and separate the thyroid gland and adjacent important structures. In RFA, a Cool-tip RFA system (Covidien, Mansfield, Mass) with a 17-gauge electrode and a 1-cm active tip would be used percutaneously in the procedures. With real-time US guidance, the ablation was performed in a trans-isthmic approach, using moving-shot technique. The ablation power was set as 40w. High temperature produced necrosis in target nodule. The instrument shut down when the impedance increased to a certain degree. In MWA, the procedures were similar. A KY-2450B MWA system (Kangyou Medical, Nanjing, China) with a 16-gauge needle antenna and a 1.1-cm active tip was inserted into the nodule in a trans-isthmic way with the guidance of US. The ablation power was 30w. As heat built up at the target area, microbubbles would appear on US images. The procedure was continued by moving the antenna towards the other parts of the nodule. The procedure reached the end when the nodule appeared

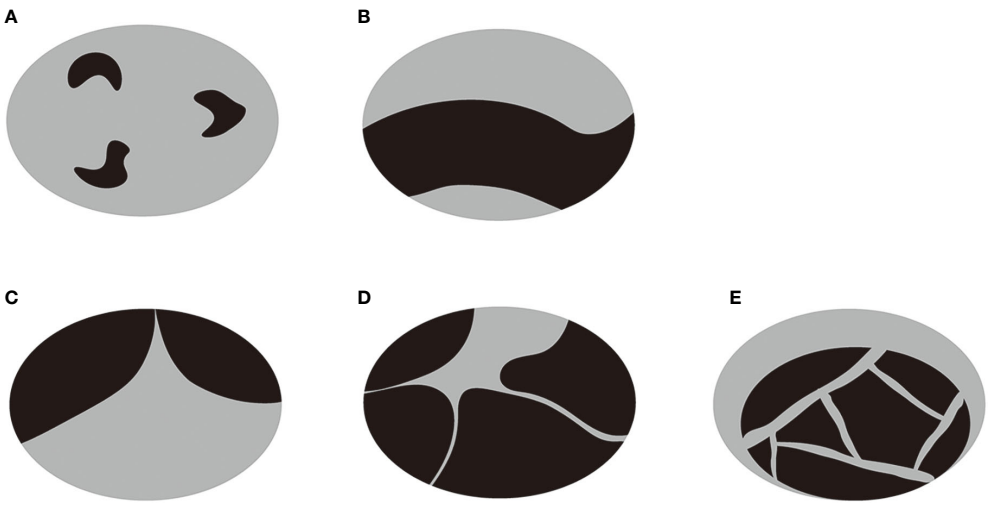

FIGURE 1
A diagram of the classification of PTCNs. **(A)** One or more small cysts were scattered among continuous solid component. **(B)** Discontinuous solid components were adhered to the wall separated by cystic component. **(C)** There was one septum in the largest cyst. **(D)** There were several septa in the largest cyst with a radial pattern. **(E)** There were several septa in the largest cyst with a random pattern. The type A and B were non-segregated nodules, and type C, D and E were segregated nodules.

completely hyperechoic. Then we drew back the electrode and cauterized the pathway to avoid bleeding and seeding.

The technical variables, including power applied, number of electrode and times of power release, and duration of ablation, were recorded during the procedure.

Post-ablation evaluation

After ablation, CEUS was performed immediately and one day later to assess ablated field. Additionally, the level of thyroid stimulating hormone (TSH), serum free triiodothyronine (fT3) and serum free thyroxine (fT4) were tested within 24 hours after treatment. The same follow-up routine was performed 1 month or later after ablation. Any short- and long-term complications were recorded. On CEUS, the ablated nodule would be presented as non-enhanced area if the ablation was complete and there was no residual vital tissue. In contrast, if enhanced area at the ablative margin was observed at any follow-up≥1month later, residual vital tissue was considered to be present. To accurately judge the ablative margin, the sonographers should scan the nodules on three perpendicular planes dynamically and consider 3 aspects comprehensively. First, they compared the images of the nodules after ablation with those obtained before ablation. If the original nodule was completely non-enhanced, it would be considered to be non-residual. Otherwise, residue may exist. Second, the completely ablated area is usually a regular and smooth ellipse. In contrast, the residual part usually has an inward tendency and turns the area to an irregular part with projections on the margin. Third, they evaluated the nodules combining US and CEUS. The solid part of most nodules has mixed echo that can be distinguished from normal thyroid parenchyma. For the nodules that were isoechoic on US and iso-enhanced on CEUS, they usually have a clear boundary with the surrounding normal thyroid parenchyma. That's to say, as long as the nodules can be detected before ablation, the residual part can also be distinguished from normal tissue. We did not include the follow-up within one month because the non-enhanced portion could not represent the actual necrotic tissue immediately (22). According to the results of follow-up CEUS examinations, the nodules were categorized into non-residue group and residue group. Figures 2, 3 shows the representative cases of the two groups.

Statistical analysis

All statistical analyses were performed using SPSS version 26.0 software (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.) and R software version 4.2.1. Continuous variables were presented as mean ± SD. Mann–Whitney U test was used to compare age, level of TSH, FT3, FT4, volume and proportion of cystic component between the two groups. Categorical variables were reported as frequencies (percentages) and Chi-square test was used to compare them

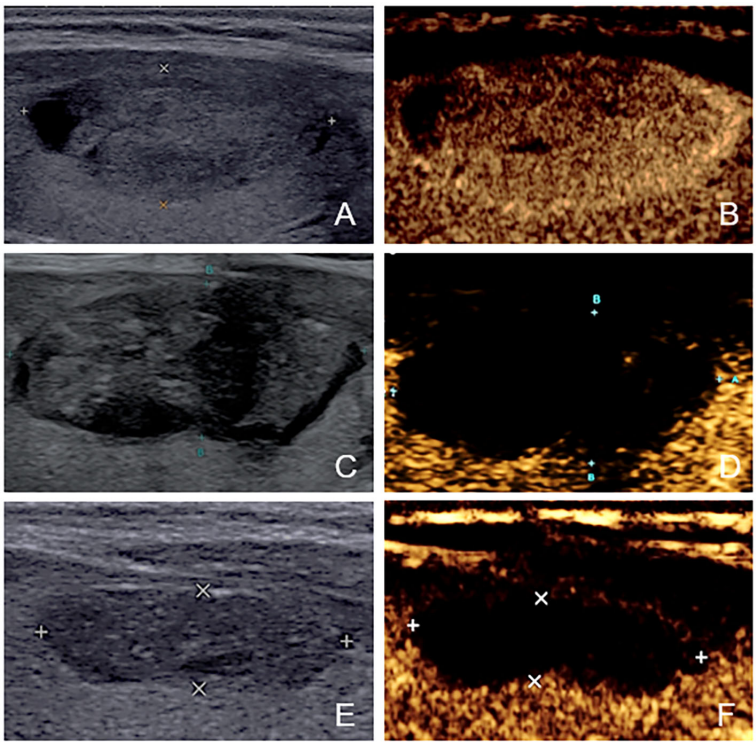

FIGURE 2
The US and CEUS feature of a case in non-residue group—a 41-year-old female. **(A, B)** Before ablation, the initial volume of the target nodule was 3.22 ml. There was no septum inside the nodule. Her residue rate according to the nomogram was 8.1%. **(C, D)** At one-month follow-up, no enhanced area was observed at the ablative margin under CEUS. **(E, F)** At four-month follow-up, the volume was 0.62ml, and VRR was 80.75%.

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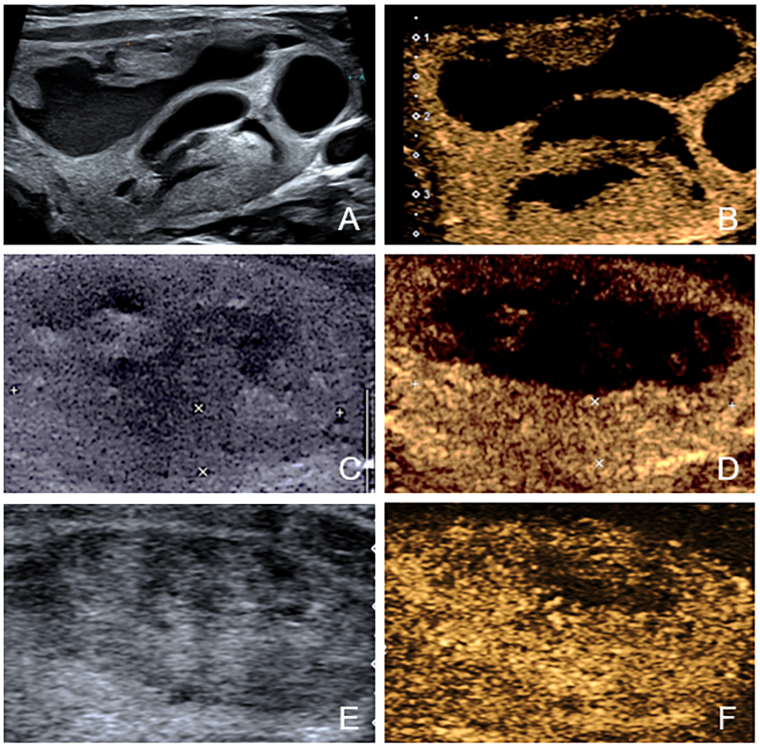

FIGURE 3
The US and CEUS feature of a case in residue group—a 34-year-old female. **(A, B)** Before ablation, the initial volume of the target nodule was 21.21 ml. There was obvious septum in the nodule. Her residue rate according to the nomogram was 58.5%. **(C, D)** At one-month follow-up, an enhanced area (about 3.0cm×0.6cm) was observed at the ablative margin under CEUS. **(E, F)** At four-month follow-up, the volume was 12.90ml, and VRR was 39.18%.

between two groups. Then univariate and multivariate logistic regression analysis were constructed to confirm whether independent variables existed. Based on these independent factors, a nomogram was constructed to predict the probability of residue after ablation. The performance of the model was tested by calibration, discrimination and clinical utility. The Hosmer–Lemeshow test was used to assess the goodness of fit of this model. A calibration curve was also produced to visualize the calibration of the nomogram. The discrimination was presented as C-index, equivalent to the area under the receiver operating characteristic (AUROC) curve. The Youden index was used to identify the best cutoff value of the ROC curve. To evaluate the clinical usefulness of the model, decision curve analysis (DCA) was constructed with 1,000 bootstrap resamples of the study group. Additionally, internal stratified fivefold cross-validation was performed to demonstrate the generalizability. All the statistical analyses were two-tailed and a P value less than 0.05 was considered statistically significant.

Results

Totally 97 patients (90 females, 17males, age 40.78 ± 12.61years) with 107 PCTNs were included in this study (Table 1). 89 out of these 97 patients had 1 nodule, 6 patients (6.2%) had 2 nodules and 2 patients (2.1%) had 3 nodules. Among all patients, 3 patients had hyperthyroidism while the other had normal thyroid function. The initial largest diameter and volume of the nodules were 3.38 ± 1.23 cm and 11.20 ± 11.11ml, respectively. The proportion of cystic component was 41 ± 27%. About half of the nodules had septum. Among the non-segregated nodules, 40 nodules were classified as type A and 10 nodules as type B. As for segregated nodules, 26, 14 and 17 nodules were respectively classified as type C, D and E. The mean follow-up was 16.0 months (range from 1 to 82 months) after ablation.

27 out of 97 patients (27.8%) reported adverse events after ablation. Mild local pain was the most common side effect that occurred in 17 patients (17.5%). Slight swelling of the neck can be seen in 13 patients (13.4%). 4 patients and 1 patient, respectively, experienced dysphonia and dysphagia. Most of these symptoms resolved within 3 days after treatment with oral analgesics, neurotrophic methycobal and dexamethasone. One patient suffered from dysphonia that resolved after 3 months.

Residue was found in 30 out of 107nodules (28.0%). Among them, 2 patients received a second ablation 3 and 5 months respectively after the initial ablation and reached complete ablation. The principle of supplementary ablation was based on patient's desire and presence of compression or cosmetic problems. The comparison of the two groups was presented in Table 2. The initial volume (19.7 ± 12.9 ml *vs* 7.9 ± 8.3 ml, P <0.001) was significantly larger in the residue group than that in the non-residue group. 76.7% (23/30) of the nodules in the residue group had

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TABLE 1 The basic and ultrasound characteristics.

Variables	Data
Clinical parameters	
No. of patients/nodules	97/107
Age (years)	40.78 ± 12.61
Sex (F/M)	90/17(84.11/15.89)
Thyroid function	
Euthyroidism/Hyperthyroidism	94/3(96.91/3.09)
Ultrasonic parameters	
Location	
Right lobe/Left lobe/Isthmus	51/52/4(47.66/48.60/3.74)
Largest diameter (cm)	3.38 ± 1.23
Initial volume (ml)	11.20 ± 11.11
Proportion of cystic component	0.41 ± 0.27
Septum No/Yes	57/50(53.27/46.73)
Type A/B/C/D/E	40/10/26/14/17(37.38/9.35/24.30/13.08/15.89)
Vascularity	
Grade 1/2/3/4	13/44/27/23(12.15/41.12/25.23/21.50)
Echogenicity of cystic component Echoless/mixed echo	64/43(59.81/40.19)
Arterial phase enhancement hypo-/iso-/hyper-enhanced	5/91/11(4.67/85.05/10.28)
Venous phase enhancement hypo-/iso-/hyper-enhanced	30/70/7(28.04/65.42/6.54)

Data are presented as mean ± SD or number of nodules (percentages).

septum, while only 44.2% (34/77) in the non-residue group had septum (P=0.002). The ablation time and energy were also statistically related to residues. Longer ablation time and larger ablation energy were related with higher possibility of residue. This is because when the initial volume of the nodule was larger, the operators would decide to ablate for longer time with larger energy. Because we aimed to construct a pre-ablative model for better decision of treatment before ablation, we did not select ablation time and energy as independent factors in our model. For other parameters collected in this study, there were no significant difference between the two groups (all P >0.05).

Univariate and multivariate binary logistic regression analyses were conducted to identify factors associated with the residue of PCTNs after ablation. After univariate logistic regression analysis, the parameters with P<0.05 were selected into multivariate logistic regression analysis. As shown in Table 3, initial volume (P<0.001, OR=1.10) and septum (P=0.040, OR=2.97) were independent factors significantly associated with residue of PCTNs. Based on these two factors, a nomogram was established to predict the residue of PCTNs (Figure 4). Each factor had a corresponding score on the uppermost axis and the two scores were summed up to get a total point. Its location on the axis of the total point suggested a predicted residue rate. The p-value of the Hosmer–Lemeshow test was 0.639 >0.05 and indicated that the model fits well. A calibration curve was shown in Figure 5, which was closely fitted to the diagonal dotted line. It means the modal had a good ability of prediction. The C-index of the model was 0.832, equal to the AUC of the ROC curve (Figure 6). The optimal cutoff value by Youden method was 0.2 with a sensitivity of 86.7% and a specificity of 68.8%. Additionally, we performed internal stratified fivefold cross-validation to demonstrate the generalizability of the model (AUC =0.822, 0.750, 0.833, 0.900, 0.800; mean AUC=0.821). According to the decision curve analysis (DCA) of the model (Figure 7), we can see that at a risk threshold of 0.07-0.75, the net benefit of applying the nomogram was more than “treat all” and “treat none”, implying that the nomogram had a considerable clinical usefulness.

Discussion

In our study, we found that the initial volume and presence of septum inside the nodule were independent factors associated with residue of PCTNs after ethanol and thermal ablation. Then we

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TABLE 2 The comparison between the non-residual and residual groups.

Variables	Non-residual group	Residual group	*P* value
Pre-treated parameters			
Age (years)	41.2 ± 12.4	39.6 ± 13.3	0.492
Sex (Male/Female)	12 (15.6%)/65 (84.4%)	5 (16.7%)/25 (83.3%)	1.000
TSH (uIU/mL)	1.6 ± 1.2	1.3± 1.0	0.101
FT3 (pmol/L)	4.8 ± 0.6	5.1 ± 1.4	0.317
FT4 (pmol/L)	11.4 ± 2.0	12.1 ± 4.7	0.854
Initial volume(ml)	7.9 ± 8.3	19.7 ± 12.9	<0.001
Adjacent critical structures (No/Yes)	5 (6.5%)/72 (93.5%)	1 (3.3%)/29 (96.7%)	0.865
Septum (No/Yes)	43 (55.8%)/34 (44.2%)	7 (23.3%)/23 (76.7%)	0.002
Non-segregated type(type A/B)	36 (83.7%)/7(16.3%)	4 (57.1%)/3(42.9%)	0.262
Segregated type (type C/D/E)	19 (55.9%)/7(20.6%)/8(23.5%)	7 (30.4%)/7(30.4%)/9(39.1%)	0.165
Proportion of cystic component	0.4 ± 0.3	0.4 ± 0.2	0.981
Echogenicity of cystic component (Echoless/mixed echo)	50 (64.9%)/27 (35.1%)	14 (46.7%)/16 (53.3%)	0.083
Vascularity(Grade 1/2/3/4)	12 (15.6%)/32 (41.6%)/ 16 (20.8%)/17 (22.1%)	1 (3.3%)/12 (40.0%)/ 11(36.7%)/6 (20.0%)	0.181
Arterial phase enhancement(hypo-/iso-/hyper-enhanced)	4 (5.2%)/67 (87%)/ 6 (7.8%)	1 (3.3%)/24 (80.0%)/ 5 (16.7%)	0.458
Venous phase enhancement(hypo-/iso-/hyper-enhanced)	24 (31.2%)/49 (63.6%)/ 4 (5.2%)	6 (20.0%)/21 (70.0%)/ 3 (10.0%)	0.395
Ablation-related parameters			
Hydrodissection (No/Yes)	41 (53.2%)/36 (46.8%)	15 (50.0%)/15 (50.0%)	0.763
Ablation type(RFA/MWA)	71 (92.2%)/6 (7.8%)	25 (83.3%)/5 (16.7%)	0.316
Ethanol ablation(No/Yes)	56 (72.7%)/21 (27.3%)	21 (70.0%)/9 (30.0%)	0.778
Ablation time(min)	13.2 ± 8.5	23.5 ± 10.2	<0.001
Ablation energy(kJ)	31.2 ± 20.6	54.5 ± 24.8	<0.001

Continuous variables were presented as mean ± SD and categorical variables were reported as frequencies (percentages).

TABLE 3 Multivariate logistic regression analysis of predictors of residue of PCTNs after ablation.

Variables	OR (95%CI)	*P* Value
Initial volume(ml)	1.10 (1.05-1.16)	<0.001
Septum	2.97 (1.05-8.41)	0.040

developed a nomogram for predicting the probability of residue based on the two pre-ablative factors. The nomogram had good calibration, discrimination and clinical utility with an AUC of 0.832, indicating that it can identify patients with high risk of residue before therapy and improve treatment strategies. For the high-risk nodules, we should take aggressive ablation plan and intensive follow-up or even suggest for surgery.

Previous studies on efficacy of ablation of benign thyroid nodules focused on the volume reduction of treated nodule (23–25), probably because the main purpose of thermal ablation was to relieve compressive symptoms. Successful treatment was defined as a VRR≥50% after ablation. And regrowth was defined as an increase of the volume of the nodule≥50% of the minimum volume before ablation (12), which needs additional treatment. However, the nodule volume was influenced by aspiration of fluid and highly time-dependent. The rate of regrowth could increase with time until more than 5 years later (16, 18). This calls for long follow-up time to detect potential regrowth and cannot help time additional treatment early. In the study of Sim et al. (18), the total volume of nodule was composed of and affected by two parts according to US, the ablated volume (Va) and vital volume (Vv). During follow-up, Va would decrease gradually because of the absorption of necrotic tissue while Vv might increase due to regrowth. Therefore, the decrease of the nodule volume may consist of larger decrease of Va and smaller increase of Vv. Only tracing the change of total volume may miss an early sign of regrowth so the follow-up of Vv was more important in early detection of regrowth

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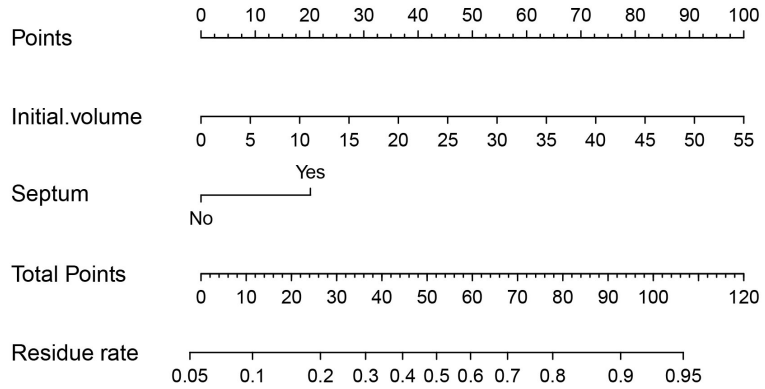

FIGURE 4
A nomogram to predict residue rate of PCTNs after ablation.

(3, 18, 26). The concept of Vv was consistent with the volume of residual vital tissue in our study. The widely used CEUS has a more reliable detection of residue (27) which was defined as enhanced area at ablative margin at one-month follow-up here. Zhao et al. found that nodules completely ablated had larger VRR compared to those incompletely ablated at 6-month follow-up, assessed with CEUS (28). Thus, in our study, we chose the presence of residual vital tissue as an outcome indicator.

In this study, we found that the larger the thyroid nodule was, the higher was the probability of residue after ablation. This finding was consistent with previous studies on predictive factors of efficacy of thyroid nodule ablation (15, 19, 29, 30). This was easy to understand because it was difficult to completely cauterize the margin of a large nodule in a single session. Meanwhile, the presence of septum was also associated with a higher residue rate. The presence of septum divided the nodule into several cysts containing fluid. Different from the cystic or solid nodules, the thermal ablation of PCTNs was conducted after simple aspiration of internal fluid with or without EA. Aspiration of fluid can not only greatly relieve the symptoms but was also helpful for a more effective ablation. For nodules with septum, it might be difficult to aspirate all the fluid completely. This fluid serves as a heat insulator, impeding the transmission of heat to all the parts of the nodule, especially those deeper than the septum. As a result, higher ablation power, longer duration and more release times of energy or multiple sessions were needed to achieve complete ablation. Thus the presence of septum was related to incomplete ablation or residue of the nodule regardless of the type of the septum. Moreover, we found that the adjacency to critical structures and vascularity were not associated to the residue of the PCTNs, which did not agree with a previous similar study (3). It was possibly because we adopted ethanol ablation as a combined therapy and hydrodissection to achieve a more complete ablation and avoid injury of the critical structures at the same time. Besides, our study focused on PCTNs so the heat-sink effect of vasculature was not so prominent and could be overcome by higher power.

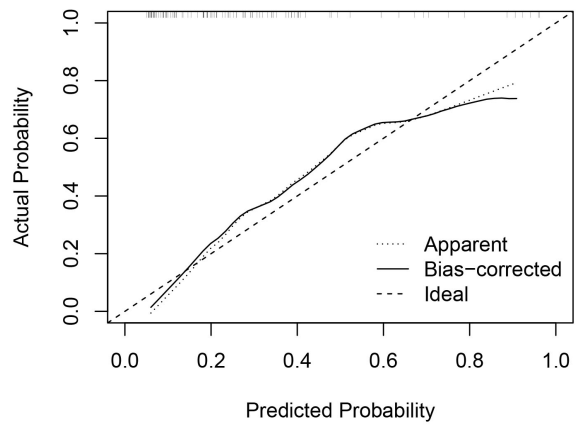

FIGURE 5
Calibration curve of the nomogram. The x axis represented the predicted probability of residue and the y axis represented the actual probability. The diagonal dashed line means an ideal model that can perfectly predict the residue. The solid line indicated the apparent predict performance of the nomogram.

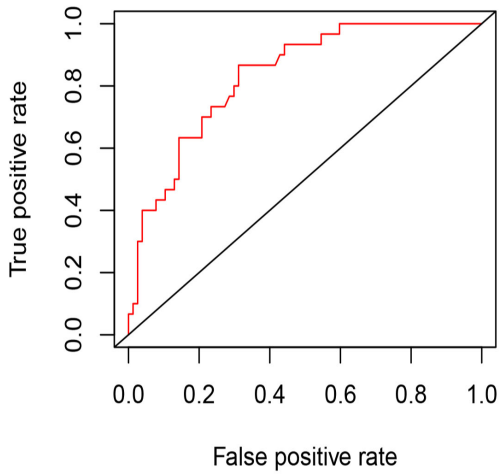

FIGURE 6
The ROC curve of the model for predicting residue of PCTNs after thermal ablation. AUC=0.832, optimal cutoff value=0.2 (specificity: 68.8%, sensitivity: 86.7%).

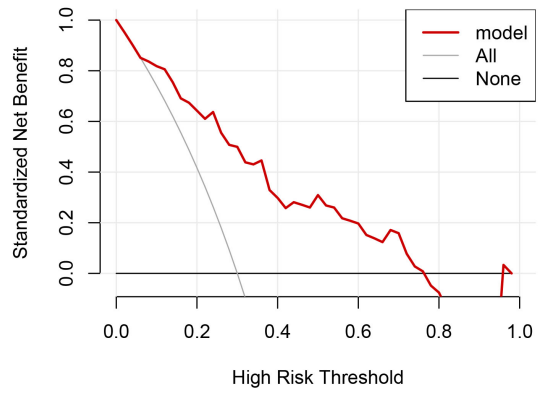

FIGURE 7
DCA of the model for predicting residue of PCTNs after thermal ablation.

There were several limitations in our study. First, it was a retrospective study. Second, this study was only conducted at single center with internal validation using stratified fivefold cross-validation. To assess the generalizability of the model more reliably, external validation was needed. Third, the follow-up time of some patients was short so we could not obtain the long-term VRR which was more convincing than short-term VRR to validate the correlation between short-term residue and long-term regrowth of nodules.

Conclusion

In this study, we developed a nomogram model for the pre-ablative prediction of residue of PCTNs undergoing ethanol and thermal ablation based on initial volume and presence of septum in the nodules. The model could discriminate patients with high or low residue possibility with good calibration. It was useful in the decision of treatment and follow-up for different patients in clinical practice.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by IEC for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

DL and XZ interpreted the data and wrote the manuscript. YZ produced figures and tables. TH, RZ and WZ collected the patient data and performed the statistical analysis. XX and MX designed the study and performed ablation procedure. DL and XZ contributed equally to this work and share first authorship. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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The length of FOXE1 polyalanine tract in congenital hypothyroidism: Evidence for a pathogenic role from familial, molecular and cohort studies

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Introduction: *FOXE1* is required for thyroid function and its homozygous mutations cause a rare syndromic form of congenital hypothyroidism (CH). *FOXE1* has a polymorphic polyalanine tract whose involvement in thyroid pathology is controversial. Starting from genetic studies in a CH family, we explored the functional role and involvement of *FOXE1* variations in a large CH population.

Methods: We applied NGS screening to a large CH family and a cohort of 1752 individuals and validated these results by *in silico* modeling and *in vitro* experiments.

Results: A new heterozygous *FOXE1* variant segregated with 14-Alanine tract homozygosity in 5 CH siblings with athyreosis. The p.L107V variant demonstrated to significantly reduce the FOXE1 transcriptional activity. The 14-Alanine-FOXE1 displayed altered subcellular localization and significantly impaired synergy with other transcription factors, when compared with the more common 16-Alanine-FOXE1. The CH group with thyroid dysgenesis was largely and significantly enriched with the 14-Alanine-*FOXE1* homozygosity.

Discussion: We provide new evidence that disentangle the pathophysiological role of FOXE1 polyalanine tract, thereby significantly broadening the perspective on the role of *FOXE1* in the complex pathogenesis of CH. FOXE1 should be therefore added to the group of polyalanine disease-associated transcription factors.

KEYWORDS

FOXE1, congenital hypothyroidism, athyreosis, polyalanine tracts, forkhead transcription factor, next generation sequencing

1 Introduction

Congenital hypothyroidism (CH) is one of the most common preventable causes of intellectual disability (1). In the last two decades, the refinement of the neonatal screenings toward lower blood TSH cutoffs brought the estimated incidence of CH from 1:4000 to 1:2000 (1–4), with significant variations depending on geographic location, ethnicity, gender and pregnancy conditions (3). CH can be due to either defects in thyroid organogenesis, collectively called thyroid dysgenesis (TD), or in thyroid hormonogenesis, called dyshormonogenic defects, in the presence of a normal or enlarged gland *in situ* (GIS) (3, 5). Consistent with such heterogeneity, CH was considered a puzzle of monogenic diseases, but the molecular mechanisms responsible for CH, particularly in TD, are still largely undefined (5, 6). Interestingly, the evidence of frequent oligogenic defects (6, 7) and the increased risk for CH in particular conditions (3, 4) proposed a more complex pathogenesis for CH, which might also explain the sporadic appearance of the disease in one family (5–8).

*Forkhead Box E*1 (*FOXE1)* is one of the candidate genes for TD, but its involvement in the CH pathogenesis has not been yet clarified. Homozygous point mutations in the DNA-binding domain (DBD) of this transcription factor are reported to be responsible for the rare Bamforth-Lazarus syndrome characterized by TD together with bifid epiglottis, cleft palate and spiky hair (9–11). FOXE1 is composed by highly conserved DBD, followed by a polyalanine (poly-Ala) tract of variable length and by a C-terminal disordered region of yet unknown significance (11, 12). During development and adult life, FOXE1 may act both as a pioneer transcription factor and as a co-regulator of the actions of other transcription factors through its DBD, poly-Ala domain or *via* its unstructured portion (13, 14). In particular, FOXE1 together with HEEX, NKX2.1 and PAX8 constitute a finely tuned system that regulates the expression of genes involved in thyrocyte precursors migration, differentiation and finally thyroid hormone production (13, 14).

Interestingly, some studies suggested a possible predisposing role for the FOXE1 poly-Ala tract in CH, but this is still highly controversial due to the limited size of the population studies or variable experimental conditions (12, 15–19).

Here, we bring new evidence about the role of *FOXE1* in thyroid pathogenesis. Our data, obtained from Next Generation Sequencing (NGS) genetic screening of a large family and of a large CH cohort, *in silico* modelling and *in vitro* experiments, indicate that variations in the *FOXE1* poly-Ala tract length may affect both its own transcriptional activity as well as its synergic action with PAX8 and NKX2-1 on the thyroglobulin (*TG*) promoter.

2 Materials and methods

2.1 Enrolment of the family

The family was enrolled by AKG who made the diagnosis and collected the clinical and biochemical data (Table 1). AKG obtained informed consent of the parents for genetic studies and publication of the family pictures (Figures 1A–F).

2.2 NGS patients database

Our database was composed of 1752 individuals analysed in our laboratory by NGS from 2015 to 2019. The CH patients were 299, 224 of which with complete clinical information available that allowed classification as GIS (66%) or TD (34%, of which 34% athyreosis, 39% ectopy, 27% hypoplasia); 1453 individuals without history and biochemical evidence of thyroid disease were used as control group. Written informed consent was obtained from all participants recruited in this study (Ethic Committee of Istituto Auxologico Italiano approvals 05C002_2010 and 05M001_2012).

2.3 Nextera rapid capture enrichment

A custom NGS panel covering all exons and adjacent intronic regions of the *DUOX2*, *DUOXA2*, *FOXE1*, *GLIS3*, *IYD*, *JAG1*, *NKX2-1*, *NKX2-5*, *PAX8*, *SLC26A4, SLC5A5*, *TG*, *TPO* and *TSHR* genes was designed using the GenomeStudio software (Illumina, San Diego, CA). NGS procedures and data analysis were performed as previously described (7). The total coverage of the target genes by the designed amplicons was 94%; these regions were covered at least by 20x. The uncovered sequences were amplified and sequenced by Sanger sequencing Big Dye Terminator Kit (Life Technologies) or by Nextera XT DNA Library Preparation kit (Illumina, San Diego, CA).

2.4 Bioinformatics analyses

NGS sequence data were aligned to the human reference genome (UCSC hg 19) and processed with MiSeq Reporter (Illumina) and wANNOVAR software. The visual inspection of the mapped data was performed using the Integrated Genomics Viewer 2.3 software (IGV; Broad Institute, Cambridge, MA, USA). The variants with the minor allele frequency (MAF) > 0.1% and annotated as benign in public or licensed databases (NCBI-dbSNP, NCBI-CliVar, Ensembl, GnomAD, ExAC Browser, NHLBI GO Exome Sequencing Project and HGMD professional, CLINVITAE) were excluded.

The different variants were analyzed according to ACMG/AMP 2015 guidelines (20) by the Varsome (https://varsome.com/) (21) and wIntervar (http://wintervar.wglab.org/) (22) bioinformatics tools for clinical interpretation of genetic variants together with the review of the scientific literature and 12 predictive software for interpretation of nonsynonymous variants (SIFT, Polyphen2_HDIV, Polyphen2_HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, MetaSVM, MetaLR, M-CAP, Fathmm-MKL).

2.5 Structural modelling

FOXE1 local secondary structure prediction was calculated with the software FELLS (23). The resulting plot shown in Figure 1

TABLE 1 Clinical and laboratory data in the Indian family.

	I-1 Father	I-2 Mother	II-3 CH	II-4 CH	II-7 CH	II-8 CH	II-9 normal	II-10 CH
Sex (M/F)	M	F	M	M	F	M	M	F
Age (years)	60	53	25	22	19	17	12	10
Weight (kg)	–	–	20	24	37	28	35	15
Height (cm)	–	–	100	113	122	124	142	98
Serum TSH at CH diagnosis (nv: 0.34-4.25 mU/L)	2.60	18.64	424.0	478.0	470.0	318.0	2.32	398.0
Total T4 at CH diagnosis (nv: 70-150 nmol/L)	–	–	21.5	15.8	18.4	25.5	–	25.3
Total T3 at CH diagnosis (nv: 1.2-2.1 nmol/L)	–	–	<0.1	<0.1	<0.1	<0.1	–	<0.1
Free T4 (nv: 8-16 pmol/L)	11.6	8.2	–	–	–	–	12.2	–
Free T3 (nv: 3.7-6.5 pmol/L)	5.2	4.0	–	–	–	–	6.1	–
Tg at CH diagnosis (nv: 13-118 µg/L)	–	–	5.3	3.2	4.3	5.8	–	6.4
Anti-TPO Abs (nv: <35kIU/l)	<10	<10	<10	<10	<10	<10	<10	<10
Anti-Tg Abs (nv: <40kIU/l)	–	–	<15	<15	<15	<15	–	<15
Thyroid US and scintiscan	–	GIS	Athy	Athy	Athy	Athy	–	Athy

CH, congenital hypothyroidism; nv, normal values; Tg, thyroglobulin; Athy, athyreosis; GIS, gland-in-situ.

indicates the probability of a defined secondary structure to be adopted by protein sequence residue.

Human FOXE1 structured region (residues 56-181) *in silico* model was obtained with AlphaFold (24) and superimposed to the experimental crystal structure of a homologue transcription factor bound to DNA (HNF-3/forkhead, PDB ID: 1VTN).

2.6 Cell line, mutagenesis and transfections

HEK293 (RRID : CVCL_0045) cells were grown in DMEM while NTHY-ORI 3-1 cells (RRID : CVCL_2659) were grown in RPMI-1640 medium, both supplemented with 10% fetal bovine serum and 1:100 penicillin-streptomycin (Life Technologies). They were cultured at 37°C in humidified 5% CO_2 environment and were routinely tested for Mycoplasma.

p3xFlag-CMV-7.1 *FOXE1* cDNA expression vectors were previously described (25), p.Leu107Val variant was introduced by site-directed mutagenesis with primers designed with NEBaseChanger version v1.3.0 software (Forward: 5'-CAACCTCACAGTCAACGACTGC, Reverse: 5'- TGGCGG ATGCTGTTCTGC). The mutagenesis was performed using NEB's Q5 Hot Start High-Fidelity Polymerase (cat. no. M0493S), the reaction was supplemented with 20% Q-solution (Qiagen) to facilitate amplification of FOXE1's GC-rich sequence.

Transient transfection was performed with Lipofectamine™ 2000 Transfection Reagent (Life Technologies) following manufacturer's instructions. All experiments were performed 24 hours post-transfection.

2.7 Western blotting

Cells were lysed with RIPA buffer (Sigma) supplemented with Complete Mini protease and phosphatase inhibitor cocktails (Roche). 5 µg of proteins were loaded on NuPAGE 4–12% Bis-Tris gel and transferred on Nitrocellulose membranes with iBlot system (Life Technologies).

After blocking in 5% milk-TBST, membranes were incubated overnight with primary antibodies anti-FLAG (M2, Sigma RRID: AB_259529) and anti-GAPDH (sc-25778, Santa Cruz Biotechnology RRID : AB_10167668).

After 1 hour incubation in the appropriate HRP conjugated secondary antibodies (Merck Millipore), detection was performed with ECL Star (Euroclone) with Azure Biosystem C400 camera. Densitometric quantification was performed with FIJI (RRID : SCR_002285) (26).

2.8 Immunofluorescence and confocal microscopy

Samples were fixed in 4% PFA for 10 minutes and permeabilized with 0.3% Triton-X/PBS for 5 minutes. After

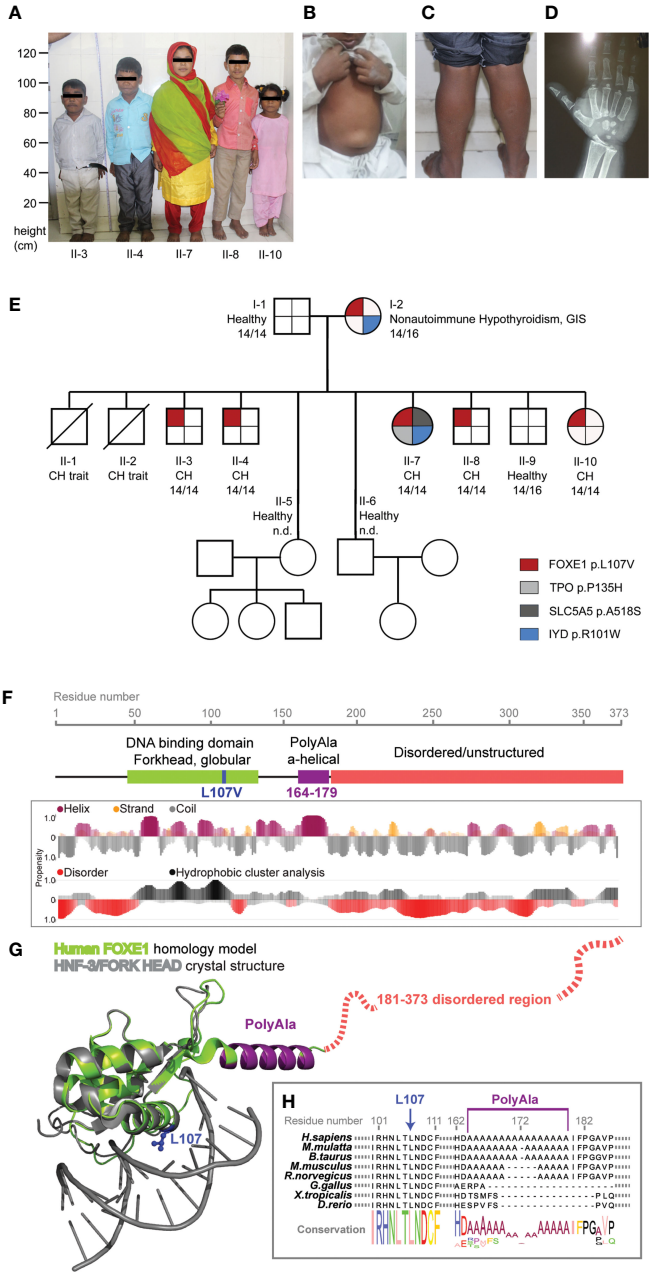

FIGURE 1
FOXE1 variants segregation in the family and protein structural modeling. **(A)** picture of the five affected siblings showing severe short stature, puffy facies with dry skin, sparse fragile hair, lateral madarosis and short round nose. **(B)** umbilical hernia in patient II-3. **(C)** calf muscle pseudo-hypertrophy in patient II-4. **(D)** X ray of left hand revealing delayed bone age and epiphyseal dysplasia in patient II-7. **(E)** family tree showing *FOXE1* and other genetic variants co-segregation with CH (14/14, Ala-14/14 FOXE1; 14/16, Ala-14/16 FOXE1; 16/16, Ala-16/16 FOXE1; n.d., not determined). **(F)** linear representation of FOXE1 sequence and local secondary structure prediction adopted by the FOXE1 aminoacidic residues calculated with the software FELLS. The plot indicates the probability of a defined secondary structure or disordered coiled to be adopted per sequence residue. **(G)** AlphaFold *in silico* model of the human FOXE1 structured region (residues 56-181), superimposed with the experimental crystal structure of a homologue transcription factor bound to DNA (HNF-3/forkhead, PDB ID: 1VTN). FOXE1 model shows a conserved DNA binding domain (green), followed by a poly-alanine alpha-helical region (purple) and by and unstructured disordered region (red dotted line). The relative position of L107 residue and polyalanine tract in respect to DNA binding suggest proximity but not apparent direct binding to DNA. **(H)** FOXE1 alignment showing the highly conserved L107 residue in the DNA binding domain and the relatively late evolutionary emergence of the polyalanine tract.

blocking with 5% donkey serum/PBS at RT for 20 minutes, samples were incubated at 37°C for 1 hour with 1:100 anti-FLAG M2 mouse monoclonal antibody (Sigma). Samples were incubated for 1 hour with 1:500 AlexaFluor-488 secondary antibody (Thermo-Fisher). Samples were mounted on microscope slides with Vectashield Hard Set with DAPI (DAKO). Images were acquired with Nikon EclipseTi-E inverted microscope with IMA10X Argon-ion laser System (Melles Griot). For FOXE1 expression pattern, DAPI was

used to determine ROI for further analysis and batch level thresholding was applied to the green channel originating a grayscale image utilized for further analysis. Each transfected cell was manually assigned to one of the 3 nuclear signal pattern categories. The analysis was performed in parallel by three independent operators (ESG, TdF and GR). All images processing and analysis were performed with FIJI (26).

2.9 Luciferase assay

FOXE1 activity was measured with the Dual-Luciferase Reporter Assay System (Promega).

250 ng of Luciferase reporter plasmid TG-Luc (27) were co-transfected with 80 ng of pRL-TK Renilla construct (Promega, Madison, Wisc., USA), 250 ng of *FOXE1* constructs, and/or 250 ng of NKX2.1, PAX8 (27), empty vector as indicated in Figure 2 and Supplementary Figure 1. 24 hours post transfection luminescence was measured with Dual Luciferase kit (Promega) with Fluoroskan Ascent FL multiplate reader.

2.10 Statistical analysis

All experiments were independently repeated at least three times, as indicated in the figure legends; data represent mean ± SEM. All analyses were performed with software R version 4.1.2. Contingency was evaluated by Chi-square, Chi-square test for trend and Fisher's exact test. For *in vitro* experiments, after normal distribution and variance similarity evaluation by Bartlett's test, one-way ANOVA followed by Bonferroni *post-hoc* test or Kruskal-Wallis test were applied.

3 Results

3.1 Clinical data

Five siblings of a large family presented to the endocrinology outpatient clinic with complaints of lethargy, constipation, short stature and intellectual disability. All children were born from a non-consanguineous marriage with uneventful pregnancies and

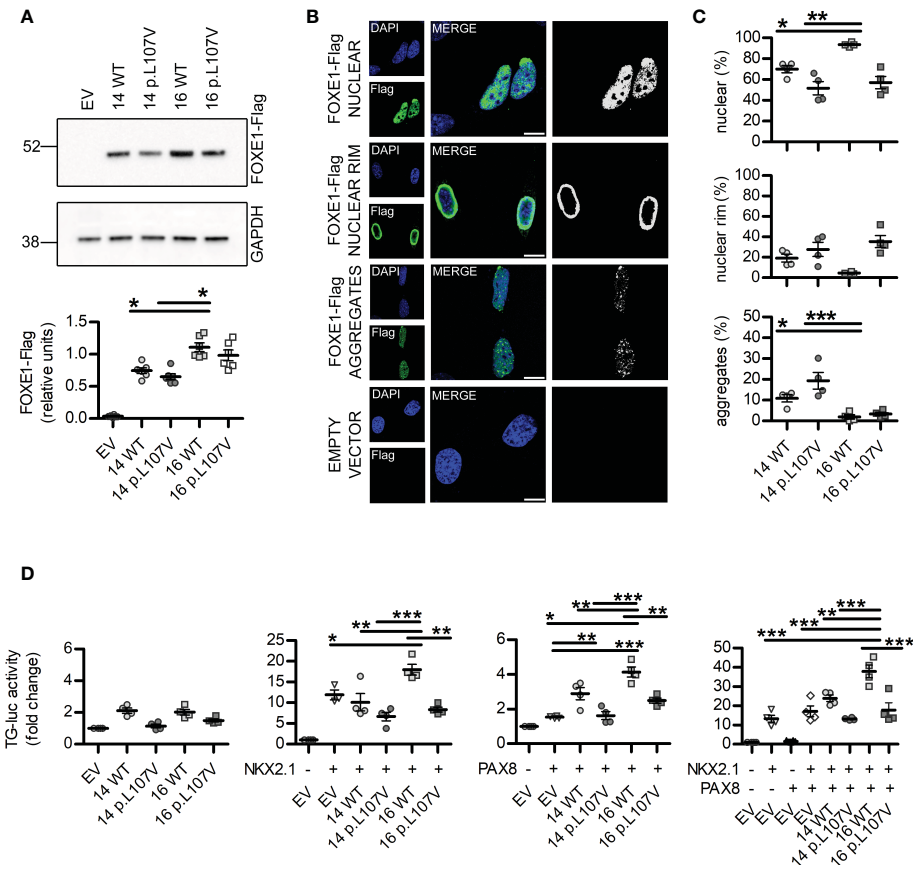

FIGURE 2
In vitro studies of FOXE1 variants reveal different subcellular localization and transcriptional activity. **(A)** representative images and quantification of western blot experiments showing FOXE1 variants expression levels in NTHY-ORI cells (n=6). **(B)** confocal microscopy images representative of the different FOXE1 nuclear morphologies in NTHY-ORI cells and corresponding signal thresholding images. **(C)** relative quantification of FOXE1 variants nuclear morphologies in NTHY-ORI cells (n=4; 1711 nuclei analyzed, of which 16 WT 295, 14 WT 258, 16 p.L107V 269, 14 p.L107V 295); scalebars 25µm. **(D)** functional assays showing FOXE1 variants activity with TG-luc reporter when *FOXE1* was transfected alone, with *NKX2.1, PAX8* or all together (n=4). EV, Empty vector. Statistical significance was determined with one-way ANOVA followed by Bonferroni *post-hoc* test **(A, D)** or Kruskal-Wallis **(C)**. *p<0.05, **p<0.01, ***p<0.001.

deliveries. All the affected siblings were reported to have had feeding difficulties and delayed developmental milestones. Family history revealed premature death of 2 additional siblings at the ages of 2 and 32 years for infectious diseases, but with complaints similar to the CH affected siblings. The remaining 3 out of the 10 siblings had no stigmata of thyroid disease.

On general examination all the affected siblings had typical signs of untreated severe CH (Figures 1A–D). The five siblings presented extremely short stature, typical puffy hypothyroid facies with cold dry skin, sparse fragile hair, lateral madarosis, short round nose (Figure 1A), umbilical hernia (Figure 1B), calf muscle pseudo-hypertrophy (Figure 1C) macroglossia, myxedema, hoarseness of voice, delayed relaxation of deep tendon reflexes, pseudomyotonia and bradycardia. X ray examination revealed delayed bone age and epiphyseal dysplasia (Figure 1D). Thyroid was not detectable at neck palpation or ultrasonography as well as on neck/mediastinum ^{99}Tc scintiscan. Biochemical investigations revealed extremely high level of TSH along with markedly low T4, T3 and Tg. Anti- thyroid peroxidase (Anti-TPO) and Anti-TG antibodies were negative (Table 1). Lab tests also showed normal hemogram, kidney and liver function. Electrolytes, LH, FSH and Prolactin were in the normal range for age.

Maternal laboratory testing revealed high TSH, negative anti-thyroid antibodies and normally located thyroid gland on ultrasonography. Father's thyroid function tests were all normal.

All five siblings were diagnosed with severe CH associated with athyreosis and started on thyroxine replacement and dose titrated by periodic laboratory investigation.

3.2 NGS sequencing analysis revealed co-segregation of the CH phenotype with FOXE1 variants

The NGS sequencing of all available members revealed genetic alterations only in the affected patients: the mother carried a *IYD* variant (NC_000006.12(NM_203395.3):c.301C>T, rs121918138) that was transmitted to only one affected daughter. This same patient had two additional *de novo* variants in *SLC5A5* (NC_000019.10 (NM_000453.3):c.1552G>T, rs147583297) and *TPO* (NC_000002.12(NM_000547.6):c.404C>A, rs61758083) (Figure 1E). The *IYD* variant had previously been described (28) and classified as pathogenic while the ones identified in *TPO* and *SLC5A5* are rare variants annotated as of unknown significance according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines (20) (Supplementary Table 1).

The mother and the five affected siblings were all found to carry a novel heterozygous *FOXE1* variant (Figure 1E) [NC_000009.12 (NM_004473.4):c.319C>G, p.Leu107Val].

A subsequent in-depth analysis of the *FOXE1* gene revealed an association between the p.Leu107Val variant, the polyalanine region length of 14 alanines (Ala-14) and the CH phenotype in the family. The homozygous Ala-14 and the heterozygous p.Leu107Val *FOXE1* variants were present in all the five siblings with athyreosis. The hypothyroid mother carried the Ala-14/p.Leu107Val combined with the Ala-16 wild-type allele, while the euthyroid father carried the homozygous Ala-14 *FOXE1* but no additional variants in the CH candidate genes.

Moreover all the family members carried the SNP (NC_000009.12:g.97786731A>G rs7850258) in homozygosity, previously described as predisposing factor for CH (29).

3.3 *In silico* analysis suggests a possible effect of the p.Leu107Val and the Ala-14 *FOXE1* variants

I*n silico* predictions classified the p.Leu107Val variant as possibly pathogenic (Supplementary Table 1).

Using AlphaFold (24) we predicted the structure of FOXE1 (Figures 1F–H) and superimposed it with the experimental structure of a transcription factor of the same Forkhead family, bound to DNA (PDB ID: 1VTN). In the predicted FOXE1 model by comparison to the reference structure bound to a short linear DNA fragment, the highly conserved L107 appears located in a hydrophobic pocket relatively distant (about 7 Å) from DNA to be directly involved in its binding, as also the alpha-helical polyalanine tract. The same L107 structural position is occupied by a phenylalanine in the reference structure within a highly hydrophobic protein region. We hypothesize that the replacement of leucine with a similarly hydrophobic but shorter valine side chain, could potentially affect the folding of FOXE1, perhaps destabilizing it locally and impacting the transcriptional activity (Figures 1F–H). These alterations may become more pronounced in combination with variations of the polyAla tract in proximity of the DBD. Indeed, it has been observed that the amplifications of polyalanine tracts that are acquired late in evolution as *FOXE1* ones (Figure 1H) may have a fine, yet unclear transcriptional regulatory role (30, 31).

It has to be considered that many pioneer transcription factors as FOXE1 bind to non-linear DNA on nucleosomes and have large disordered regions. Our analysis is limited to short DNA linear fragments present in the Protein Data Bank and we don't consider the highly C-terminal disordered region for which is impossible to reliably predict. Nonetheless, the current *in silico* analysis is important to highlight the non-obvious and complex molecular role of Poly-Ala tracts and L107V variants, which deserve finer molecular investigations in the future.

3.4 *In vitro* studies revealed negative effects of the p.Leu107Val and the Ala-14 *FOXE1* variants

We moved to *in vitro* experiments to evaluate possible alterations in FOXE1 expression and functionality. We performed transient transfection experiments on HEK293 and NTHY-ORI 3-1 cells with *FOXE1* either with Ala-14 or Ala-16, each one with (14 p.L107V, 16 p.L107V) or without (14 WT, 16 WT) the p.Leu107Val variant.

Western blotting experiments did not reveal variations among the different FOXE1 variants in HEK cells (Supplementary Figure 1)

but showed a reduced expression of the Ala-14 FOXE1s when compared to the Ala-16 ones in the thyrocyte-derived NTHY-ORI cells (Figure 2A). These differences among the two cell lines are probably due to their different origins. NTHY-ORI are the only available cell line of immortalized human thyrocytes and may provide a more accurate model for thyroid transcription factors studies. Though they exhibit a partial loss of differentiation due to adherent cell culturing conditions, they possess the adequate biological machinery for FOXE1 expression and functionality and for this reason are probably more sensitive than HEK cells to FOXE1 alterations.

Confocal microscopy experiments indicated that the length of the polyalanine tract and the presence of p.Leu107Val variant can influence FOXE1 nuclear localization. In all the different FOXE conditions we identified three main different patterns, evenly diffuse nuclear signal (nuclear), uneven nuclear signal with significantly higher intensity at nuclear rim (nuclear rim), and the presence of nuclear puncta aggregates (aggregates) (Figures 2B, C, Supplementary Figures 1B, C, 2).

The quantification of the different patterns revealed that while the Ala-16 WT protein has significantly higher proportion of cell that display an evenly diffuse nuclear pattern, the Ala-14 WT is more prone to form nuclear aggregates. The presence of the p.Leu107Val variant significantly reduced the percentage of cells with even nuclear signal while increasing the frequency of nuclear aggregates and nuclear rim in both poly-Ala backgrounds (Figures 2B, C, Supplementary Figures 1B, C).

Functional assays performed in NTHY-ORI cells revealed that the different FOXE1 variants have variable activities on the TG promoter (Figure 2D). First of all, when FOXE1 is expressed alone, the Ala-14 and Ala-16 have similar activity, and this is negatively affected by the presence of the p.Leu107Val variant. As during thyroid development and adult life FOXE1 is concomitantly expressed with PAX8 and NKX2.1 and it is expected to modulate their transcriptional activity (13), we performed different co-transfection experiments. Under these conditions, Ala-14 and Ala-16 FOXE1s have significantly different activities. In particular, only the Ala-16 FOXE1 significantly enhances NKX2.1 transcriptional activity, while PAX8 can be induced by both Ala-16 and Ala-14 FOXE1s, although the latter with significantly lower efficiency (Figure 2D). Moreover, when the three transcriptional factors are co-expressed, a significantly higher activity is detected only in the presence of the Ala-16 FOXE1 (Figure 2D). The introduction of the p.Leu107Val variant decreased the transcriptional activity in all the different experimental settings (Figure 2D).

Altogether these data indicate that both the presence of 14 alanines and p.Leu107Val variant in the DBD may negatively affect FOXE1 expression patterns and functionality, further supporting their role in the development of congenital hypothyroidism.

3.5 FOXE1 heterozygous variants and polyalanine region role in CH predisposition

We then evaluated the distribution of the different poly-Ala *FOXE1* tracts in 299 CH patients and 1453 controls.

We identified nine different alleles generating sixteen different combinations of genotypes present in controls and CH groups (NCBI refSNP: rs71369530). In both groups the most frequent alleles were Ala-14 and Ala-16 (Supplementary Table 2), but the Ala-14/14 homozygous genotype was predominant in CH patients, either in absolute numbers and in percentages (Supplementary Table 2, Figure 3A), with a highly significant X^2 test for trends ($p<0.0001$). The allelic combinations different from Ala-14/14, Ala-14/16 and Ala-16/16 represented less than 5% of cases and were not included in further evaluations.

The Ala-14/14 genotype showed a significant association with CH, when compared to Ala-14/16 (odds ratio (OR) 2.031), Ala-16/16 (OR 2.645) as well as to the sum of the latter ones (OR 2.360) (Table 2).

Next, we investigated the distribution of the poly-Ala FOXE1 alleles in the different CH subgroups, TD and GIS. Although the Ala-14/14 was the most frequent genotype in both CH subgroups (Figure 3B), it was significantly associated with TD (OR 3.909 vs Ala-16/16) (Table 2). No significant differences in the poly-Ala tract distribution were detected among the three TD subtypes (Figure 3C). At last, among the few patients with FOXE1 heterozygous mutations that we previously identified (7) only the Ala-14/14 genotype was associated with athyreosis (Supplementary Table 3).

4 Discussion

In this study, we report for the first time that a novel heterozygous point mutation (p.Leu107Val) affecting *FOXE1* DBD may be sufficient to cause thyroid dysgenesis and CH only when associated with homozygous Ala-14-*FOXE1*. The analysis of our large NGS cohort revealed a significant enrichment of the biallelic Ala-14-*FOXE1* genotype in CH, and particularly in TD. In agreement with this data, functional studies showed a significantly impaired transcriptional activity of the p.Leu107Val variant. At variance with the more common Ala-16-*FOXE1*, the 14-Alanine isoform was also found to modify the expression pattern and localization of the transcription factor and to significantly impair the synergic effects that FOXE1 has on the transcriptional activities elicited by NKX2.1 and PAX8.

This study started after the observation that the heterozygous *FOXE1* variant p.Leu107Val segregated with severe CH and athyreosis in 5 siblings of our family (Figure 1E). Interestingly, our structural mapping indicates that Leu107 is not in direct contact with DNA. In addition, despite the p.Leu107Val introduces a conservative aminoacidic change in the DBD, we propose that it could modify the interaction with the surrounding hydrophobic regions, indirectly compromising protein stability and affecting its transcriptional functionality (Figures 1F, G). All the CH siblings had the same phenotype indicating a minor, if any, pathogenic role for the additional rare variants identified in only one of these patients. The p.Leu107Val variant was inherited from the mother, who presented a gland-*in-situ* with adult-onset nonautoimmune hypothyroidism. This may suggest that, although contributing to alterations in thyroid functionality, this variant is not sufficient *per se* to cause CH. We thus focused on other genetic variants that were

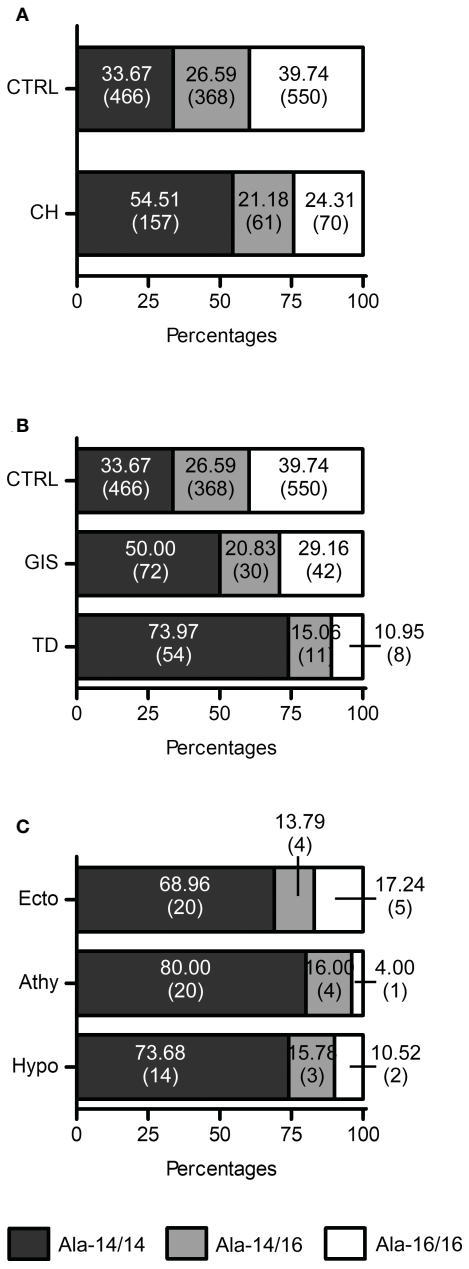

FIGURE 3
FOXE1 polyalanine genotypes distribution indicates a role of Ala-14/14 in CH and TD. **(A)** Graphical representation of the distribution of the Ala-14/14, Ala-14/16 and Ala16/16 *FOXE1* genotypes among control group (CTRL) (n=1384) and CH cases (n=288); **(B)** graphical representation of *FOXE1* main genotypes distribution among control group (n=1384), GIS (n=144) and TD (n=73) cases. **(C)** graphical representation of FOXE1 main genotypes distribution among TD subgroups (Ecto, ectopy, n=29; Athy, athyreosis n=25, Hypo, hypoplasia, n=19). Contingency was evaluated by Chi-square, Chi-square test for trend and Fisher's exact test and is reported in the results and discussion section of the article. CH mut, patients with mutations in CH genes.

present in the 5 affected siblings and found the recurrence of homozygosity for Ala-14 *FOXE1*. This genotype is also present in the euthyroid father, but not in the mother who is carrier of the heterozygous Ala-14/16 *FOXE1*. The co-segregation of an additional non-FOXE1 defect together with the FOXE1 p.L107V variant in all the 5 athyreotic siblings has a low likelihood and this probability would be further lowered if we consider the other two siblings with a CH-like phenotype that died before this study. For these reasons, we propose the concomitant inheritance of the p.L107V variant and the 14-Alanine stretch, together with the CH predisposing background of the family indicated by FOXE1 SNP rs7850258, represents the most likely explanation for the athyreotic phenotype of the 5 siblings.

Variations in the poly-Ala tract length of several nuclear transcription factors emerged late in evolution and were shown to play a fundamental role in their activity (30–32). Accordingly, the poly-Ala tract of FOXE1 is present only in mammals (Figure 1H). Interestingly, FOXE1 was reported to affect the transcriptional activity of other thyroid transcription factor, such as NKX2.1 (13, 14). NKX2.1, together with HHEX, PAX8 and FOXE1 strictly regulates in a spatial and temporal manner the complex multiphase process of thyroid development. How these actors interact to finely tune the thyroid function is far to be understood, but they are required for the adequate expression of genes involved in thyrocyte precursors migration, differentiation, proliferation and finally thyroid hormone production (33, 34).

Our data indicate that, although both Ala-14- and Ala-16-*FOXE1* alleles are common in the general population, the two variants have a different modulatory activity on the complex transcription factors network that regulates thyroid development and functionality (Figure 2D). From *in silico* structural analysis based on models bound to short DNA fragments, the FOXE1 polyAla predicted helix, although close to the DBD, seems not directly involved in DNA binding (Figure 1H), at least considering short DNA linear fragments. Its molecular impact on transcriptional activity, together with that of the large disordered region, is far to be solved at molecular level. Nonetheless, in our cellular studies we report that variations in the poly-Ala length cause alterations in the protein expression and nuclear localization. From here, we hypothesize that the poly-Ala may partially mediate the aggregation or local concentration in the nucleus, by potentially binding and recruiting other transcription factors. These two actions were previously described for other transcription factors containing poly-Ala regions (32, 35, 36) and we propose this might be also the case for FOXE1. Our experiments confirm that functional differences between Ala-16- and Ala-14-FOXE1 become evident and significant only when these isoforms are co-expressed with the other thyroid transcription factors. Notably, when Ala-14-FOXE1 is expressed together with NKX2.1 and/or PAX8, the transcriptional activation of TG promoter is significantly lower than that seen with Ala-16-FOXE1 (Figure 2D).

Although the homozygous Ala-14-*FOXE1* genotype significantly increases the risk of TD, as shown by our CH cohort (Figure 3B), variations in FOXE1 polyalanine repeats are not sufficient to induce CH *per se*, as around one third of the healthy population has the homozygous Ala-14 tract (Figure 3A) (15, 17, 19, 37). Nevertheless, in the few CH patients that have heterozygous

TABLE 2 Association of poly-Ala-FOXE1 isoforms with CH and TD.

Polyalanine alleles frequencies in healthy subjects and CH patients					
FOXE1	CH (n)	Healthy Subjects (n)	Fisher's exact test	Relative Risk (95% CI)	Odds ratio (95% CI)
Ala-14/14	157	466	–	–	–
Ala-14/16	61	368	< 0.0001	1.772 (1.359 to 2.323)	2.031 (1.453 to 2.865)
Ala-16/16	70	550	< 0.0001	2.232 (1.727 to 2.893)	2.645 (1.928 to 3.625)
Ala-14/16 and 16/16	131	918	< 0.0001	2.018 (1.636 to 2.487)	2.360 (1.809 to 3.080)
Polyalanine alleles frequencies in TD and GIS patients					
	TD (n)	GIS (n)	Fisher's exact test	Relative Risk (95% CI)	Odds ratio (95% CI)
Ala-14/14	54	72	–	–	–
Ala-14/16	11	30	0.096	1.597 (0.970 to 2.821)	2.037 (0.895 to 4.926)
Ala-16/16	8	42	0.0008	2.679 (1.445 to 5.282)	3.909 (1.639 to 10.448)
Ala-14/16 and 16/16	19	72	0.0008	2.053 (1.334 to 3.240)	2.829 (1.479 to 5.580)

CH, congenital hypothyroidism; HS, Healthy Subjects; TD, Thyroid Disgenesys; GIS, gland-in-situ.

FOXE1 variants, athyreosis was present only in the patient with Ala-14/14 genotype, while the Ala-14/16 and 16/16 genotypes were associated with GIS and hypoplasia (Supplementary Table 3). Moreover, reports associating *FOXE1* poly-Ala variations with low fT4 levels (38, 39) indicate that the Ala-14 allele may favor the onset of hypothyroidism in combination with other genetic, epigenetic and environmental factors, in the context of a complex origin of CH (6, 7).

In conclusion, the NGS analysis of a large family affected with CH together with the experimental and association studies indicate that homozygous Ala-14-*FOXE1* genotype may contribute to the complex pathogenesis of TD and CH, particularly when combined with heterozygous loss-of-function FOXE1 variant. Therefore, we propose that from now on, the status of *FOXE1* polyalanine tract should be taken in consideration when investigating the genetic origin of CH patients.

Hence, then propose to include *FOXE1* in the group of transcription factors linked to a disease associated with variations of their polyalanine tract (31–33).

Further molecular and cellular studies are needed to elucidate the possible regulatory role of FOXE1 domains other than the DBD and fully understand its role in thyroid development and function.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: Harvard Dataverse, https://doi.org/10.7910/DVN/KGUPED. Data was collected from human subjects who provided informed consent for its use in the original study, but did not consent for its release to the public. Data access can be required to MD LP (luca.persani@unimi.it), upon reasonable request.

Ethics statement

The studies involving human participants were reviewed and approved by Istituto Auxologico Italiano. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

AG enrolled the CH family and provided clinical and biochemical data. LP conceived and supported the study. DG, EC, and TF performed the bioinformatical analysis of NGS data. EG, GR, SU, TF, MB, and RC-B performed the *in vitro* experiments. FC performed the *in silico* predictions and analysis. EG, GR, and LP interpreted the data; EG, GR, AG, TF, MB, and FC contributed to draft of the manuscript; EG and LP revised the draft and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1127312/full#supplementary-material

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Emerging trends and hot spots in subacute thyroiditis research from 2001 to 2022: A bibliometric analysis

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Background: Subacute thyroiditis (SAT) is the most prevalent self-limiting thyroid disease that causes pain, accounting for about 5% of all clinical thyroid disorders. Numerous clinically noteworthy results have been published in this area over the last 20 years. However, no article has comprehensively assessed the relevant literature yet. We conducted a bibliometric analysis of SAT to provide light on the dynamic nature of scientific advancement and aid researchers in gaining a global perspective while examining research core themes and hotspots.

Methods: SAT-related articles and reviews from 2001 to 2022 were retrieved from the Science Citation Index-Expanded of Web of Science Core Collection (WoSCC). We analyzed current research trends and hotspots in this area using CiteSpace and Vosviewer.

Results: A total of 568 studies associated with SAT research were published in 282 academic journals by 2,473 authors in 900 institutions from 61 countries/regions. The United States was a crucial link in inter-country/region collaboration and was the most frequently involved country in international cooperation. The University of Missouri System was the top organization, and Braley-Mullen H. was the most productive researcher. *Thyroid* published the most papers, with 36 publications. The most co-cited article was "Clinical features and outcome of subacute thyroiditis in an incidence cohort: Olmsted County, Minnesota, study" (by Fatourechi V., 2003). The clustered network and timeline view of keywords showed that the prevalence, diagnosis, and treatment of SAT were the research core themes during the past 20 years. Analysis of keyword bursts indicated that the clinical characteristic and the influence of COVID-19 on SAT appeared to be the current research hotspots.

Conclusion: This bibliometric analysis conducted a thorough review of the SAT research. The clinical characteristics and the genetic background of SAT under the influence of COVID-19 are current research hotspots. However, there is still a need for further study and global collaboration. Our findings can aid researchers in understanding the current status of SAT research and immediately pinpoint new directions for further investigation.

KEYWORDS

subacute thyroiditis (SAT), bibliometric analysis, CiteSpace, VOSviewer, hot spots

1 Introduction

Subacute thyroiditis (SAT) is the most prevalent self-limiting thyroid disease that causes pain, accounting for about 5% of all clinical thyroid disorders (1). Most patients are middle-aged, and women are four to seven times more likely than males to have the condition (2, 3). SAT manifests as anterior neck pain and tenderness during the physical examination with various systemic symptoms, including fever, chills, palpitation, weight loss, and malaise (4), typically characterized by three clinical processes: thyrotoxicosis, hypothyroidism, and return to normal thyroid function. Since SAT is a self-limiting disease, nonsteroidal anti-inflammatory medications (NSAIDs) and corticosteroids are suggested therapies to treat the signs and symptoms and lessen inflammation (5, 6). However, even among properly treated patients, the recurrence rate for SAT is relatively high, varying from 1.6% to more than 20%, which is associated with the presence of specific types of human leukocyte antigens (HLA) (7). High recurrence rates and prolonged treatment time have become severe problems in treating SAT (8).

Although the cause and pathogenesis of SAT have long been unknown, viral infection or allergic reaction following viral infection are frequently implicated. The onset of SAT is related to several viruses, including the Coxsackie virus, Echovirus, influenza virus, parvovirus B19, mumps, rubella virus, HIV, Epstein-Barr virus, hepatitis E, measles, and dengue virus (9, 10). Since the winter of 2019, the novel coronavirus (COVID-19) outbreak has spread globally. As the pandemic spreads, there is more and more proof that SAT and SARS-CoV-2 infection are related. Numerous SAT cases involving COVID-19 infection or vaccination have already been published (11–13). However, it remains an open question whether SAT is a complication of COVID-19 or a side effect of vaccination based on available research data.

Over the past two decades, there have been many published research findings on SAT. New methods for reviewing and analyzing trends are required because of the literature's increasing growth. Bibliometrics is a method for comprehensively assessing a research field that measures scientific data distribution, traits, and regulations from various angles, displaying the field's macro knowledge structure and development trend (14). No bibliometric study has been reported to evaluate the associated literature comprehensively, summarize the latest trend, and predict research hotspots of SAT. Our study aims to analyze research core themes, explore the frontier issues of SAT from 2001-2022 and provide a comprehensive perspective and guidance for other researchers.

2 Methods

2.1 Data sources and search strategies

We thoroughly searched the Science Citation Index-Expanded (SCI-E) of Web of Science Core Collection (WoSCC) database for the period 2001–2022. All searches were finished and independently verified by two authors on December 31, 2022, in order to remove the bias introduced by daily database changes. The selection criteria were as follows: (1) timespan: 2001.01.01–2022.12.31; (2) document type: article or review; (3) language: English. The details of the search strategy are presented in Figure 1.

2.2 Bibliometric analysis

For further analysis and to describe all aspects of the literature about SAT research, we converted all WoSCC data that complied with requirements to TXT format and imported it into CiteSpace (V6.1 R6 Basic) and VOSviewer (V1.6.18). CiteSpace is a freely available program written in Java for visualizing and examining trends and patterns in scientific publications (14). The version of this software is constantly updated, and the latest version V6.1R6 Basic was used in this study. CiteSpace was utilized in this study to analyze and display data on the annual growth patterns of publication outputs, countries/regions, institutions, authors, and co-cited authors, the occurrence of keywords, and reference burst. VOSviewer, a Java-based bibliometric mapping program created by Leiden University, excels in handling sizable bibliometric maps based on network data and displaying scientific knowledge (15). Vosviewer was used to study and visualize the analysis of co-cited references, journals, and co-cited journals.

3 Results

3.1 Annual growth trend of publications

Following the criteria for data selection, 568 SAT studies were found in WoSCC between 2001 and 2022, including 468 original

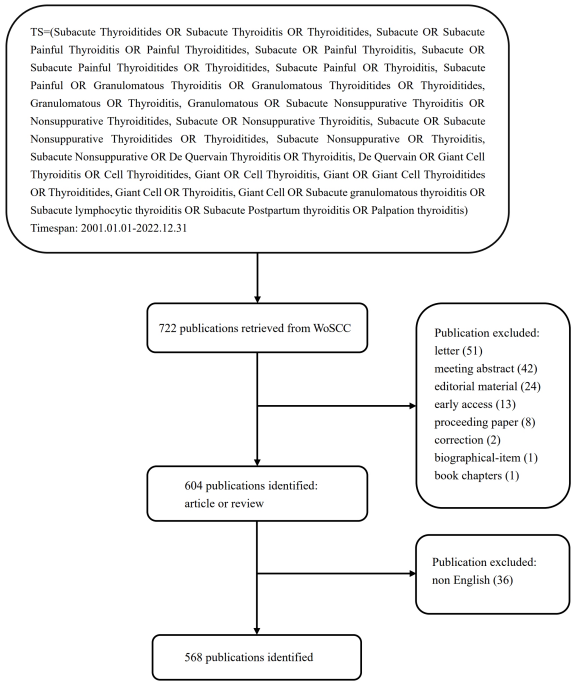

FIGURE 1
Flowchart for including and excluding literature studies.

articles (82.4%) and 100 reviews (17.6%). Figure 2 illustrates that from 2001 to 2018, the volume of publications relating to SAT research tended to be steady. However, there has been an increasing trend in the number of publications since 2019.

3.2 Analysis of country/region and institutions

Between 2001 and 2022, 568 studies were co-authored by 900 institutions across 61 nations and regions. United States (n = 126, 22.18%) placed first in terms of publications, followed by Japan (n = 75, 13.20%), Turkey (n = 64, 11.44%), China (n = 63, 11.09%), and Italy (n = 52, 9.16%). Moreover, The United States has the highest centrality (0.36) (Figure 3A), indicating its importance as a link in international and regional cooperation (16). As for the institutions, first place went to the University of Missouri System (n = 19, 3.35%), followed by Kuma Hospital (n = 16, 2.82%), University of Pisa (n = 15, 2.64%), University of California System (n =14, 2.47%), and Harvard University (n = 11, 1.94%). However, network density was only 0.0031(Figure 3B), indicating that institutions did not cooperate well enough (16).

3.3 Analysis of journals and co-cited journals

The 568 SAT research publications were printed in 281 academic journals. Table 1 lists the top 10 journals by productivity and co-citations. *Thyroid* (n = 36, 6.34%), which had an IF of 6.506 in 2022, published the most studies in this field, followed by *Journal of Endocrinological Investigation* (n = 26, 4.58%), *Endocrine Journal* (n = 18, 3.17%), *Journal of Clinical Endocrinology & Metabolism* (n = 14, 2.47%), and *Endocrine* (n = 12, 2.11%). There were three journals in the Q1 JCR (Journal Citation Reports) division, and *Thyroid* had the highest impact factor (IF) (IF = 6.506). The top 50 journals with the highest overall link strength out of all the included journals were chosen to create the density map, which clearly shows the productive journals (Figure 4A). As for the most frequently co-cited journals in Table 1, *Journal of Clinical Endocrinology & Metabolism* (n = 1350) ranked first, followed by *Thyroid* (n = 980), *Journal of Endocrinological Investigation* (n = 578), *New England Journal of Medicine* (n = 387), and *Clinical Endocrinology* (n= 382). Seven journals were also found in the Q1 JCR division, with Lancet having the highest IF (IF = 202.731). The density map in Figure 4B shows the top 50 co-cited journals selected among the publications with the highest overall link strength.

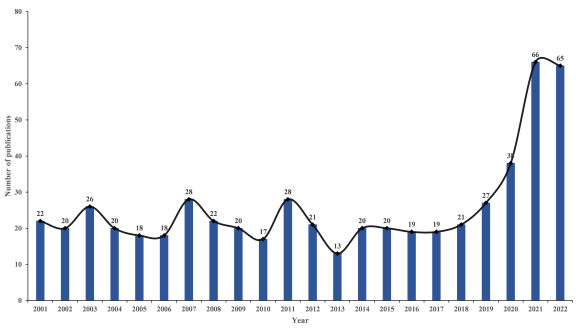

FIGURE 2
Trend of publications about the research of SAT.

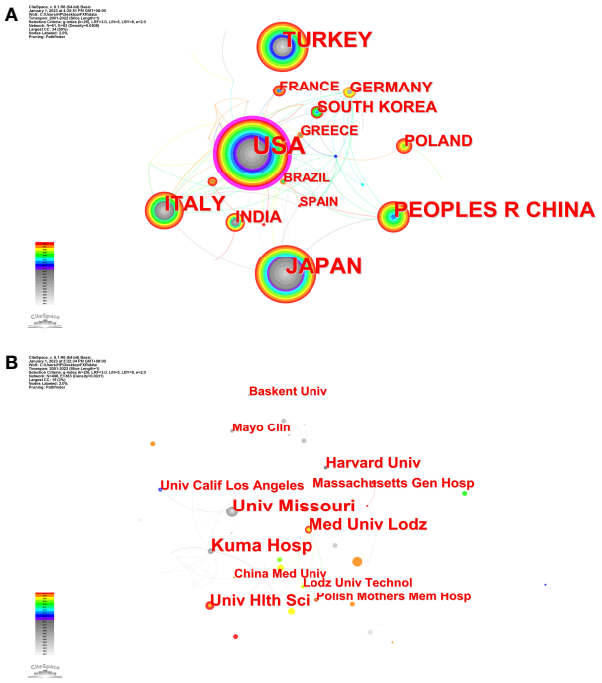

FIGURE 3
The network maps **(A)** Countries; **(B)** Institutions. The size of node represents the co-occurrence frequencies while the links reflect the co-occurrence relationships. The color of node and line indicates different years.

3.4 Analysis of core author distribution and co-authorship network

The overall number of authors who contributed to SAT research output was 2,468. Table 2 lists the authors who are the most productive. The author Braley-Mullen H had the most publications (17), followed by authors Miyauchi A (16) and Sharp GC (16), all of whom had 16 publications. The fourth spot was shared by Cakal E (11), Lewinski A (11), and Stasiak M (11). Figure 5A depicts the network visualization map of the authors' cooperation. Co-cited authors are those who have their work referenced in multiple studies at the same time. Figure 5B displays the network visualization map for the co-cited authors. The most often co-cited authors are represented by the largest nodes, including Stasiak M (156), Fatourechi V (127), Nishihara E (124), Brancatella A (96), and Bartalena L (94). The most commonly co-cited authors included two of the top ten most productive authors (Stasiak M, Braley-Mullen H).

TABLE 1 Top 10 productive journals and co-cited journals of SAT research.

Rank	Productive Journal	Count	IF	JCR (2022)	Co-cited journal	Citation	IF	JCR (2022)
1	Thyroid	36	6.506	Q1	Journal of Clinical Endocrinology & Metabolism	1350	6.134	Q1
2	Journal Of Endocrinological Investigation	26	5.467	Q2	Thyroid	980	6.506	Q1
3	Endocrine Journal	18	2.86	Q4	Journal of Endocrinological Investigation	578	5.467	Q2
4	Journal of Clinical Endocrinology & Metabolism	14	6.134	Q1	New England Journal of Medicine	387	176.079	Q1
5	Endocrine	12	3.925	Q3	Clinical Endocrinology	382	3.523	Q3
6	Clinical Endocrinology	11	3.523	Q3	Lancet	240	202.731	Q1
7	Diagnostic Cytopathology	11	1.39	Q4	Endocrine Journal	236	2.86	Q4
8	Frontiers In Endocrinology	10	6.055	Q1	European Journal of Endocrinology	227	6.558	Q1
9	Internal Medicine	9	1.282	Q4	Internal Medicine	190	1.282	Q4
10	Archives Of Endocrinology Metabolism	7	2.032	Q4	Endocrine	176	3.925	Q3

3.5 Analysis of document co-citation

Document co-citation is a technique for elucidating co-cited literature by several authors. Precisely, this technique visualizes the co-occurrence of citations in two publications to assess their link (18). Vosviewer examined a total of 568 articles and their 12,163 references that were retrieved from WoSCC during the years of 2001 and 2022 to determine typical homogeneity. Figure 6 displays a map of co-citation references for SAT research. The findings revealed that the most highly cited reference is a cohort study of clinical features and outcomes of SAT published by *Journal of Clinical Endocrinology & Metabolism* in 2003 (17). This cohort study found that early transient hypothyroidism is common in SAT, and corticosteroid therapy might relieve symptoms but could not prevent early-onset or late-onset thyroid dysfunction. The second-ranked paper was a retrospective clinical study published by *Internal Medicine* in 2008 (19). The study reviewed the medical records of 852 SAT patients from 1996 to 2004, and evaluated the characteristics of SAT at onset, recurrent episodes, and abnormal laboratory findings. The third-ranked paper examined virological data for each form of thyroiditis at various degrees of proof and offered concrete evidence of the existence of viruses or their byproducts in the thyroid gland. However, it was still unknown whether these viruses were the cause of thyroid illness (9). The top 10 co-cited works, which are presented in Table 3, have made significant contributions to SAT research and are perhaps the most well-known works in this area.

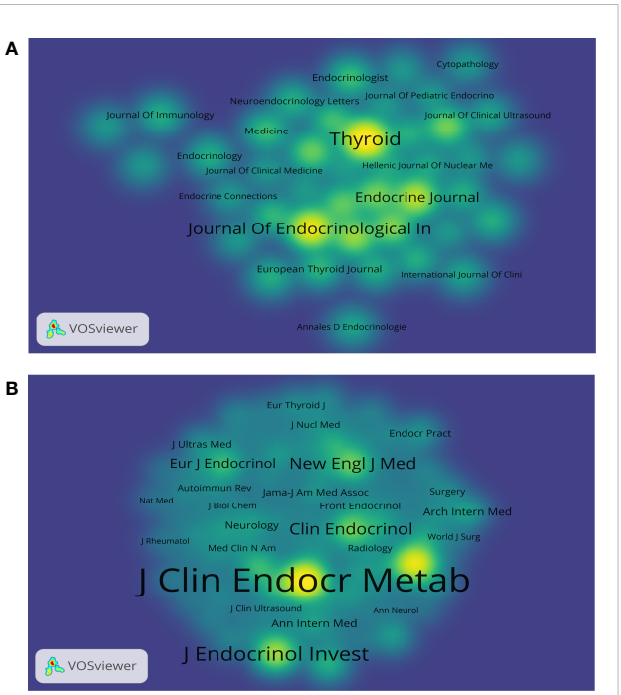

FIGURE 4
The density maps **(A)** Journals; **(B)** Co-cited journals. The size of the word and round, and the opacity of yellow are positively associated with the cited frequency **(A)**. The size of the word and round, and the opacity of yellow are positively associated with the co-cited frequency **(B)**.

3.6 Analysis of keyword co-occurrence, clusters, and bursts

Keyword co-occurrence and network cluster analysis are both available through CiteSpace. 502 keywords were retrieved in total. Table 4 and Figure 7A show the most frequently occurring keywords, indicating the hotspots of the SAT research. The most relevant terms in the SAT research were identified by the keyword co-occurrence clusters using the hierarchical cluster labeling method, which includes "prevalence" (cluster #0), "papillary thyroid carcinoma" (cluster #1), "effector cell" (cluster #2),

TABLE 2 Top 12 productive authors and co-cited authors in SAT research.

Rank	Author	Count	Rank	Co-cited author	Citation
1	Braley-Mullen H	18	1	Stasiak M	156
2	Miyauchi A	16	2	Fatourechi V	127
2	Sharp GC	16	3	Nishihara E	124
4	Cakal E	11	4	Brancatella A	96
5	Lewinski A	11	5	Bartalena L	94
6	Stasiak M	11	6	Volpe R	90
7	Amino N	10	7	Pearce EN	80
7	Chen KM	10	8	Braley-mullen H	74
7	Sencar ME	10	9	Ross DS	70
10	Wei YZ	10	10	Desailloud R	67
10	Fukata S	9	11	Martino E	66
10	Unsal IO	9	12	Bogazzi F	56

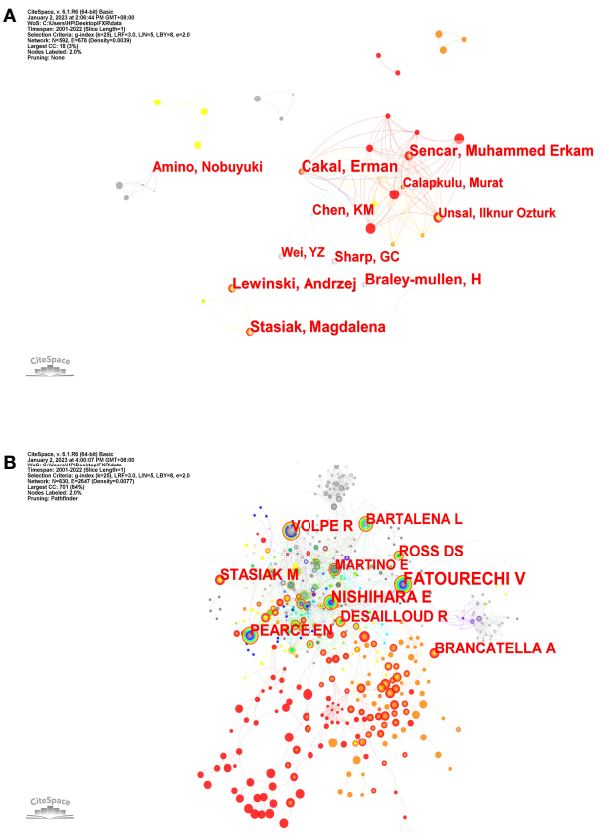

FIGURE 5
The network maps **(A)** Authors; **(B)** Co-cited authors. The size of node indicates the number of articles published by a certain author, and the links between two nodes mean a collaboration between each other **(A)**. The size of node indicates the co-cited frequency of a certain author, and the links between two circles mean a co-citation relationship between authors **(B)**.

"graves disease" (cluster #3), "recurrence" (cluster #4), "children" (cluster #5), "ace2" (cluster #6), "autoimmune thyroid disease" (cluster #7), "subacute thyroiditis" (cluster #8), and "liver dysfunction" (cluster #9) (Figure 7B). The amount of cluster labels is inverse to the number of articles each cluster contains. Therefore, cluster #0 has the greatest number of papers. Supplementary Table S1 contains a list of clusters in summary form. In order to depict the development of high-frequency keywords within each cluster, CiteSpace developed a keywords timeline viewer that could cluster keywords and take time into consideration. The viewer might also make it straightforward to pinpoint the time frame for a specific subject and the development of this research area. Each stage and evolution path of the SAT research's concentration could be intuitively understood, as shown in Figure 7C. We used CiteSpace to find burst keywords to track the hotspots and research boundaries over time. Figure 8 displays the top 10 keyword bursts from SAT research from 2001 to 2022 that had the most robust strength. The keyword bursts among them that persisted through the end of 2022 included "clinical characteristics" (with a burst strength of 4.93), "covid 19" (with a burst strength of 4.38), "guideline" (with a burst strength of 4.12), "case report" (with a burst strength of 3.42), and covid-19 vaccine (with a burst strength of 3.19), which represented the hot spots in recent years.

4 Discussion

4.1 General information

It is comparatively challenging to fully grasp the focus of a certain topic, access cutting-edge information, and identify research trends and hot spots in the age of the information boom (20).

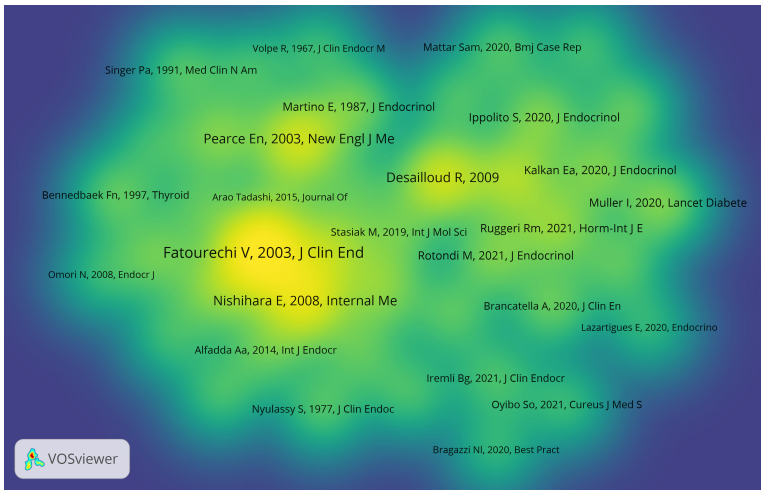

FIGURE 6
The density map of co-cited documents. The size of the word and round, and the opacity of yellow are positively associated with the co-cited frequency.

Bibliometric analysis is often used as a method to solve these problems. As SAT is a self-limiting disease that may resolve spontaneously, there is little research before 2019. However, the COVID-19 outbreak has resulted in a sharp rise in the number of relevant studies, demonstrating that the global scientific community is interested in learning more about the connection between SAT and the COVID-19 infection or vaccination. Since COVID-19 patients' clinical characteristics of SAT may differ from those of

TABLE 3 The top 10 high co-cited documents in SAT research.

Rank	Title	Journal	Year	Author	Co-citation counts	DOI
1	Clinical features and outcome of subacute thyroiditis in an incidence cohort: Olmsted County, Minnesota, study	Journal of Clinical Endocrinology & Metabolism	2003	Fatourechi V	112	10.1210/jc.2002-021799
2	Clinical characteristics of 852 patients with subacute thyroiditis before treatment	Internal Medicine	2008	Nishihara E	73	10.2169/internalmedicine.47.0740
3	Viruses and thyroiditis: an update	Virology Journal	2009	Desailloud R	66	10.1186/1743-422x-6-5
4	Thyroiditis	New England Journal of Medicine	2003	Pearce EN	66	10.1056/nejmra021194
5	Subacute Thyroiditis After Sars-COV-2 Infection	Journal of Clinical Endocrinology & Metabolism	2020	Brancatella A	50	10.1210/clinem/dgaa276
6	2016 American Thyroid Association Guidelines for Diagnosis and Management of Hyperthyroidism and Other Causes of Thyrotoxicosis	Thyroid	2016	Ross DS	44	10.1089/thy.2016.0229
7	Subacute thyroiditis in a patient infected with SARS-COV-2: an endocrine complication linked to the COVID-19 pandemic	Hormones-International Journal of Endocrinology and Metabolism	2021	Ruggeri RM	40	10.1007/s42000-020-00230-w
8	SARS-CoV-2-related atypical thyroiditis	The Lancet Diabetes & Endocrinology	2020	Muller I	39	10.1016/s2213-8587(20)30266-7
9	Subacute thyroiditis: clinical characteristics and treatment outcome in fifty-six consecutive patients diagnosed between 1999 and 2005	Journal of Endocrinological Investigation	2007	Benbassat CA	37	10.1007/bf03347442
10	SARS-CoV-2: a potential trigger for subacute thyroiditis? Insights from a case report	Journal of Endocrinological Investigation	2020	Ippolito S	37	10.1007/s40618-020-01312-7

TABLE 4 The top 20 keywords associated with SAT research.

Rank	Keyword	Count	Rank	Keyword	Count
1	Subacute Thyroiditi	226	11	Antibody	26
2	Graves Disease	83	12	Thyrotoxicosis	23
3	Disease	66	13	Feature	22
4	Diagnosis	56	14	Nodule	19
5	Management	44	15	Fine Needle Aspiration	17
6	Hyperthyroidism	42	16	Carcinoma	16
7	Hashimotos Thyroiditi	41	17	Autoantibody	16
8	Clinical Characteristics	39	18	Hypothyroidism	16
9	Association	33	19	Prevalence	16
10	Autoimmune Thyroiditi	33	20	Benign	14

normal SAT patients, and numerous new clinical trends have emerged (21), it is still a promising subject for research and merits financial and human resources investment, which is congruent with the actual clinical situation.

Teamwork and global collaboration in a specific field are made easier with the aid of distribution analysis of countries/regions and institutions. Figure 3A demonstrates that the United States and Japan were the two major nations that contributed considerably to SAT research. The United States had the highest centrality (0.36), showing that it was crucial in bridging international cooperation (16), but it is a pity that there was not much cooperation among other countries/regions. Additionally, the fact that three of the top five universities are from the United States shows that American academics have dominated SAT research for the past 20 years and have the greatest influence. This distribution has to do with academic funding and economic growth. At the same time, most institutional cooperation is domestic scientific cooperation, and inter-national institutional cooperation is insufficient (Figure 3B). Given the above, it is essential to strengthen international relations and institutional collaboration to encourage the ongoing growth of this field and help more SAT sufferers.

An analysis of journals and co-citations of journals can demonstrate their contribution to the field, and researchers may use these results to choose appropriate journals for manuscripts relating to SAT. The journal *Thyroid* (n = 36) published the most papers on this topic. *Thyroid*, an official journal of the American Thyroid Association, is the top journal in the field, whose publications cover thyroid diseases from cellular molecular biology to clinical management. Among the top 10 most co-cited journals, *Journal of Clinical Endocrinology & Metabolism* (n = 1350) possessed the most co-citations. It is a journal associated with research on the clinical practice of endocrinology and metabolism. Five of the top 10 co-cited journals, including *Lancet* (IF = 202.731) and *New England Journal of Medicine* (IF = 176.079), are in the Q1 JCR division, proving that some high-caliber and high-impact journals values SAT research.

Among the authors who contributed to the research of SAT from 2001-2022, Braley-Mullen H. from the University of Missouri System published 18 articles in this field. Prof. Braley-Mullen H. was an expert in the field of thyroid diseases and was committed to the molecular mechanism research of various types of thyroiditis. Stasiak M. from Polish Mother's Memorial Hospital Research Institution was the most co-cited author in this field. Prof. Stasiak M. was engaged in clinical research on thyroid diseases and conducted in-depth research on genetic susceptibility for SAT.

Among the top 10 high co-cited documents in SAT research, 5 were published before 2010 (4, 9, 17, 19, 22), which mainly focused on the clinical characteristics and pathogenesis of SAT, while 4 articles published after 2019 were studies on SAT in relation to the COVID-19 pandemic (23–26). The number of co-citations reached the top 10 within 2 years, reflecting that the research of SAT under the COVID-19 pandemic is a current research hotspot. However, the absence of basic research among the top 10 high co-cited articles suggests that basic research on the SAT is not a trend for the next few years.

The clustered network and timeline view of keywords display the evolution of high-frequency keywords and show the research progression path evolution in the research of SAT (Figure 7). Three research core themes can be distilled into the following characteristics using these analyses: 1. Prevalence (cluster #0, cluster #5 and cluster #6); 2. Diagnosis (cluster #1, cluster #3, and cluster #7); 3. Treatment (cluster #2, cluster #4, and cluster #9). The keyword bursts are thought to be signs of modern subjects or developing trends (Figure 8). The keyword bursts among them that lasted until the end of 2022 included "covid 19", "covid-19 vaccine", "clinical characteristics", "case report", and "guideline", which represented the hot spots in recent years. The clinical characteristics of SAT have been changing significantly in recent years, and findings are usually initially presented in the form of case reports. COVID-19 and COVID-19 vaccination can be potent SAT-triggering factors, and the clinical course of SAT in patients affected by them is different from a typical one. It is imperative to explore new guidelines for the diagnosis and treatment of SAT. Stasiak M. et al. have proposed new diagnostic criteria for SAT that complement new aspects related to the COVID-19 pandemic and may help improve the effectiveness of diagnosis and treatment of the disease (21).

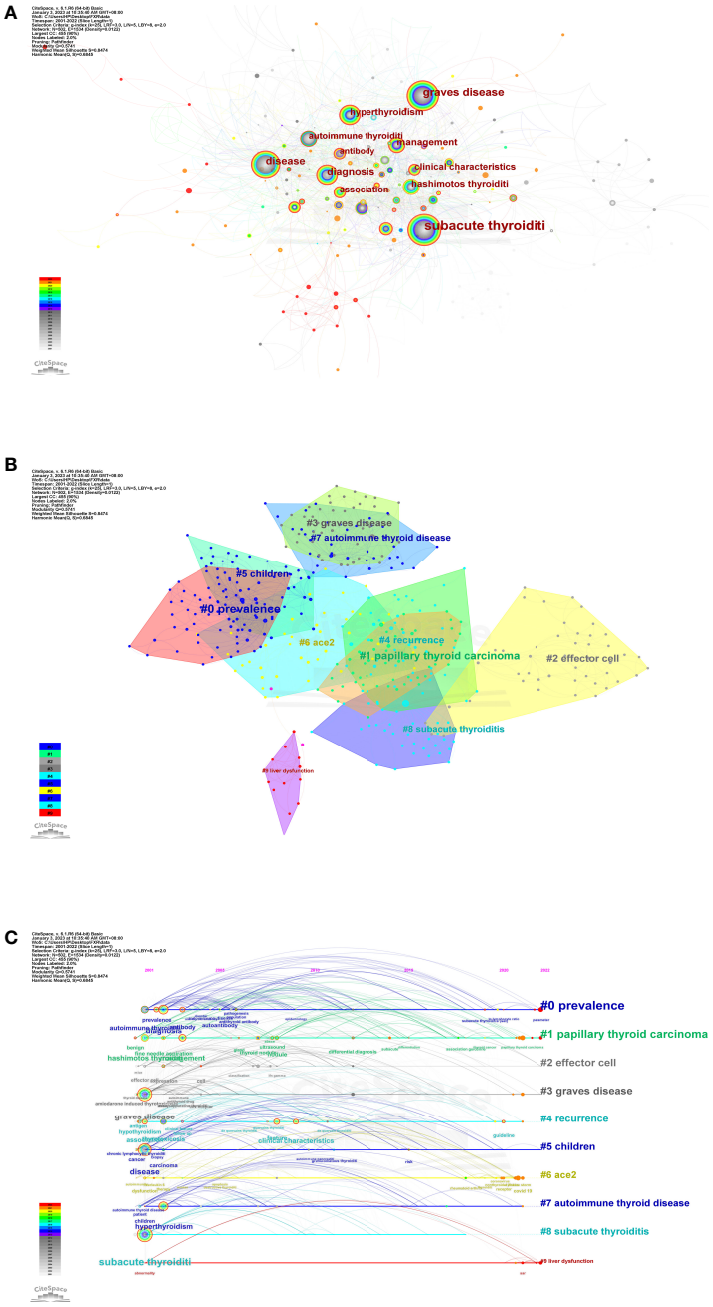

FIGURE 7
(A) The network map of keywords. **(B)** Clustered network of keywords. **(C)** The timeline view of keywords.

4.2 Research core themes

4.2.1 Prevalence

Middle-aged women had the highest SAT incidence rate, accounting for 75% to 80% of all SAT patients (3). It is worth noting that there have been recent reports of SAT in kids, despite the fact that SAT in children is thought to be incredibly rare (27, 28). Clinicians should be aware that SAT may exist in children.

As a result of the COVID-19 outbreak, up to 10% to 20% of COVID-19 patients who are hospitalized also have symptoms of SAT (25, 29). However, the impact of COVID-19 on the prevalence of SAT is still up for discussion. The results of a retrospective single-center study conducted in Turkey from 2018 to 2020 found increases in seasonal variation and an increase in the number of men who had SAT but no changes in the prevalence or clinical course of the illness (30). On the contrary, a large cross-sectional study conducted in South Korea revealed that the incidence of SAT was much greater in 2020 than it was from 2017 to 2019, and corticosteroids were prescribed more frequently, but the peak age and sex ratio of onset were no different from previous years (31). The generalization of incidence rate statistics is constrained by the current study's single-center, retrospective design and potential

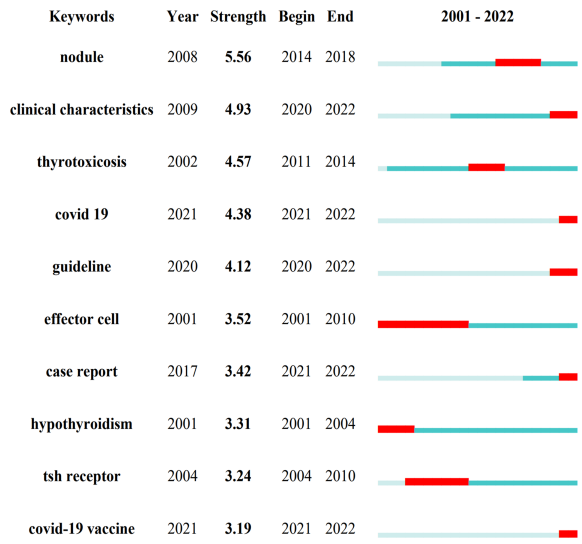

FIGURE 8
Keywords with the strongest burst strength. The blue line represents the period from 2001 to 2022 and the red line represents the burst's maintenance period.

cross-national and regional variances. It is essential to perform a multicenter study based on the general population to evaluate the existing results.

4.2.2 Diagnosis

The symptoms and signs of SAT are not typical. Despite advancements in diagnostic methods, new changes in the clinical presentation make the diagnosis much more challenging and more likely to result in a false negative. Patients frequently see multiple doctors before receiving the diagnosis of SAT, and the time it takes can vary from two weeks to six months (32). In a retrospective study, an upper respiratory tract infection was the initial diagnosis for one-third of SAT patients (33). Misdiagnosis of infection leads to overuse of antibiotics, with fever, elevated C-reactive protein (CRP) levels, and white blood cell (WBC) being the most common features in patients treated with antibiotics.

False negative SAT diagnoses cause therapy delays and poor quality of patient's life, but they do not pose a life-threatening risk. However, the false positive diagnosis of thyroid primary and metastatic malignancies as SAT will delay treatment and endanger patients' life (34). It was thought to be extremely rare for SAT and thyroid carcinoma to coexist, and such cases were typically reported as case reports (35, 36). Following up with 710 SAT patients for a long time demonstrated that initial ultrasound screening for thyroid nodules had a sensitivity of 72.4%, specificity of 89.0%, positive predictive value of 80.4%, and negative predictive value of 83.8% in SAT patients (37). In 3.1% of individuals with SAT, thyroid papillary carcinoma (PTC) was found, and up to 30% of PTC instances go unreported at the initial scan and are only found at a subsequent ultrasound (37). Therefore, ultrasound retesting should always be performed after SAT-related thyroid lesions have subsided. Fine needle aspiration biopsy (FNAB) tests should be carried out to rule out malignancy if the ultrasonography results are questionable (34).

4.2.3 Treatment

Pain relief and inflammation management are the primary goals of SAT therapy. NSAIDs and steroids have long been recommended for the treatment of SAT. Observational findings suggested that NSAIDs were less effective in SAT treatment than steroids, which were considered protective factors in reducing recurrence (6, 38, 39). Recurrence of SAT and steroid dependence remain essential issues in the treatment of SAT. The danger of recurrence from reducing glucocorticoid doses and the risk of consequences from glucocorticoid dependence must be balanced carefully. Evidence shows that a higher prevalence of hypothyroidism is linked to large cumulative dosages of prednisolone (40).

The optimal steroid treatment for SAT is still controversial. A high risk of recurrence is known to be linked to a too fast tapering of steroid dose. A randomized controlled trial, however, has revealed that short-term prednisone therapy is comparable to long-term efficacy and has a better safety profile (39). Additionally, a cohort study discovered that SAT recovery might be possible with low-dose steroid therapy (41). The results of these related studies should warrant further in-depth clinical trials involving more patients to assess how to avoid long-term steroid therapy.

In addition, it has been reported that ultrasound-guided intrathyroid administration of corticosteroids can significantly reduce the duration of SAT therapy compared to oral administration (42, 43), but further evidence-based medical evidence is needed. For SAT patients who have relapsed and are resistant to prednisolone, colchicine has been reported to have a potential therapeutic benefit (44). Nevertheless, solid proof will require a large, double-blind, controlled, prospective multicenter trial, and therefore it needs to be used with caution.

4.3 Research hotpots

4.3.1 Clinical characteristic

The clinical characteristics of the disease have seen various alterations in recent years. The frequency of painless SAT has increased, reaching 6.25% (3), as more and more cases have been documented (45, 46). Fever was also observed to occur less frequently than previously believed and was commonly associated with microhematuria (3). It was once believed that the absence of thyroid antibodies constituted a distinctive feature of the SAT. However, elevated levels of anti-thyroid antibodies, such as thyroid peroxidase antibodies (aTPO), thyroglobulin antibodies (aTG), and even thyrotropin receptor antibodies (TRAb) are more often present (3, 47).

A typical late SAT symptom is persistent hypothyroidism. According to earlier research, the extent of inflammation and thyroid hormone levels in SAT patients may be reflected by ultrasound, but it is challenging to forecast permanent hypothyroidism (48). However, a recent study indicated that the probability of chronic hypothyroidism is connected to the decrease

in thyroid volume shown by ultrasound within one month of beginning (49). Thyroid-stimulating hormone (TSH) and CRP levels were revealed to be risk factors for hypothyroidism in SAT patients, particularly in those with TSH levels less than 0.10 mIU/L and CRP levels greater than 97.80 mg/L (50).

The relationship between SAT susceptibility and specific HLA categories has been discovered (21). In 70% of SAT patients, HLA-B*35 was detected (51), but other genotypes were also discovered to be connected to the genetics of SAT. Along with the connection to HLA-B*35 that has already been discussed, SAT is also linked to the presence of HLA-B*18:01, HLA-DRB1*01, and HLA-C*04:01 (52). Recent research has demonstrated that the risk of SAT recurrence is HLA-dependent, with the presence of both HLA-B*18:01 and HLA-B*35 serving as the decisive factor (7), revealing that SAT recurrence may be genetically related. Additionally, it was discovered that the sonographic pattern of the SAT was related to HLA (53). Multiple hypoechoic hazy lesions, which are common, were infrequently detected in HLA-B*18:01 positive patients. Most of the patients with HLA-B*18:01 alone had a unilateral, homogenously hypoechoic single SAT region that filled the entire affected lobe and resembled a large thyroid nodule (53). The form of the SAT lesions, which were spotty or spherical, imitating true thyroid nodules, was the main departure from the expected pattern in patients with co-presence of HLA-B*18:01 and HLA-B*35 (53).

4.3.2 Influence of COVID-19

In May 2020, the first SARS-CoV-2 infection-related SAT case was reported (23). The original report's two-week window between PCR positive and SAT incidence rate was typically accepted. However, SAT caused by SARS-CoV-2 may diverge dramatically from the classic one. The majority of the stages are painless, but tachyarrhythmias and worsening of a general condition are frequently regarded as the main symptoms, especially in patients hospitalized with COVID-19 (21, 25). In certain patients, SAT usually occurs a few weeks after COVID-19 (21, 24), although in other cases it may take a few days for SAT to occur following the commencement of COVID-19 (26), and the two conditions may even manifest at the same time (21, 25, 29). This phenomenon can be HLA-dependent, and the presence of homozygosity at HLA-B*35 may be a potential major contributor to the early onset of SAT symptoms (54). Clinicians need to be aware that COVID-19 infection may result in thyroid dysfunction. Early detection and prompt anti-inflammatory therapy contribute to successful treatment.

Previous viral infection is thought to be the trigger for the SAT. Significant histotropism is seen by SARS-CoV-2, including a strong affinity for thyroid tissue. Angiotensin-converting enzyme 2 (ACE2), a possible receptor that allows the virus to enter cells, is a significant component of new coronavirus infection, and thyroid cells are abundant in ACE2 (55, 56). A study has demonstrated that thyroid follicular cells exhibit significant levels of the mRNA encoding ACE2 receptor, making them a possible entry point for COVID-19 (57). Many SAT cases directly associated with SARS-CoV-2 infection have been described (23, 58–61). Although the scale and quality of the published COVID-19 related SAT data are insufficient, in view of the development of the epidemic, we should still consider that SARS-COV-2 is the most important trigger factor for SAT at present, and its relevant mechanism needs further study.

Finding a therapeutic vaccination that is both effective and safe has become a top priority as COVID-19 spreads to become a pandemic. The vaccine contains adjuvants, which cannot avoid adverse reactions and can cause autoimmune/inflammatory syndrome induced by adjuvants (ASIA) (62). SARS-CoV-2 vaccination can lead to subacute thyroiditis as a phenomenon of ASIA syndrome. The first report of SAT as a phenomenon of ASIA syndrome after inactivated COVID-19 vaccination was reported in August 2021 (63). These cases shared diagnostic characteristics and clinical courses with the classic form of SAT. The mechanism of thyroid dysfunction caused by COVID-19 vaccines has yet to be clearly elucidated at present. Possible mechanisms are considered to include molecular mimicry caused by exposure to abnormal reactivity of adjuvants and/or viral proteins. Adjuvants are substances that promote the immunogenicity of vaccines, and SAT is assumed to be brought on by adjuvant-dependent autoimmune inflammatory alterations. However, it is becoming evident that the cause of SAT may be more complex than just the adjuvant as more cases of SAT associated with unadjuvanted COVID-19 vaccinations are reported (64, 65). The immune response to mRNA and whole viral vaccinations frequently uses spike protein as a stimulant. By binding to HLA-B*35 molecules in macrophages and activating cytotoxic T cells, spike proteins can cause SAT in susceptible individuals (66). Since spike protein-binding ACE2 receptors are abundant in thyroid follicular cells, their activation may be why thyroid follicular cells are being destroyed.

SAT occurrence after COVID-19 vaccination was also HLA-dependent and associated with a specific HLA profile covering the simultaneous presence of HLA-B*35:03 and HLA-C*04:01 (67, 68). Thyrotoxicosis and a more intense inflammatory response were related to homozygosity for HLA-B*35 and HLA-C*04 (67). According to a recent study, which is the first to suggest that the frequency of the HLA-A*11 allele is associated with SAT, it was found that SAT caused by the SARS-CoV-2 vaccine had a higher frequency of the HLA-A*11 allele and the A*11-B*35-C*04 haplotype than in the group unrelated to the SARS-CoV-2 vaccine (69). These findings suggest that HLA-related susceptibility may play a significant role in the development of SAT after COVID-19 vaccination, and the results need to be confirmed in a larger patient population with complete HLA genotyping results available.

Most SAT patients caused by vaccination have a mild clinical course that improves with the use of NSAIDs or steroids (70). It was recommended that patients be treated with NSAIDs in order to obtain adequate vaccine antibody response (66). A systematic review has shown that thyroid diseases may occur within 2 months after COVID-19 vaccination, and SAT is the most common of all thyroid diseases (71). Revaccination in COVID-19 vaccine-induced SAT cases currently appears to be safe (72), but the quantity and quality of published data on thyroid discomfort following COVID-19 vaccination are limited, and further evidence is needed on COVID-19 vaccine-induced SAT.

4.4 Strengths and limitations

According to our knowledge, this study is the first bibliometric analysis of SAT research to offer researchers guidance. Compared to a typical review, an analysis based on bibliometric tools, such CiteSpace and VOSviewer, offers a better depiction of changing research trends and hotspots and a relatively thorough and objective data analysis. However, this study has some limitations. First, despite recent increases in the number of publications published in SAT research, the aggregate total is still rather low. Second, owing to CiteSpace's format constraints, we only counted publications in the WoSCC database, which may have disregarded papers only in other databases such as PubMed, Medline, and Scopus. Due to the extensive cross-replication of records in other databases and the specialized authority of the WoSCC database, this study may still be utilized to illustrate the general situation and overall trend in this field. Third, non-English publications were not included in the study since English was the most often used language, which might have influenced the results due to source bias.

5 Conclusion

We conducted the first bibliometric analysis utilizing tools like CiteSpace and VOSviewer to examine the trends and hotspots in SAT research. Due to the COVID-19 pandemic, there has been a sharp rise in the number of publications in the SAT field in recent years, indicating a growing interest among researchers in the field. The clinical characteristics and the genetic background of SAT under the influence of COVID-19 are currently research hotspots. Our study clarified the fundamental scientific understanding of SAT and offered crucial hints for emerging research trends and hotspots. We hope that this study will aid researchers in better grasping the general trend in this field and offer guidance for future research.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

CX conceived and designed the study. RJ and J-yL contributed to data collection. CX, RJ, and J-yL conducted the data analysis and interpretation. CX drafted the initial manuscript. CX revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1144465/full#supplementary-material

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Selenium and thyroid diseases

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Selenium, a non-metallic element, is a micronutrient essential for the biosynthesis of selenoproteins containing selenocysteine. In adults, the thyroid contains the highest amount of selenium per gram of tissue. Most known selenoproteins, such as glutathione peroxidase, are expressed in the thyroid and are involved in thyroid hormone metabolism, redox state regulation, and maintenance of cellular homeostasis. Some clinical studies have shown that lack of selenium will increase the prevalence of several kinds of thyroid diseases. Selenium treatment in patients with Graves' orbitopathy has been shown to delay disease progression and improve the quality of life. Selenium supplementation in Hashimoto's thyroiditis was associated with the decreased levels of anti-thyroid peroxidase antibody and improved thyroid ultrasound structure. In thyroid cancer, various selenium supplements have shown variable anticancer activity. However, published results remain the conflicting and more clinical evidence is still needed to determine the clinical significance of selenium. This article reviews the strong association between selenium and thyroid disease and provides new ideas for the clinical management of selenium in thyroid disease.

KEYWORDS

selenium, selenoprotein, thyroid disease, oxidative stress, iodine

1 Introduction

In 1817, the Swedish chemist Berzelius discovered a non-metallic element and named it selenium (Se). Se is an essential trace element for human body (1). In the 1980s, it was found that supplementation with sodium selenite could improve chondrodystrophy (Kashin-Beck disease) and juvenile cardiomyopathy (Keshan disease) which were caused by Se deficiency. That was the first time Se was found to be useful in clinical treatment. With the gradual increased understanding of Se, it has been proposed that there is a U-shaped curve between Se status and the health status of the organism (2). Patients with Se deficiency can benefit from Se supplementation, while Se supplementation in people with adequate Se levels can exacerbate the risk of certain diseases (3). The thyroid is one of the highest content of Se in the body organs, it is interesting to note that in the case of Se deficiency, the Se content of thyroid gland is also high (4). Se is present in selenoproteins in the form of selenocysteine, which is involved in constituting the active center of selenoproteins. It plays an important role

in the metabolism of thyroid hormones and in the fight against oxidative stress (5). This highlights the uniqueness of human thyroid and the importance of Se to the thyroid gland. Although the relationship between Se and thyroid diseases is not well established and needs to be explored in depth. Low Se levels are currently considered to be one of the independent risk factors for thyroid diseases and Se supplement treatments for patients with low Se levels are thought to be generally beneficial for thyroid diseases.

2 Se is closely related to the metabolism of thyroid hormones

2.1 Se and selenoprotein

Se is absorbed by the body and involved in the synthesis of selenoproteins. It exerts biological functions such as antioxidant and metabolic regulation through selenoproteins, which are key biomolecules. To date, we have identified 25 genetically encoded selenoproteins in human, including glutathione peroxidase (GPx), thioredoxin reductase (TXNRD), and iodothyronine deiodinases (DIOs), which have a wide range of functions, from anti-inflammatory and antioxidant activities to thyroid hormone metabolism.

2.2 Selenoproteins are involved in the metabolism of thyroid hormones

After entering the thyroid cells, iodine ions are activated by H2O2 under the action of thyroid peroxidase (TPO). The activated iodine binds to tyrosine residues on thyroglobulin molecules under the action of TPO to produce monoiodotyrosine (MIT) and diiodotyrosine (DIT), which are subsequently coupled to produce T3 or T4 (Figure 1). Activation and deactivation of thyroxine need the participation of DIOs to complete (6–8).

When iodine is sufficient in the body, the production of H2O2 is the step that limits the synthesis of thyroid hormones; when iodine is lacking, under the stimulation of high TSH, thyroid cells produce more H2O2, whose accumulation gradually damages thyroid cells. Selenoproteins such as GPXs and TRs can scavenge H2O2, protect cell membrane structure and function, repair the site of molecular damage, achieve anti-oxidative stress and local protective effects against oxidative stress or inflammation. In Se deficiency, GPx activity decreases, degradation of H2O2 is reduced, thyroid cells are less resistant to oxidative stress, apoptosis and cell death occur (9). On the other hand, the activity of DIOs is reduced in Se deficiency, thyroxine is not activated and affects the thyroid hormones to perform their biological functions.

3 Se deficiency is one of the risk factors for many thyroid diseases

3.1 Se and Graves' disease

The main clinical manifestation of Graves' disease (GD), also known as toxic diffuse goiter, is thyrotoxicosis caused by excessive production of thyroid hormones. In this hypermetabolic state, the body releases a large number of reactive oxygen species (ROS), which can lead to thyroid epithelial cell damage, autoantigen activation of the immune system, and induction of autoimmune deterioration. Graves' orbitopathy (GO) is the most prominent and common extrathyroidal manifestation of GD, characterized by the production and accumulation of glycosaminoglycans (especially hyaluronic acid) in the retrobulbar and periorbital tissues causing protrusion of the eyeball and restriction of ocular muscle movement (10). A large number of clinical trials have demonstrated the efficacy of Se in the treatment of Graves' hyperthyroidism, but the results have been somewhat contradictory. We conducted a screening of clinical controlled trials on GD using PubMed and Cochrane library databases with the search terms: "(Graves' disease OR hyperthyroidism) AND Se". After analysis of 11 clinical trials with full text that are eligible, 9 trials confirmed that Se supplementation resulted in faster achievement of normal thyroid function in patients with hyperthyroidism, but 2 still did not show an adjuvant effect of Se. The details are shown in Table 1.

3.1.1 The role of Se in GD

A large number of studies have now confirmed Se deficiency as a risk factor for GD in areas with adequate soil Se levels (21–26).

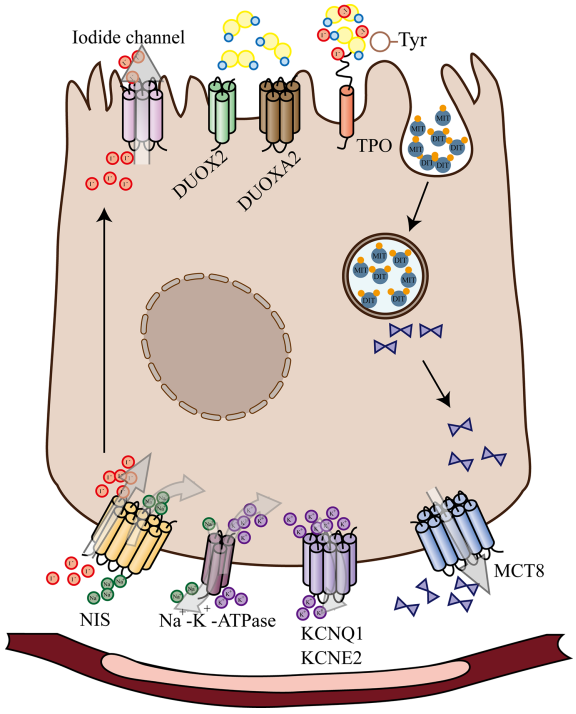

FIGURE 1
Schematic Diagram of Thyroid Hormone Biosynthesis and Release. After entering the thyroid cells, iodine ions are activated by H2O2 under the action of thyroid peroxidase. The activated iodine binds to tyrosine residues on thyroglobulin molecules under the action of TPO to produce monoiodotyrosine and diiodotyrosine. NIS, sodium/iodide symporter; KCNQ1 and KCNE2, Voltage-gated K^+ channels; MCT8, SLC16A2 monocarboxylate transporter 8; DUOX2, dual oxidase 2; DUOXA2, maturation factor of dual oxidase 2; TPO, thyroid peroxidase; MIT, monoiodotyrosine; DIT, diiodotyrosine.

TABLE 1 Characteristics of 11 included studies.

First author Year	Country	Characteristics of participants	Interventions dose	Number of participants, n		Mean age, year		Male (%)		Follow-up time	Outcome index	Outcome
				Se	control	Se	control	Se	control			
Bacic Vrca, V. 2004 (11)	Croatia	newly detected GD	capsule of antioxidants (include 60ug Se)	29	28	–	–	14	4	2 months	FT4, FT3, TSH, ferritin, transferrin glucose, uric acid and TAS	Experimental group reached normal thyroid function faster
Bacic Vrca, V. 2004 (11)	Croatia	newly detected GD	capsule of antioxidants (include 60ug Se)	29	28	–	–	14	4	2 months	FT4, FT3, TSH, serum selenium GPx activity	Experimental group reached normal thyroid function faster
Bacic Vrca, V. 2005 (12)	Croatia	newly detected GD	capsule of antioxidants (include 60ug Se)	27	28	44.0± 12.0	41.0± 14.0	15	4	2 months	FT4, FT3, TSH, SOD activity, Cu and Zn concentrations in erythrocyte lysate	Experimental group reached normal thyroid function faster
Marcocci, C. 2011 (13)	Holland	mild signs or symptoms of GO of less than 18 months' duration	sodium selenite (100ug twice/day)	54	50	43.0± 11.0	44.6± 10.7	11	18	12 months	overall ophthalmic assessment and the GO-QOL score	Selenium treatment is associated with improved quality of life and reduced ocular involvement
Vrca, V. B. 2012 (14)	Croatia	newly detected GD	capsule of antioxidants (include 60ug Se)	27	28	44.0± 12.0	41.0± 14.0	15	4	2 months	FT4, FT3, TSH, triglyceride, LDL- and HDL-cholesterol	Experimental group reached normal thyroid function faster
Calissendorff, J. 2015 (15)	Sweden	newly diagnosed and untreated GD (aged 18–55)	Se yeast(200ug/ day)	19	19	35.0	44.0	21	16	36weeks	FT4, FT3, TSH, TRAb, TPOAb and self-rated symptoms	Selenium supplementation promotes biochemical recovery
Kahaly, G. J. 2017 (16)	Germany	untreated GD patients	sodium selenite (300ug/day)	35	35	44.5 (13.8)	44.5 (13.4)	20	26	36weeks	the response rate at week 24 and the remission/recurrence rate at week 36	Selenium supplementation did not affect the response or recurrence rate of GD
Leo, M. 2017 (17)	Italy	newly diagnosed hyperthyroid GD	L-selenomethionine (166ug/day)	15	15	43.0± 11.0	38.0± 11.0	7	13	90days	control of hyperthyroidism, clinical and biochemical manifestations of it	No auxiliary role of selenium in GD was shown
Xu, B. 2019 (18)	China	newly diagnosed hyperthyroid GD	selenium tablets (150ug twice/day)	44	50	38.89 ±11.59	40.20 ±12.63	32	38	6months	FT4, FT3, TSH, TRAb, TPOAb, TGAb	Combined use of selenium improves thyroid viability in patients
Almanza-Monterrubio, M. 2021 (19)	Mexico	mild and active GO by CAS > 3(≥18years old)	selenium tablets (100ug twice/day)	15	15	40.7± 10.5	42.5± 11.8	20	27	6months	visual acuity in LogMAR scale, palpebral aperture (mm), proptosis measured with Hertel exophthalmometer and CAS	Oral Selenium Improves Disease Activity in Patients with mild GO
Gallo, D. 2022 (20)	Italy	newly diagnosed GD with serum Se <120 mcg/l and plasma VitD <30 ng/ml	selenomethionine 83 mcg + selenium yeast 17 mcg(180 days stop)	21	21	45.8± 9.3	47.7± 11.4	19	5	270days	FT4 levels mean decrease from baseline to 180 days.biochemical marker, clinical parameters and QoL scores at 45, 180 and 270 days.	Achieving optimal Se and VitD levels can improve the early efficacy of MMI treatment

GD, Graves' disease; GO, Graves' orbitopathy; CAS, clinical activity score; Se, selenium; FT4, free thyroxine; FT3, free triiodothyronine; TSH, thyroid stimulating hormone; TAS, total antioxidant status; GPx, glutathione peroxidase; SOD, superoxide dismutase; TRAb, thyrotropin receptor antibody; TPOAb, thyroid peroxidase antibody; TGAb, thyroglobulin antibody; GO-QOL, Graves' orbitopathy–specific quality-of-life questionnaire.

Notably, a cross-sectional study conducted in an area with poor soil Se showed that Se levels in patients in GD with or without GO were lower than in normal healthy controls (27), which would seem to be able to suggest that relatively low Se level is an independent risk factor for GD.

Based on the damage to thyroid cells caused by low Se level, researchers have raised the possibility that Se supplementation may be beneficial in GD and have conducted a series of clinical studies (16–18, 20, 28–30). In these studies, patients in the experimental group were often treated with Se supplements or antioxidants containing Se in combination with antithyroid drugs (generally methimazole, MMI). Interestingly, these results were not always consistent. During the study by Nordio, M., subjects took one tablet containing 500 mg of L-carnitine and 83ug of Se (L-Carn + Se) orally daily for 1 month. It showed significant relief of symptoms associated with subclinical hyperthyroidism and improved the quality of life of the patients, but no significant effect was seen in terms of thyroid function (28). In contrast, the results of Gallo, D. showed that MMI + antioxidants (83 ug selenomethionine + 17 ug Se yeast + vitamin D) treatment for 6 months improved thyroid activity more effectively than MMI use that alone (20). We speculate that this difference in results might be related to the type of Se supplementation, dose, duration of treatment and the status of other nutrients in the subject's organism. In studies which only additional Se was added to experimental groups, the findings were also variable. The results of Leo, M. showed that serum Se levels and selenoprotein concentrations were not associated with shortterm control of GD (17), while a randomized controlled trial by Xu, B. demonstrated that Se supplementation improved thyroid activity (18). The former study was conducted in an area with sufficient Se and therefore failed to show a short-term therapeutic effect of Se in hyperthyroidism. This difference in study premise may explain the difference in results.

Although the current findings are ambiguous and evidence from clinical trials does not favor the use of Se as a routine treatment option in GD, nor does the use of Se supplementation affect the remission and recurrence rates of GD (16). However, it is undeniable that correction of moderate to severe Se deficiency has a positive impact on the prophylaxis of GD.

3.1.2 The role of Se in GO

Current studies suggest that fibroblasts are distributed in the posterior globular connective tissue and ocular myofilament. They are target and effector cells of GO autoimmune response. In a study by Rotondo Dottore, G., it was mentioned that H2O2 has a dual effect on fibroblast cell proliferation, with low concentrations of H2O2 inducing proliferation and releasing cytokines, while high concentrations of H2O2 are cytotoxic, when cell viability decreases (9, 31). By further treating adipose/connective tissue in the orbital region with selenocysteine (SeMCys), Rotondo Dottore, G. and his team found that SeMCys appeared to reduce the toxic effects of H2O2 by reducing cell necrosis and apoptosis. In another study, Kim, B. Y. confirmed the beneficial effects of Se on orbital fibroblasts by primary culturing of orbital specimens from GO patients and healthy subjects with selenite (32). These findings seem to be interesting and they seem to indicate that Se also has a dual role in orbital fibroblasts, i.e., under conditions of oxidative stress without cytotoxicity, Se can inhibit the release of pro-inflammatory factors and hyaluronic acid; under conditions of cytotoxic oxidative stress, Se can prevent cellular damage and the release or exposure of autoantigens as well as reduce the toxic effects of reactive oxygen radicals. Excitingly, in addition to improving the antioxidant capacity of the body, Se is also thought to directly affect the sympathetic tone of opercular muscles and reduce inflammation in the muscles of the eyelids (33).

The effect of Se on patients with mild GO was further confirmed by a randomized clinical controlled trial conducted by Marcocci, C. et al. (13). In this study, subjects were arbitrarily assigned to one of the sodium selenite (100 micrograms twice daily), pentoxifylline (600 mg twice daily), or placebo (twice daily) therapy groups for a duration of 6 months. It was then followed up for 6 months after treatment was stopped. At the end of treatment, the investigators found that more patients in the Se group had improved the quality of life, while significantly fewer patients had disease progression. Encouragingly, Se also expressed a sustained beneficial effect on GO during the follow-up period. Notably, this study was conducted in different regions of Europe with different Se levels, so it is unclear whether the effectiveness of Se and the generalizability of the result were confounded by other confounding factors such as the effect of baseline Se levels.

Although the actual efficacy of Se in GO is uncertain, it is generally considered to be beneficial in the treatment of mildly active GO (19) and has been used in clinical practice (17, 34, 35). It is instructive that the 2021 EUGOGO guidelines include Se supplementation in the treatment regimen for mild GO, recommending a 6-month treatment with Se preparations for patients with mild GO of short duration to prevent the progression to more severe forms of GO (34).

3.2 Se and Hashimoto's thyroiditis

In recent years, the incidence of Hashimoto's thyroiditis (HT) has been increasing with the wide application of thyroid ultrasound, fine needle puncture biopsy and other testing techniques. However, due to the strong occult nature of the disease and atypical clinical symptoms, a large number of HT have not been diagnosed. Its epidemiological details are still very limited. HT is known to be the leading cause of primary hypothyroidism in areas where iodine is abundant. The prevalence of hypothyroidism varies from 0.2% to 5.3% in different regions. This proportion varies with geography, genetic factors, gender and age. The prevalence of overt hypothyroidism in the general population ranges from 0.2% to 5.3% in Europe and only 0.3% to 3.7% in the United States. A meta-analysis based on the human genome showed that high-risk individuals were 2.5 times more likely to have hypothyroidism than those at low genetic risk. Stratified analysis of gender shows that women are 10 times more likely to suffer from hypothyroidism than men. The prevalence increased to more than 20% for women in the higher age group (≥75 years) (36, 37). Due to the increased prevalence of HT (38–40), numerous studies have been conducted by researchers to address its etiology and treatment.HT occurs when the immune system

produces autoantibodies that attack the thyroid gland and some thyroid follicular cells are destroyed, resulting in insufficient thyroid hormone secretion and compensatory proliferation of undamaged thyroid follicular cells to produce more thyroid in order to maintain the normal function of organism hormones. The pathological manifestations of HT are often lymphocyte infiltration, follicular cell atrophy and glandular fibrosis. A 2021 review of autoimmune thyroiditis (AITD) clearly identified Se as an important risk factor for HT (41). Some cross-sectional studies have also confirmed low Se levels in HT patients (27, 42). Some researchers have detected a general deficiency of the antioxidant Se in HT patients with subclinical hypothyroidism in the area of Ankara, Turkey, which is iodine-rich. In the case of the cross-sectional study conducted in a Se-deficient area, although the difference between HT and controls was not statistically significant, Se levels of HT patients were lower than those of controls, and we speculate that the results may be limited by the size of the study with too few sub-jects. In conclusion, at this stage of the study, we still consider Se deficiency as a risk factor for HT. This seems to be related to the reduced activity of Se-dependent enzymes such as GPx, which has strong antioxidant activity to scavenge excess superoxide in the thyroid and maintain the integrity of cell membranes.

Se deficiency is often accompanied by a loss of immune function (43, 44). In cellular immunity, Se may reduce thyroid antibodies by upregulating activated Treg cells (45). Se deficiency may upregulate Th1/Th2 effectors and enhances immune responses. The possible therapeutic effect of Se in HT to improve immune function was validated in a prospective study conducted in 2022, which showed that Se supplementation with 100ug per day improved thyroid function and the quality of life of patients by decreasing interferon gamma concentrations and increasing interleukin 1β concentrations (46).

In addition, back in 2017, researchers studied the immunological effects of selenomethionine (SeMet) in 21 patients who had normal thyroid function with HT (47). The patients were treated with myoinositol plus Se (600mg/83ug) tablets twice daily for 6 months. Excitingly, in addition to the significant decrease in TSH levels, there was also a lever reduction in serum CXCchemokineligand-10 (CXCL10) chemokine which were induced by IFN-γ. CXCL10 is released by thyroid cells in response to IFN-γ stimulation. Its serum level is often proportional to the percentage of lymphomonocyte infiltration in thyroid tissue and the degree of thyroid destruction (48, 49). The immunoregulatory effect of myoinositol combined with Se on CXCL10 indicated that it could reduce the immune response of the body (50). However, the specific mechanism of this process remains unclear, and the specific role of the antioxidant Se in this process needs further research to clarify.

The autoimmune process of HT has a specific elevation of thyroid peroxidase antibodies (TPO-Ab) in addition to the chronic lymphocyte invasion of the gland. This specific elevation suggests that disorders of our humoral immunity may be one of the risk factors associated with HT. Based on characteristic serological markers of HT, a prospective clinical trial demonstrated that Se supplementation reduced TPO-Ab titers and improved the quality of life of patients (45), which is consistent with the results of several intervention studies or meta-analyses (51–55). It is worth exploring that, the high antibody group (TPO-Ab>200) also had a decrease in thyroglobulin antibody (TG-Ab) at 6 months, which seems to indicate that the high antibody group could benefit more significantly from treatment with Se supplementation. Karanikas did not observe inhibition of TPO-Ab titers by Se in their patients (56). The reason for this is unclear, but the result of this study does not deny the therapeutic effect of Se on HT.

Notably, women during pregnancy and delivery are a special population of HT patients. Pregnant women with TPO-Ab-positive have a higher risk of preterm delivery and miscarriage, as well as the development of postpartum thyroid dysfunction (PPTD) and eventually permanent hypothyroidism (57). A prospective controlled study showed that supplementation with 200ug SeMet per day during pregnancy and postpartum, reduced the incidence of PPTD and hypothyroidism (58).

In HT patients with severe hypothyroidism, levothyroxine (L-T4) is the usual treatment strategy. In an open controlled trial of 60 patients with chronic lymphocytic thyroiditis (59), the combination of L-T4+ Se possessed better efficacy than L-T4 monotherapy compared to the L-T4 group, TPO-Ab and TG-Ab were significantly reduced. However, in another intervention trial with combination therapy (53), the risk of adverse effects was significantly higher in the Se supplementation group, which we speculate may be related to unclear baseline Se levels in the subjects and a narrower safety window during Se treatment.

In summary, Se deficiency appears to be a risk factor for HT and the current evidence does not justify the use of Se supplementation as part of the treatment of HT, despite its ability to improve immune function. If supplementation is indeed required, it must be preceded by a careful consideration of the patient's Se baseline status, gender, weight and so on (60).

3.3 Dual role of Se deficiency in cretinism

Cretinism often occurs in areas where endemic goiter is prevalent, with mucinous edema cretinism being the main manifestation of hypothyroidism without goiter. Researchers' intervention studies on cretinism originated from the emergence of familial aggregates of cretinism in Central Africa (northern Zaire). Contempré, B. and his team constructed a model of the epidemiology of mucinous edema cretinism (61) (Figure 2). In this model, iodine deficiency and Se deficiency exacerbate H2O2 accumulation, Se deficiency decreases cellular defense, promotes thyroid cell fibrosis, increases the inflammatory response following thyroid cell necrosis, and thiocyanate overload triggers follicular cell necrosis. The interaction and mutual promotion of these three factors are thought to be responsible for the concentrated epidemic of cretinism in Central Africa (62–66).

It is thought-provoking that Se supplementation in iodine-deficient populations in this region can have serious and incalculable consequences (64, 67). In 1988, Contempre, B. and his team treated 26 children with cretinism living in northern Zaire with 50ug of Se per day for 2 months, but the results of the study showed a further decrease in the already impaired thyroid function of the subjects. This result may be due to the low percentage of

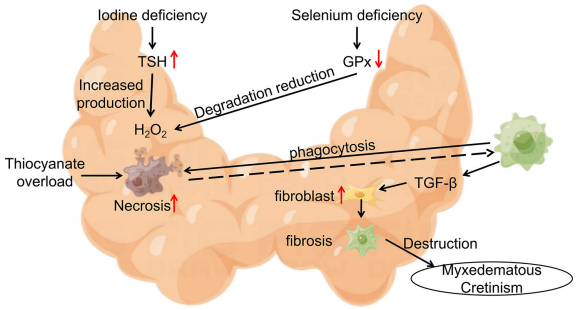

FIGURE 2
Epidemiological Model of Myxedematous Cretinism. Iodine deficiency, Se deficiency and thiocyanate overload are involved in the pathogenesis of myxedematous cretinism. TSH, Thyroid Stimulating Hormone; GPx, glutathione peroxidase; TGF-β, transforming growth factor-β.

functional thyroid tissue in children with cretinism in which T4 is converted to active T3 at the periphery in the presence of Se supplementation only. At this time, the body's demand for thyroid hormones increases, and the small amount of iodine stored in the gland is consumed more quickly without being replaced, resulting in a more rapid loss of thyroid status. In contrast, in patients with congenital hypothyroidism in areas without iodine deficiency treated with 20-60 ug selenomethionine for 3 months (68), Se supplementation was found to reduce serum thyroglobulin. In addition, Se supplementation improved hypothalamic-pituitary feedback to thyroid hormones, and thyroid stimulating hormone (TSH) returned to within the normal range, thereby reducing the stimulatory effect of TSH on thyroid tissue. The comparison of the results of these two studies seems to indicate that Se deficiency protects to some extent the thyroid function in patients with cretinism in Central Africa.

This enlightens us that Se supplementation should not be done indiscriminately in patients with congenital hypothyroidism (CH). For cretinism in iodine-deficient areas, Se supplementation should not be promoted prior to iodine supplementation or when there is no plan for iodine supplementation, as stated by the American Thyroid Association, "The differential intakes of iodine, Se, or both in different regions must be considered before any action for Se supplementation is taken" (69).

3.4 Se and thyroid tumors

The incidence of thyroid cancer has been on the rise in the past decades, increasing faster than any other cancer (70, 71). In addition to unchangeable external conditions such as age, gender, race and genetic susceptibility to thyroid cancer (72–74), the role played by the trace element Se in thyroid cancer has attracted the attention of researchers. Although most findings suggest a positive association between low Se level and the risk of thyroid cancer (24, 75–77), a prospective study conducted by Xu, X. and his team did not seem to find this association (78). From 1993-1998 researchers recruited 147,348 postmenopausal women through the Women's Health Initiative (WHI) database and investigated the subjects' dietary habits (Se intake) through a dietary questionnaire, and over the following 16 years, 442 cases of thyroid cancer were identified among the subjects. After adjusting for multiple covariates in the study, the researchers found no significant association between Se intake and thyroid cancer. This result is not consistent with previous studies that have investigated the link between low Se levels and thyroid cancer. In this regard, we speculate that the irrelevance of this study may stem from the method of Se measurement, which is available in whole blood, serum (79, 80), urine (81), nails (82), and questionnaires (83). The Se levels of the subjects in this study were inferred from the diet, so it has an information bias. Secondly, it was found that dietary Se intake was weakly correlated with peripheral Se levels such as whole blood and serum (84), and the intake of Se did not accurately reflect the relationship of serum Se and thyroid carcinoma, which may be another reason for the inconsistent results of this experiment. Finally, Xu, X.'s study was conducted in postmenopausal women whose Se intake was greater than or equal to baseline levels, and the subject's condition was another major limitation of this trial. In conclusion, we suggest that Se deficiency promotes the risk of thyroid cancer, but more conclusive research evidence is still needed to conclusively determine this relationship (75).

In addition to increasing the risk of disease, Se deficiency is also strongly associated with the progression of thyroid cancer. Some findings showed that serum Se concentration was inversely correlated with disease stage (P=0.011), and low levels of serum Se were potentially associated with high stage of thyroid cancer (80). Although 25 selenoproteins are tissue-specific finetuned in Se deficiency (85) and deiodinase is a highly conserved selenoprotein, the content activity of deiodinase also decreases in severe Se deficiency, when the body synthesizes significantly less T3 and the inhibitory effect on TSH is diminished. Increased TSH fosters cAMP synthesis, which in turn initiates cAMP-dependent protein kinase signaling systems, to potentiate EGF-mediated cell proliferation, stimulating thyroid cell growth and promoting tumor cell proliferation, invasion and metastasis (86–88).

At the molecular level, researchers conducted a multiomics data mining study and found that multiple selenoproteins are lowly expressed in thyroid cancer (89), which is associated with reduced selenoproteins content and activity due to Se deficiency and is consistent with the results of several previous studies (90, 91).GPx3 is known to be the only member of the GPx family that can be secreted into the plasma and therefore it can play an important role in extracellular oxidative stress (92), which enhances the antioxidant defense of cells *via* a blockage of redox DNA destruction, thus reducing as well a reduction in the abundance of damaged cells (93). On the other hand GPx3 is negatively correlated with MAPK oncogenic signaling pathway (94), which suggests a potential antitumor effect in thyroid cancer, while a decrease in GPx3 levels predisposes to an increase in the size of primary tumors and the number of metastatic lymph nodes (95, 96).

At the cellular level, Erdamar, H. examined 41 tissue samples (including 9 papillary thyroid cancer tissues) (97) and found that their Se levels and GPx activity were lower than those of non-cancerous tissues, while malondialdehyde (MDA) concentrations were increased. This may be due to the vicious cycle of increased

lipid peroxidation and free radicals in cancer cells, while the decrease in GPx activity makes cancer tissues more susceptible to the damaging effects of free radicals. In conclusion, Se can "boost" the immune and antioxidant capacity of the body and strengthen the immune defense.

In view of the role of Se deficiency in promoting thyroid tumorigenesis and progression, appropriate Se supplementation for differentiated thyroid cancer has been proposed to delay the disease progression and improve the prognosis (98).Kato, M. A. et al. treated thyroid cancer cells of different cell lines with selenomethionine (SeMet) (99) and found that SeMet could timedependently upregulate the expression of GADD family genes and arrest cells in cell cycle S phase or G2/M phase to inhibit the proliferation of thyroid cancer cells. This provides another great evidence for the clinical treatment of Se against cancer.

For differentiated thyroid cancer, surgical resection, radioiodine therapy, and TSH suppression therapy are often the three steps of its treatment. Radiation inflammation is the most common long-term complication during iodine therapy, often manifesting as dry mouth, altered taste, and dental caries (100, 101). Excitingly, Se supplementation may protect patients' salivary glands from radiation (102). On the other hand, the use of antioxidants containing Se may reduce the oxidative stress state of the body during iodine treatment (103).

Although there are no recommendations for the addition of compounds containing Se to the medical therapy of thyroid cancer, their anticancer properties have attracted attention. It is worth noting that there is still a need for careful consideration as to whether to use Se-containing compound to intervene in the development and progression of cancer. Apart from possible selenosis caused by excessive Se accumulation, intervention with micronutrient Se in subjects with adequate Se levels failed to reduce the incidence of thyroid cancer (82).

4 Se supplementation and precautions

The daily intake of Se in human is determined by a combination of the Se value in dietary content, the intake of food and the configuration of the diet. The Se content of food is strongly dependent on the Se content of the soil in which plants and animals grow (104–106). In China, about 51% of the regions are deficient in Se (107) Se. A study by Dinh, Q. T. and his team also found that 39-61% of the Chinese population had daily Se intakes below standards according to WHO/FAO recommendations. It is known that Se cannot be synthesized in the human body and daily supplementation is required to meet the body's Se requirements. Therefore, the need for Se supplementation to reduce disease incidence and delay disease progression in people with low baseline Se status has attracted the interest of researchers.

4.1 Types of Se supplements

Se occurs by two modes naturally: both inorganic Se and phytoactive Se. Inorganic Se has a greater cumulative toxicity and is not readily absorbed (108), and is not suitable for use in human. phytoactive Se generally exists as selenomethionine, which is biotransformed to synthesize specific selenoproteins and is not directly toxic even at a high doses (109). Ingested selenomethionine can bind to tissue proteins, especially muscle proteins, forming a reservoir that is slowly released according to the protein turnover rate in the body (42).SeSeSeSe

4.2 Factors affecting the absorption of Se supplements

I. Vitamin E: Adequate vitamin E improves the body's ability to utilize Se and can multiply the Se accumulation in the liver (110).
II. Iron: Patients with iron deficiency anemia are often accompanied by a decrease in glutathione peroxidase activity. In this case, iron should be supplemented along with Se in order to restore its activity to normal levels in a timely manner (111–113).
III. Thiamine-containing amino acids: Thiamine-containing amino acids are the raw materials for the synthesis of glutathione. It directly affects the synthesis of glutathione when the dietary content of thiamin-containing amino acids is low. Therefore, vegetarians with low protein intake have lower levels of both amino acids and Se in their bodies.
IV. Intestinal bacteria: Thyroid disease and intestinal disease commonly coexist, probably because of disruption of the intestinal barrier, where it is easier for antigens to pass through and react with the immune system or with extra-intestinal tissues to intersect and destroy thyroid cells. Lactobacillus and Bifidobacterium are positively associated with Se absorption, and these bacteria are reduced in autoimmune thyroid disease. Escherichia coli, Streptococcus faecalis, Clostridium difficile and certain Salmonella species can bind Se from the body to their own enzymes to use amino acids instead of thiamine-containing amino acids, and can also convert the soluble form of Se to an insoluble form, making it unavailable for absorption by the body (114).

4.3 Manifestations of Se toxicity

A study conducted in China showed that the lowest level of harmful effects observed for Se toxicity was 900-1000ug/day (115). Excessive Se intake can lead to adverse health problems and Se toxicity. The clinical features of Se toxicity mainly include brittle hair and nail loss, gastrointestinal disorders, rash, rickets and neurological disorders (116).

Based on the facts described above, Se status at baseline is the determining element for the assessment of Se supplementation needs. Serum Se measurements should be performed in patients

before, during and after Se supplementation to assess the organism's Se status.

4.4 Assessment of Se status

Se exists in the body in many forms and can be excreted in urine, feces and lungs. Common methods for determining Se include urine (81), blood (79, 80) and hair (117). About 50-60% of ingested Se is known to be excreted in urine (2, 118). By measuring it we can estimate Se intake over a short period of time.

Se levels in the blood often represent the state in which the body uses and accumulates. It is important to note that serum Se (S-Se) does not directly reflect the concentration of Se in tissues, and even normal S-Se content does not exclude the possibility of Se deficiency in the thyroid (119, 120). Selenoprotein P (Se-P) may be a better biomarker of Se nutritional status than S-Se. As the main transportation and storage protein of Se, low levels of Se-P will also ensure Se concentration in various tissues in the event of Se deficiency and disease (121). In the case of Se deficiency, Se-P will decrease first. After Se supplementation, the body will firstly increase the level of selenoproteins such as GPx and then Se-P to normal levels (122).

The Se in hair often represents the nutritional status of Se in the body over a period of weeks or even months. A study conducted in China showed that the Se content of hair is <0.20 mg/kg for Se deficiency, 0.20-0.25 mg/kg for marginal Se deficiency, 0.25-0.50 mg/kg for medium Se nutrition level, and ≥0.50 mg/kg for high Se nutrition level (117). However, whether the universality of this criterion is limited by ethnicity, region and gender remains to be further studied.

5 Summary

The microelement Se performs an essential role in maintaining normal body functions as well as the function of the thyroid axis. Se deficiency is a risk factor for many thyroid disorders, and Se supplementation offers new ideas for clinical practice in thyroid disorders. Although current recommendations for Se therapy extend only to GO patients, Se supplementation has been widely used by clinicians for a variety of other thyroid disorders. More reliable clinical evidence is still needed to determine the role of Se supplementation in thyroid disorders.

Author contributions

FW: Literature retrieval, Paper writing and Paper submission. CL: Literature retrieval. SL: Literature retrieval. LC: Literature retrieval. JZ and LL: Article guidance, Paper revision and Paper submission. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Predicting skip metastasis in lateral lymph nodes of papillary thyroid carcinoma based on clinical and ultrasound features

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Background: Skip metastasis in papillary thyroid cancer (PTC), defined as lateral lymph node metastasis (LLNM) without the involvement of central lymph node metastasis (CLNM), is generally unpredictable. Our study aimed to develop a model to predict skip metastasis by using clinicopathological and ultrasound factors of PTC.

Methods: We retrospectively reviewed the medical records of patients who underwent total thyroidectomy and central lymph node dissection (CLND) plus lateral lymph node dissection (LLND) between January 2019 and December 2021 at the First Affiliated Hospital of Soochow University. Furthermore, univariate and multivariate analyses assessed the clinical and ultrasound risk factors. Receiver operating characteristic (ROC) curves were used to find the optimal cut-off values for age and dominant nodule diameter. Multivariate logistic regression analysis results were used to construct a nomogram and were validated internally.

Results: In all patients, the skip metastasis rate was 15.4% (41/267). Skip metastasis was more frequently found in patients with a tumour size ≤10 mm (OR 0.439; P = 0.033), upper tumour location (OR 3.050; P=0.006) and fewer CLNDs (OR 0.870; P = 0.005). After analysing the clinical and ultrasound characteristics of the tumour, five factors were ultimately associated with lateral lymph node skip metastasis and were used to construct the model. These factors were an age >40 years, tumour diameter <9.1 mm, upper tumour location, non-smooth margin and extrathyroidal extension. The internally evaluated calibration curves indicated an excellent correlation between the projected and actual skip metastasis probability. The nomogram performed well in discrimination, with a concordance index of 0.797 (95% CI, 0.726 to 0.867).

Conclusions: This study screened for predictors of skip metastasis in PTC and established a nomogram that effectively predicted the risk of potential skip metastasis in patients preoperatively. The method can predict and distinguish skip metastases in PTC in a simple and inexpensive manner, and it may have future therapeutic utility.

KEYWORDS

papillary thyroid cancer, skip metastasis, lateral lymph node metastasis, nomogram, factors

Introduction

Papillary thyroid carcinoma is a prevalent endocrine malignancy. It accounts for 90% of thyroid cancer cases, and its incidence is increasing worldwide (1, 2). An abundance of previous studies had reported that cervical lymph node in PTC occurs in a stepwise manner. Generally, lymph node metastasis in PTC involves the central compartment, the ipsilateral lateral compartment, and the contralateral lateral compartment (3–6). However, LLNM without CLNM is also found in PTC; this unpredictable lymph node metastasis pattern is known as "skip metastasis" (7).

In clinical practice, ultrasound is often used for the preliminary examination of cervical lymph node metastasis. Ultrasound has been reported to have poor sensitivity but good specificity in the diagnosis of CLNM. The ultrasound specialist will evaluate the central and lateral cervical regions for suspicious lymph nodes before surgery. Because the presence of thyroid reduces the visualization of the interventricular lymph nodes, it is often easier to ignore LLNM when no CLNM is found. Standard primary surgery can significantly reduce patients' risk of recurrence and distant metastasis, while secondary surgery may significantly increase the incidence of surgical risk and complications. However, preventive LLND for patients without LLNM will also increase surgical complications and medical costs to a certain extent (8–10). Therefore, it is very important to accurately evaluate the status of cervical lymph node metastasis before surgery to select a reasonable surgical method and plan an accurate range of dissection according to the condition.

It has become a major challenge for most thyroid surgeons to control localized regional recurrence (11). A precise preoperative assessment of skip metastasis aids in establishing the surgical window, lowering the risk of recurrence and reducing death rates. The present study aimed to investigate the incidence and clinicopathologic risk factors for skip metastasis. In addition, we established skip metastasis in patients with PTC based on preoperative thyroid ultrasound, laboratory examination and clinical characteristics.

Materials and methods

Patients

This retrospective analysis originally examined PTC patients who underwent total thyroidectomy with LLND plus CLND at the First Affiliated Hospital of Soochow University between January 2019 and December 2021. The pathology section of our hospital classified each case as PTC with LLNM. The skip metastatic and non-skip metastatic groups were created from each set. The exclusion criteria included (1) distant metastases already present or other cancers at the time of diagnosis, (2) neck surgery or radiation history at the time of diagnosis, and (3) limited information or an unknown clinicopathologic profile.

Surgery treatment

All patients underwent total thyroidectomy with LLND plus CLND. This study included both therapeutic and preventive cases of LLND. Therapeutic LLND is performed when LLNM is diagnosed by preoperative ultrasound, CT, and/or FNA. In addition, based on the surgeon's experience, LLND can be performed prophylactically if the patient has high risk factors. CLND was performed to remove all lymph nodes and fibro-fatty tissue from the medial border of the common carotid artery to the midline of the trachea, and from the hyoid bone to the thoracic inlet. The typical therapy for LLNM at our institution is modified LLND incorporating stages II–V with preservation of the spinal accessory nerve, internal jugular vein, and sternocleidomastoid muscle. Unless otherwise noted, level I dissection was not conducted routinely.

Data collection

Basic information, laboratory examination, thyroid ultrasound and pathological factors were collected. Basic information included the patient's sex, age at diagnosis and status of underlying disease (hypertension, diabetes, and hyperlipidaemia). The laboratory indices included thyroid-stimulating hormone (TSH), thyroglobulin antibodies (TgAb), and thyroglobulin (Tg). The characteristics of preoperative thyroid ultrasound of the largest tumour or the most suspicious dominant nodule included the following features: diameter, location, flexibility score, component, echogenicity, shape, margin, ratio of tall to wide, extrathyroidal extension, calcification, and vascularization. Multifocality, bilaterality, and extrathyroidal extension were also enrolled. Histopathologic factors analysed on postoperative pathological examination included maximum tumour size, maximum tumour location, multifocality, bilaterality, extrathyroidal extension (ETE), coexistence of nodular goitre, coexistence of Hashimoto's thyroiditis, number of central/lateral dissected lymph nodes and number of lateral metastatic lymph nodes.

Statistical analysis

All statistical analyses were performed with the SPSS 20.0 package (IBM SPSS Inc., Chicago, USA) and R software (ver. 4.1.3, Institute of Statistics and Mathematics, Vienna, Austria). The chi-square test and the independent t test were performed for categorical and continuous variables, respectively. Multivariate logistic regression analysis was performed for significant factors, and $P<0.05$ was considered to indicate that the differences were statistically significant. ROC curves were constructed to determine the optimal cut-off value. Based on the results of multiple logistic regression analysis, significant predictors were combined to develop a nomogram. The AUC values and calibration curves were used to

examine the discriminatory power and degree of consistency of our prediction model.

Results

Patient characteristics

The study ultimately enrolled 267 patients. We found that skip metastasis occurred in 41 (15.4%) of these patients, and this phenomenon was not detected in the remaining patients. We summarize the demographic and pathological tumour characteristics of these patients in Table 1. A summary of the preoperative ultrasonographic characteristics of the tumours and laboratory tests of all patients is presented in Table 2.

Clinicopathological factors for skip metastasis

Using univariate analysis, we compared the clinicopathological factors of the groups with and without skip metastases. In the group with skipped metastases, the following patient characteristics were more prevalent: age ≥55 (P = 0.022), tumour size ≤10 mm (P < 0.001), upper location (P = 0.040), bilaterality (P = 0.005), fewer CLNDs (P<0.001) and fewer LLNMs (P = 0.005). Furthermore, there were no significant differences in sex, multifocality, extrathyroidal extension, Hashimoto's thyroiditis, nodular goitre, or LLND number between the skip metastasis group and non-skip metastasis group (all P > 0.05). Additionally, multivariate analysis revealed that tumour size ≤10 mm (OR 2.276; 95% CI 1.067-4.852; P = 0.033), upper tumour location (OR 3.050; 95% CI 1.380–6.740; P= 0.006) and fewer CLNDs (OR 0.870; 95% CI 0.789–0.960; P = 0.005) were independent factors for skip metastasis, as shown in Table 1.

Preoperative examination features for skip metastasis

We initially looked at the association between preoperative clinical and ultrasound characteristics and skip metastasis using a univariate analysis to better understand the indicators of skip metastasis. The significant risk factors were as follows: age (P=0.033), tumour diameter (P=0.036), upper tumour location (P=0.035), non-smooth margin (P=0.001), extrathyroidal extension (P=0.038) and BMI ≥25 (P=0.042) (Table 2).

To further investigate the association between age and tumour diameter in PTC patients and the occurrence of skip metastasis, we created a ROC curve for 267 patients with PTC to establish the value of these parameters in predicting skip metastasis. The cut-off age was 40 years old, as shown in Figure 1 [area under the curve (AUC) = 0.586, P = 0.089], and the tumour diameter was 9.1 mm (AUC = 0.643, P=0.003).

Further multivariate analysis indicated that tumour diameter <9.1 mm (OR 4.625; 95% CI 2.092–10.227; P<0.001), upper tumour location (OR 3.025; 95% CI 1.395–6.559; P=0.005), non-smooth margin (OR 4.104; 95% CI 1.874–8.987; P<0.001) and extrathyroidal extension (OR 2.251; 95% CI 1.014–4.996; P = 0.046) were independent predictors of skip metastasis in PTC (Table 3).

Construction of an individualized prediction model

A nomogram was developed for forecasting each individual's probability of skip metastasis based on the independent characteristics assessed using multivariate analysis (Figure 2). The risk of each factor, including the diameter, location, and margin of the dominant nodule and extrathyroidal extension, was quantified in our prediction model based on the results of the multivariate analysis. As previously indicated, the univariate analysis revealed a difference in age between the skip metastasis and non-skip metastasis groups, and in the multivariate analysis, its P value was 0.053, which is close to 0.05. Therefore, we decided to include age as well. It was simple to calculate the estimated chance of skip metastases in LLNM patients by combining the scores for each variable and then drawing a straight line. The likelihood of skip metastases was often higher in the patients with higher total scores. The predicted chance of skip metastases and the actual observed skipped metastases were in good agreement, according to the internally confirmed calibration curves (Figure 3A). As shown in Figure 3B, the performance of the nomogram was validated internally, with an AUC of 0.797 (95% CI 0.726-0.867).

Discussion

When the central lymph node is found to be negative by intraoperative pathology, no further LLND will be performed unless preoperative ultrasound-guided fine-needle aspiration biopsy (FNAB) and imaging demonstrate LLNM (12). However, LLNM *via* preoperative examination is proven to have a significant false-negative rate (4, 13), and the accuracy greatly depends on the pathologists' and ultrasound operators' experience (14). Underestimating skip metastases in PTC will result in insufficient lymph node dissection during surgery, which will ultimately have a negative impact on the prognosis of PTC patients. Therefore, it is crucial for surgeons to perform an accurate preoperative evaluation and prognosis of cervical lymph nodes.

The skip metastasis rate in our study with a large sample was 15.4% (41/267), which is in the range of 0.6% to 37.5% reported in previous studies (15–22). The greater skip metastatic rate shows that the occurrence of skip metastasis in our clinical work has not gone unnoticed.

The clinicopathological characteristics and risk factors for skip lateral lymph node metastasis in PTC patients were examined in this retrospective analysis. In the univariate and multivariate analyses, the rate of skip metastasis was significantly higher in patients with a tumour size ≤10 mm (P = 0.033), upper tumour location (P = 0.006) and fewer CLNDs (P = 0.005). Previously, many studies (18, 20, 23) have reported that skip lymph node

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TABLE 1 Comparison of the clinicopathological factors of skip metastasis and non-skip metastasis in PTC patients.

Variables	Skip metastasis		P value	Multivariate analysis	P value
	Absent (N=226)	Present (N=41)		OR (95% CI)	
Sex					
Female	135	28			
Male	91	13	0.384		
Age (year)					
< 55	202	31			
≥ 55	24	10	**0.022**	2.489(0.934-6.631)	0.068
Diameter of largest tumour (mm)					
≤ 10	67	24			
> 10	159	17	**<0.001**	2.276(1.067-4.852)	**0.033**
Location of largest tumour					
Non-upper	134	17			
Upper	92	24	**0.040**	3.050(1.380-6.740)	**0.006**
Multifocality					
Absent	83	21			
Present	143	20	0.080		
Bilaterality					
Absent	112	30			
Present	114	11	**0.005**	0.491(0.219-1.098)	0.083
Extrathyroidal extension					
Absent	165	30			
Microscopic	34	3			
Gross	27	8	0.222		
Hashimoto's thyroiditis					
Absent	154	31			
Present	72	10	0.340		
Nodular goitre					
Absent	176	29			
Present	50	12	0.319		
CLND number	9.35 ± 5.681	5.83 ± 4.748	**<0.001**	0.870(0.789-0.960)	**0.005**
LLND number	24.54 ± 13.603	22.29 ± 11.858	0.321		
LLNM number	5.78 ± 4.189	3.80 ± 3.422	**0.005**	0.885(0.776-1.010)	0.07

Bold values indicate that P-value is significant.

metastasis is associated with tumour size, and skip metastasis is often found to be more common in PTC patients with a tumour size ≤1 cm. Several of these studies (15, 24, 25) found that the location of the tumour in the upper pole is one of the independent risk factors for the development of skip metastasis in patients with PTC. This could be because the upper pole of the thyroid lobe has a distinct lymphatic drainage system from that of the remainder of the thyroid lobe. Lymphatic flow through the superior thyroid artery is more likely to carry PTC cells from the upper area to the lateral lymph nodes. In addition, we discovered that the probability of skip metastasis was negatively correlated with the number of lymph nodes removed in the central neck (P = 0.005). A small number of central lymph nodes that have been removed may cause the probability of skip metastases to be overestimated (26, 27). A

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TABLE 2 Comparison of the preoperative examination features of skip metastasis and non-skip metastasis in patients with PTC.

Variable	Skip metastasis		P value
	Absent (N=226)	Present (N=41)	
Sex			
Female	135	28	
Male	91	13	0.384
Age (year)	38.95 ± 10.946	43.05 ± 12.779	0.033
US-Multifocality			
Absent	135	21	
Present	91	20	0.389
US-Bilaterality			
Absent	165	34	
Present	61	7	0.242
US-reported dominant nodule			
Diameter	18.00 ± 10.239	14.34 ± 10.317	0.036
Location			
Non-upper	146	19	
Upper	80	22	0.035
Flexibility score			
1	0	0	
2	15	5	
3	97	15	
4	97	16	
5	17	5	0.365
Component			
Solid	201	33	
Cystic-solid	25	8	0.193
Echogenicity			
Hypoechoic	221	38	
Iso/hyperechoic	5	3	0.108
Shape			
Regular	115	15	
Irregular	111	26	0.126
Margin			
Smooth	145	15	
Non-smooth	81	26	0.001
Ratio of tall to wide			
<1	168	34	
≥1	58	7	0.323

(Continued)

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TABLE 2 Continued

Variable	Skip metastasis		P value
	Absent (N=226)	Present (N=41)	
Extrathyroidal extension			
Absent	183	27	
Present	43	14	0.038
Calcification			
Absent/macrocalcification	46	12	
Microcalcification	180	29	0.219
Vascularization			
Absent	40	4	
Present	186	37	0.257
Hashimoto's thyroiditis			
Absent	157	31	
Present	69	10	0.464
Nodular goitre			
Absent	177	29	
Present	49	12	0.313
TSH			
Low	6	3	
Normal	189	31	
High	31	7	0.204
Tg			
Low	54	7	
Normal	135	30	
High	37	4	0.267
TgAb			
Positive	57	8	
Negative	169	33	0.554
BMI			
<25	123	15	
≥25	103	26	0.042
Hypertension			
Absent	196	33	
Present	30	8	0.330
Diabetes			
Absent	213	38	
Present	13	3	0.719
Hyperlipidaemia			
Absent	145	31	
Present	81	10	0.21

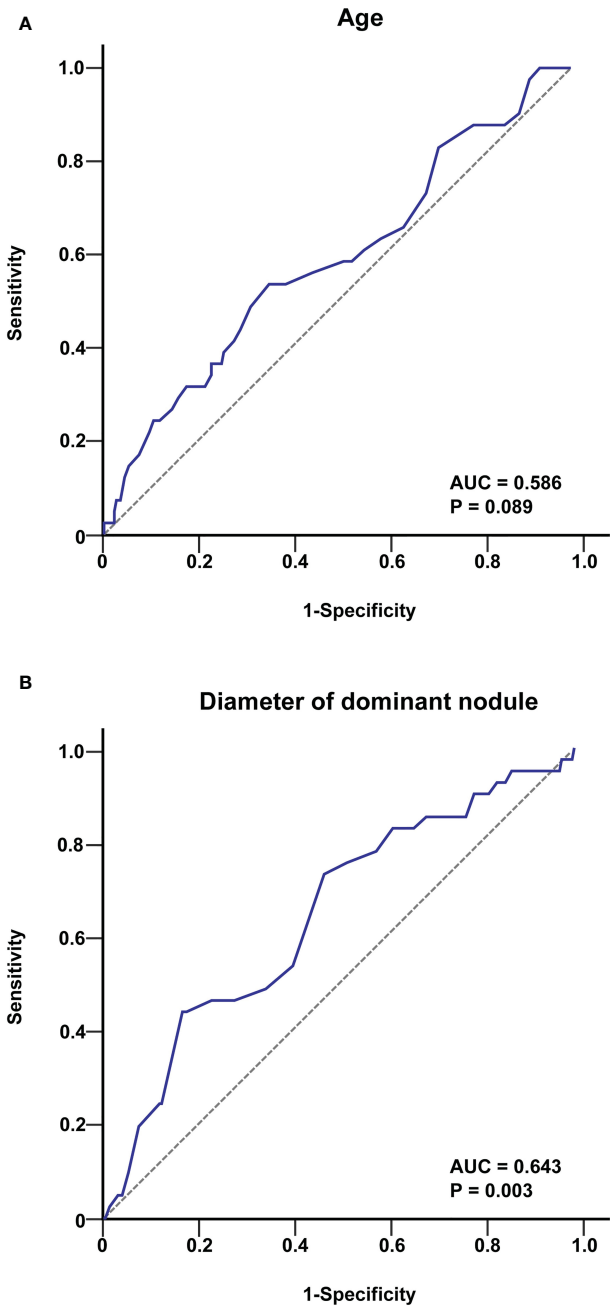

FIGURE 1
ROC curve analysis of age **(A)** and tumour diameter **(B)** for predicting skip metastasis in PTC patients. The cut-off value of age was 40 years, and that for the tumour diameter was 9.1 mm.

total CLND may eradicate all CLNMs and reduce the likelihood of false-positive skip metastasis detection.

The predicted variables associated with skip metastases in PTC were then investigated. Age, tumour diameter, upper tumour site, non-smooth margin, extrathyroidal extension, and BMI ≥25 were all associated with favourable outcomes in the univariate analysis (all P<0.05). Age and tumour diameter have been previously reported as risk factors for skip metastasis, so their relationship with skip rate was further investigated. To identify these parameters' critical levels for predicting skip metastases in 267 PTC patients, we built ROC curves. According to our findings, the tumour diameter was 9.1 mm, and the cut-off age in PTC for skip metastasis was 40 years old. Age > 40 and tumour diameter < 9.1 mm are therefore thought to be used as thresholds for skip metastasis. Further multivariate analysis indicated that tumour diameter <9.1 mm, upper tumour location, non-smooth margin and extrathyroidal extension were independent predictors of skip metastasis in PTC. Furthermore, Zhao et al. (20) discovered by multivariate analysis that an age > 45 years was an independent risk factor for skip metastasis (OR 4.37; 95% CI 1.14-16.66; P = 0.031). Hu et al. (27) discovered that an older age (OR 2.63; 95% CI 1.34-5.04, P = 0.004) was an independent risk factor for skip metastasis. In our multifactorial analysis, the p value for an age

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TABLE 3 Multivariate analysis of the predictive factors for skip metastasis in PTC patients.

Variable	OR (95% CI)	P value
Age (>40 years)	2.102(0.989-4.465)	0.053
BMI (≥25)	1.987(0.922-4.283)	0.080
US-reported dominant nodule		
Diameter (<9.1 mm)	4.625(2.092-10.227)	<0.001
Located in the upper pole	3.025(1.395-6.559)	0.005
Margin (Non-smooth)	4.104(1.874-8.987)	<0.001
Extrathyroidal extension	2.251(1.014-4.996)	0.046

>40 years was 0.053, which is close to 0.05. Therefore, we decided to include age as well.

A predictive nomogram was constructed based on the above significant factors of preoperative clinical and ultrasound features associated with PTC skip metastasis. Nomograms, which have received widespread attention in cancer research (3, 28–30), are a simple and effective tool for identifying high-risk individuals and measuring individual risk. No study has yet reported the use of nomograms to predict skip metastases using more detailed clinical data. A previous study developed several prediction models to discriminate patients with skip metastases from those with LLNM, but their clinical applicability was restricted (14, 20, 24, 27).

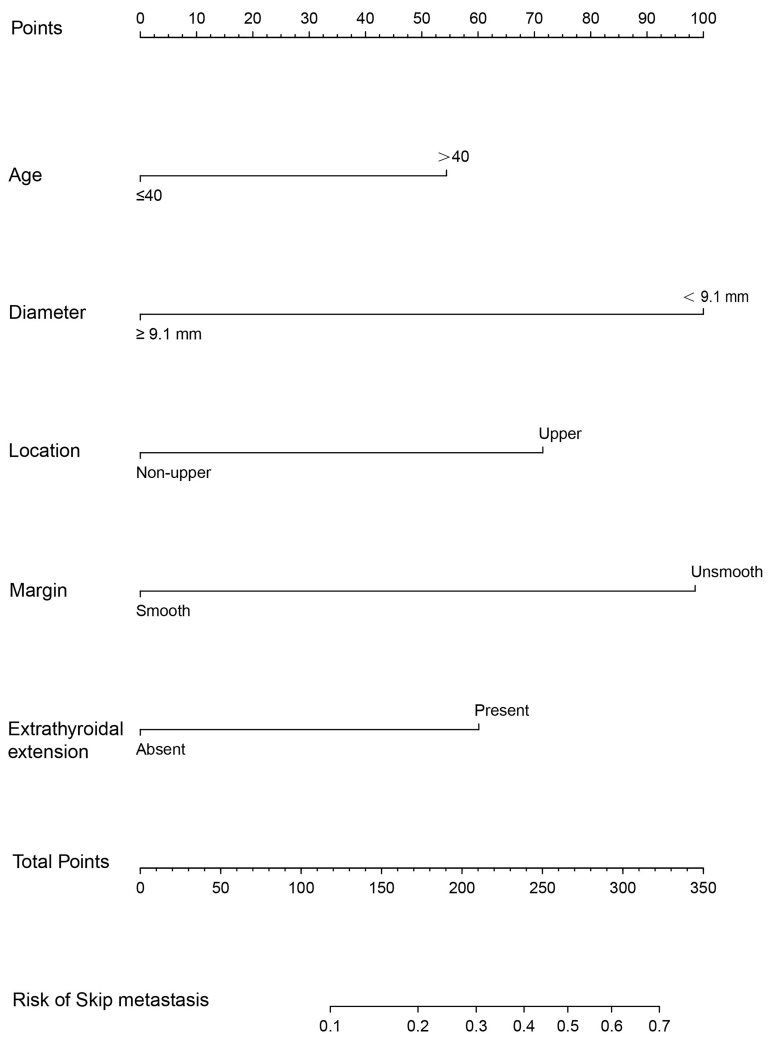

FIGURE 2
The nomogram for predicting the risk of skip metastasis in PTC patients.

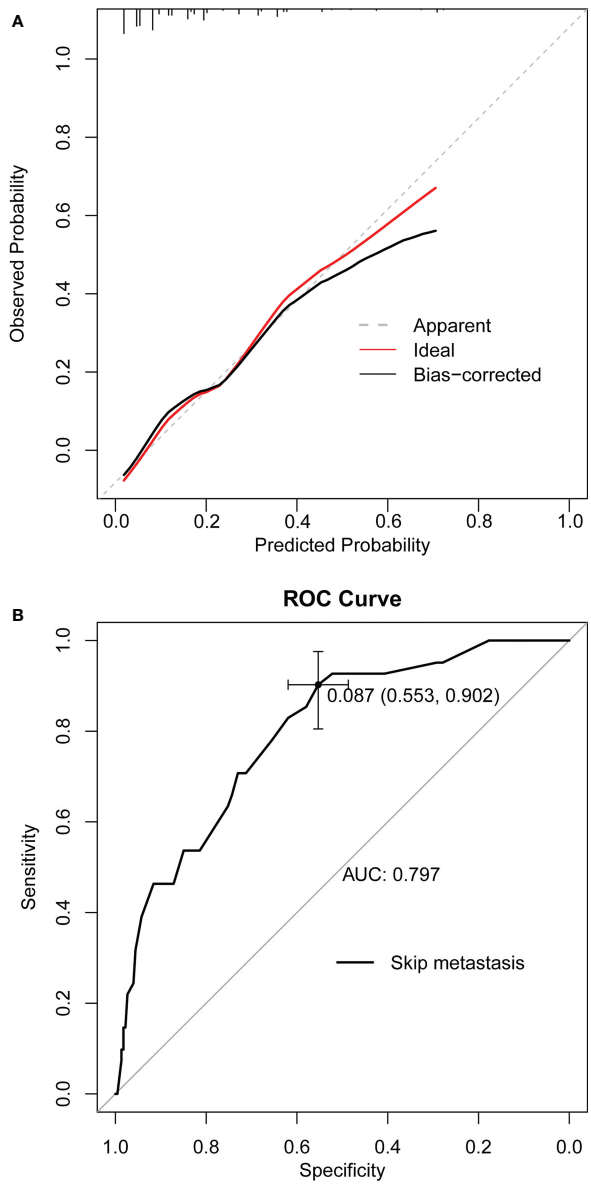

FIGURE 3
(A) Curves with internal validation for the nomogram. **(B)** ROC analysis of the nomogram. The ROC curve of nomograms for skip metastasis. The area under the ROC curve (AUC) was 0.797, 95% CI 0.726-0.867. ROC, receiver operating characteristic; AUC, area under curve.

Due to our ability to identify at-risk skip metastasis patients in the negative CLNM group, we were able to make an informed surgical choice, lessen the likelihood of additional procedures, develop an effective active monitoring plan, and other things. However, our present study has certain drawbacks. First, the current study is a retrospective single-centre investigation. Therefore, its findings can differ slightly from those of other research. To ensure better extrapolation, external validation should be performed, as our nomogram's validation was only performed internally. Last, there is a lack of long-term monitoring and research on the prognosis of skip metastasis in this study. Despite the fact that our nomogram can identify patients with high-risk skip metastases, it is still unclear whether receiving LLND will increase long-term survival. Therefore, we are conducting an intensive study on the prognosis of skip metastases, such as disease recurrence and postoperative radioactive iodine therapy studies. Notwithstanding these shortcomings, our nomogram is based on good clinical data, has sufficient discriminating power, and has been internally validated in patient populations.

Conclusion

In conclusion, we created a prediction nomogram for skip metastasis in PTC patients that can assist in identifying patients who require LLND and are at high risk of skip metastasis. Therefore, using this nomogram can help patients make treatment decisions and provide an individual risk assessment.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the First Affiliated Hospital of Soochow University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

MZ, XS, JC, and BZ conceptualized and designed the study. ZZ, RW, YL, and JL performed analysis. JC and BZ interpreted the data. MZ and XS drafted the manuscript. JC and BZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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The value of FT4/TSH ratio in the differential diagnosis of Graves' disease and subacute thyroiditis

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Objective: To explore the value of the FT4/TSH ratio in the etiological diagnosis of newly diagnosed patients with thyrotoxicosis.

Methods: The retrospective study was conducted on 287 patients with thyrotoxicosis (122 patients with subacute thyroiditis and 165 patients with Graves' disease) and 415 healthy people on their first visit to our hospital. All patients underwent thyroid function tests including the measurement of T3, T4, FT3, FT4, TSH, T3/TSH, and T4/TSH. The receiver operating characteristic (ROC) curve was employed to evaluate the value of FT4/TSH in the differential diagnosis of Graves' disease and subacute thyroiditis, and compared with other related indicators.

Results: The area under the curve of FT4/TSH for diagnosing Graves' disease and thyroiditis was 0.846, which was significantly larger than the area under the curve of T3/T4 ratio ($P< 0.05$) and FT3/FT4 ratio ($P< 0.05$). When the cut-off value of the FT4/TSH ratio was 5731.286 pmol/mIU, the sensitivity was 71.52%, the specificity was 90.16%, the positive predictive value was 90.77% and the negative predictive value was 70.06%. The diagnostic accuracy was 79.44%.

Conclusion: FT4/TSH ratio can be used as a new reference index for the differential diagnosis of thyrotoxicosis.

KEYWORDS

thyrotoxicosis, FT4/TSH ratio, Graves' disease, subacute thyroiditis, differential diagnosis

1 Introduction

Thyrotoxicosis is a series of clinical syndromes characterized by a rapid increase in thyroid hormones in the blood caused by abnormal thyroid function in patients (1). Thyrotoxicosis can be divided into two types according to the different causes: hyperthyroidism and non-hyperthyroidism. The most common cause of thyrotoxicosis

due to hyperthyroidism is Graves' disease (GD), and the most common cause of thyrotoxicosis due to non-hyperthyroidism is subacute thyroiditis (ST) (1–4).

GD is an autoimmune disease. The etiology is primarily due to the presence of circulating anti-thyroid-stimulating-hormone receptor (TSH-R) stimulating autoantibodies in the body, which leads to hyperthyroidism (5). The clinical manifestations are not limited to the thyroid gland but are a syndrome involving multiple systems, including hypermetabolic symptom, diffuse goiter, Graves' ophthalmopathy (GO), pretibial myxedema (PTM), and thyroid acromegaly. Hypermetabolic symptom including heat sensitivity, excessive sweating, weight loss, and fatigue. Menstrual cycle disturbances can occur in female and erectile dysfunction in male. ST, also known as granulomatous thyroiditis, giant cell thyroiditis, is the result of an inflammatory thyroid process of unknown etiology (6). The most common clinical manifestations include neck pain with tenderness and general fatigue. It is worth noting that compared with GD, ST can also show weight loss, heat sensitivity and hyperhidrosis.

In clinical work, there are great similarities between GD and ST in terms of clinical presentations and laboratory thyroid hormone levels. However, significantly different treatments should be chosen due to their different etiologies. Therefore, how to further differentiate GD and ST becomes a question worthy of our consideration, and differentiating GD and ST has an important guideline for the treatment of thyrotoxicosis. We usually make the diagnosis based on the patient's clinical symptoms, and laboratory examinations, combined with the results of the radioactive iodine uptake (RAIU) test and conventional ^{99m}TcO4 thyroid scintigraphy. It is important to note that there are limitations to each of these tests (1). Firstly, subacute thyroiditis usually has a history of upper respiratory tract infection, but in some patients with subacute thyroiditis, there is no history of upper respiratory tract infection or neck pain and other evidence of inflammatory infection. Secondly, thyrotropin receptor antibodies (TRAb) are positive on laboratory tests in GD patients, but in some GD patients, the test is negative. Thirdly, radiological iodine uptake experiments and thyroid imaging are of great clinical value. However, these two tests are radiological and are not permitted during pregnancy and lactation (7). Finally, many regions, especially community hospitals do not have the conditions to carry out nuclear medicine department examinations.

There are some difficulties in the differential diagnosis of patients with subacute thyroiditis and Graves' disease. It is necessary to find a clinical differential diagnosis method that is easy to carry out, rapid, accurate, and applicable to all patients. Laboratory testing has been used in recent years as an easy, rapid, and accurate differential diagnostic method that applies to all patients. Some previous studies have been used in diagnosing GD and ST, including the ratio of total triiodothyronine(T3)/total thyroxine(T4) (8), the ratio of free triiodothyronine (FT3)/free thyroxine (FT4). There are few studies on the value of the FT4/thyroid-stimulating hormone (TSH) ratio. This study aimed to evaluate the usefulness of the ratio of FT4/TSH for differentiating GD from ST, we anticipate discovering a new indicator for the etiology of thyrotoxicosis.

2 Material and methods

2.1 Patients

The Institutional Review Board of The Third Hospital, Hebei Medical University approved this retrospective study (Approval No.: W2022-003-1).

A total of 287 patients with thyrotoxicosis who visited the endocrinology clinic of our hospital for the first time from January 1, 2019, to January 31, 2020, were enrolled. In addition, 415 healthy subjects were selected as the healthy control group at the same time.

The inclusion criteria were as follows: (1) having signs and symptoms of thyrotoxicosis;(2) diffuse thyroid lesions on palpation or color doppler ultrasonography;(3) serum TSH level decreased, with or without changes in serum FT3 and FT4 levels; (4) patients first visit due to thyrotoxicosis without any treatment; (5) patients who can complete the thyroid radioactive iodine uptake (RAIU) test.

The exclusion criteria were as follows:(1) thyrotoxicosis caused by other causes was finally determined (such as pregnancy, multinodular toxic goiter, toxic adenoma, amiodarone-induced thyrotoxicosis and/or exogenous thyroxine intake); (2) concomitant diseases that may affect the measurement of thyroid function;(3) patients with hyperthyroidism complicated with thyroiditis;(4) patients with any one of FT3, FT4, TSH higher or lower than the measured value range.

2.2 Methods

2.2.1 Thyroid function laboratory tests

Peripheral venous blood was collected from all subjects. All the subjects were collected in the morning on an empty stomach, and the specimens were collected from the procoagulant tube containing separation glue. After collection, the specimens were allowed to stand for more than 30 minutes and then centrifugation for 10 minutes at 3500r/min. Specimen examination was completed within 2 hours after sample collection, and the test was carried out by the standard operating procedures of our laboratory. At the same time, low-value and high-value quality controls are used to manage the quality of results. Automated chemiluminescent immunoassays (ADVIA centaur XP; Siemens) were used to determine Serum levels of TSH, FT3, FT4, T3, and T4, and the quality control products were used Bio-Rad. The corresponding reference ranges for serum FT3, FT4, TSH, T3 and T4 were 3.5-6.5pmol/L, 11.5-22.7pmol/L, 0.55-4.78mIU/L, 0.92-2.79 nmol/L and 58.1-161 nmol/L, respectively.

2.2.2 The radioactive iodine uptake test

All subjects were required to undergo radioactive iodine uptake (RAIU) test to confirm the diagnosis, and the examination process

was carried out according to the standard examination process of RAIU test. The final diagnosis report is jointly issued by 2 nuclear medicine physicians.

2.2.3 Diagnostic criteria

According to the 2018 European Thyroid Association Guideline for the Management of Graves' Hyperthyroidism (9) and related references (10). The diagnostic criteria of Graves' disease were as follows: (1) the patient had symptoms and signs of hypermetabolism caused by thyrotoxicosis; (2) diffuse thyroid enlargement (confirmed by palpation and ultrasound), with some cases lacking goiter; (3) laboratory examination showed that the serum T4 level was increased, and the serum TSH level was decreased; (4) exophthalmos and other invasive eye signs; (5) the patient had pretibial myxedema; (6) laboratory tests showed that the TRAb was positive. (7) increased ^{131}I uptake in radioactive iodine uptake (RAIU) test or increased radioactive uptake in ^{99m}TcO4 thyroid scintigraphy. Among them, 1-3 items are essential for diagnosis, and 4-7 items can further provide the basis for the determination of the etiology.

According to the United States Guidelines for the diagnosis and treatment of thyroid diseases (1, 11) and related contents (12), the diagnostic criteria for the thyrotoxicosis stage of ST were as follows: (1) hypermetabolic symptoms and signs of thyrotoxicosis, with or without systemic symptoms of acute inflammation; (2) mild to moderate thyroid enlargement, moderate hardness, obvious or no obvious tenderness, (3) increased serum T4 level, decreased TSH level; (4) decreased ^{131}I uptake in RAIU test or decreased radioactive uptake in $^{99m}TcO_4$ thyroid scintigraphy.

Confirmation of diagnosis of Graves' disease and subacute thyroiditis, two experienced physicians (one from the department of endocrinology and the other from the department of nuclear medicine) were invited to independently make a diagnosis of Graves' disease and subacute thyroiditis based on all clinical data and the diagnostic criteria developed in this study. If the diagnosis was consistent, the diagnosis was established. In cases of disagreement, the diagnosis was made by a third experienced physician.

2.3 Statistical analysis

SPSS 25.0 statistical software was used for data statistics. Normal distribution samples were expressed as mean ± standard deviation (X ± SD), and a one-way analysis of variance was used for the comparison of multiple groups of samples. The results of skewed distribution samples were expressed as median M (interquartile range Q), and the Kruskal-Walis H test was used for the comparison of multiple samples. MedCal 19 statistical software was employed to analyze the ROC curve of the data, and the Area Under Curve (AUC), cut-off value, sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of Graves' disease and subacute thyroiditis were determined. $P<0.05$ was considered statistically significant.

3 Results

3.1 The basic information of the included population was collected

A total of 702 samples were included in this study, including 415 healthy people, 186 males, and 229 females. There were 122 patients with subacute thyroiditis, including 15 males and 107 females. There were 165 patients with Graves' disease, including 39 males and 126 females. The average age was 45.50 ± 12.15 in the healthy population, 50.23 ± 15.93 in the subacute thyroiditis group, and 48.18 ± 14.86 in the Graves' disease group. There was no significant difference in age among the three groups (P=0.206).

The FT3, FT4, TSH, T3, and T4 levels of the normal control group, ST group, and GD group were as follows: (1)FT3(5.174 (0.693) pmol/L, 7.824(4.316) pmol/L, and 15.708(11.327) pmol/L); (2)FT4(16.254(3.096) pmol/L, 25.478(12.707) pmol/L, and 43.086 (27.864) pmol/L); (3)TSH(1.886(1.356) mIU/L, 0.021(0.065) mIU/L, and 0.005(0.005) mIU/L); (4)T3(174.636(42.135) nmol/L, 235.374(163.509) nmol/L, and 435.527(257.981) nmol/L); (5)T4 (107.070(28.380) nmol/L, 163.830(88.688) nmol/L, and 241.230 (136.934) nmol/L). The differences in the levels of FT3, FT4, TSH, T3, and T4 in the three groups were statistically significant (H=374.473, 362.789, 474.602, 277.105, 304.929; $P<0.001$). The levels of FT3, FT4, TSH, T3, and T4 were significantly different between the normal control group and the ST group ($P<0.001$), the normal control group and the GD group ($P<0.001$), and the ST group and the GD group ($P<0.001$) (Table 1 and Figure 1).

The FT3/FT4, T3/T4, and FT4/TSH ratio of the normal control group, ST group, and GD group were as follows: (1) FT3/FT4(0.321 (0.063) pmol/mIU, 0.325(0.071) pmol/mIU, and 0.361(0.086) pmol/mIU); (2) T3/T4(1.643(0.434) pmol/mIU, 1.529(0.671) pmol/mIU, and 1.773(0.548) pmol/mIU); (3) FT4/TSH (8.755 (6.030) pmol/mIU, 1,307.200(3,622.580) pmol/mIU, and 8,292.857(12,177.600) pmol/mIU). The differences in FT3/FT4, T3/T4, and FT4/TSH ratios in the three groups were statistically significant (H=45.122, 27.496, 472.014; $P<0.001$). There was no significant difference in FT3/FT4 ratio between the normal control group and the ST group (P=1.000). However, there were significant differences in FT3/FT4 ratio between the normal control group and the GD group ($P<0.001$) and between the ST group and the GD group ($P<0.001$). There were significant differences in T3/T4 ratio and FT4/TSH ratio between the above 3 groups ($P<0.001$) (Table 1 and Figure 2).

The T3/TSH, and T4/TSH ratio of the normal control group, ST group, and GD group were as follows: (1) T3/TSH(89.317(78.361) pmol/mIU, 10830.913(28360.613) pmol/mIU, and 82621.000 (131762.583)pmol/mIU); (2) T4/TSH(53.978(48.978)pmol/mIU, 7413.676(19114.200)pmol/mIU, and 46440.000(60474.893) pmol/mIU). The differences in T3/TSH and T4/TSH ratios in the three groups were statistically significant (H=469.785, 472.620; $P<0.001$). The levels of T3/TSH ratio and T4/TSH ratio were significantly different between the normal control group and the ST group ($P<0.001$), the normal control group and the GD group

TABLE 1 Baseline characteristics in the patients with Graves' disease, subacute thyroiditis, and healthy control.

Total	HC	ST	GD
	415	122	165
Sex(Male/Female))	186/229	15/107	39/126
Age (years)	45.5 ± 12.15	50.23 ± 15.93	48.18 ± 14.86
FT_3(pmol/L)	5.174(0.693)	7.824(4.316)*	15.708(11.327)*#
FT_4(pmol/L)	16.254(3.096)	25.478(12.707) *	43.086(27.864) *#
TSH(mIU/L)	1.886(1.356)	0.021(0.065) *	0.005(0.005) *#
T_3(nmol/L)	174.636(42.135)	235.374(163.509) *	435.527(257.981) *#
T_4(nmol/L)	107.070(28.380)	163.830(88.688) *	241.230(136.934) *#
FT_3/FT_4	0.321(0.063)	0.325(0.071)	0.361(0.086) *#
T_3/T_4	1.643(0.434)	1.529(0.671) *	1.773(0.548) *#
T_3/TSH	89.317(78.361)	10830.913(28360.613)*	82621.000(131762.583)*#
T_4/TSH	53.978 (48.978)	7413.676(19114.200)*	46440.000(60474.893)*#
FT_4/TSH (pmol/mIU)	8.755(6.030)	1,307.200(3,622.580) *	8,292.857(12,177.600) *#

*Compared with healthy control group, $P<0.05$, #Compared with subacute thyroiditis group, $P<0.05$.
Normal control group (HC); Subacute thyroiditis group (ST); Graves' disease group (GD).

($P<0.001$), and the ST group and the GD group ($P<0.001$) (Table 1 and Figure 2).

Our study results by plotting ROC curves, the ROC area of FT3, FT4, TSH, T3, T4, FT3/FT4 ratio, T3/T4 ratio, T3/TSH ratio, T4/TSH ratio and FT4/TSH ratio in the differential diagnosis of Graves' disease and subacute thyroiditis was 0.806, 0.790, 0.818, 0.771, 0.727, 0.654, 0.658,0.843,0.831 and 0.846, respectively (Figure 3). Notably, the AUC of the FT4/TSH ratio was larger than that of all other parameters, and the differences were statistically significant ($P<0.05$). The cut-off value of 5731.286 pmol/mIU for FT4/TSH ratio for differential diagnosis of subacute thyroiditis and Graves' disease had a diagnostic sensitivity of 71.52%, specificity of 90.16%, a positive predictive value of 90.77%, a negative predictive value of 70.06%, and diagnostic accuracy of 79.44% (Table 2).

4 Discussion

As the study of Graves' disease and subacute thyroiditis progress, clinicians need to get some new clinical diagnostic indicators with the aim of better differential diagnosis. We are always looking for quick and easy methods to help diagnose thyroid disease correctly. With the advances in thyroid hormone testing methods in recent years, we have been able to study thyroid hormone levels more comprehensively, especially in terms of thyroid hormone ratios. As early as 1978, a related study concluded that the ratio of T3/T4 can be used to distinguish Graves' disease from thyroiditis. T3/T4 ratio has been considered an ancillary tool in delineating the etiology of thyrotoxicosis, with T3/T4 >20ng/mg suggesting hyperthyroidism (1, 8). However, T3 and T4 are the products of the combination of hormones and thyroid-binding globulin (TBG). Their levels are directly affected by plasma proteins, so theoretically the accuracy of TT3 and TT4 is limited. Currently, it is more commonly used to replace TT3 and TT4 with FT3 and FT4, which are less influential factors (13–15). There are various related studies showed that the value of the FT3/FT4 ratio in determining the etiology of thyrotoxicosis (13, 16). Wu (17) et al. 's study suggested that FT3/FT4 ratio was a better indicator for the differential diagnosis of Graves' disease and subacute thyroiditis, and the area under the ROC curve was 0.86 (95%CI: 0.84-0.88), the cut-off value was 1.99pmol/mIU, the sensitivity was 79%, and the specificity was 80%. Recent studies have shown that inferior thyroid artery blood flow, T3/T4 ratio and FT3/FT4 ratio are useful parameters in the differentiation between Graves' disease and Destructive thyroiditis (DT) (18). The three parameters in combination yielded a positive predictive value of 100% in the diagnosis of Graves' disease. The results of the FT3/FT4 ratio in the differential diagnosis of Graves' disease in this study are not ideal. The reason for this result may be the elevated levels of FT3 and FT4 in the patient's serum due to thyrotoxicosis caused by ST. The degree of elevation is similar to the level of FT3 and FT4 elevation caused by GD. Therefore, in our study, the results of the FT3/FT4 ratio differential diagnosis of Graves' disease need further justification. Yoshimura Noh et al. (19) concluded that there is some overlap between the FT3/FT4 ratios of patients with painless thyroiditis and those of patients with GD. When FT4 levels are much higher than FT3, this ratio can be helpful in distinguishing between painless thyroiditis and GD. Among the thyroid-based studies, the aspect of the FT3/FT4 ratio and TT3/TT4 ratio is a hot topic for scientific researchers. However, the findings of the FT3/FT4 ratio and TT3/TT4 ratio in the differential diagnosis of GD and thyroiditis are not yet uniform. Therefore it is important to find new indicators for a more reliable diagnosis (19–22).

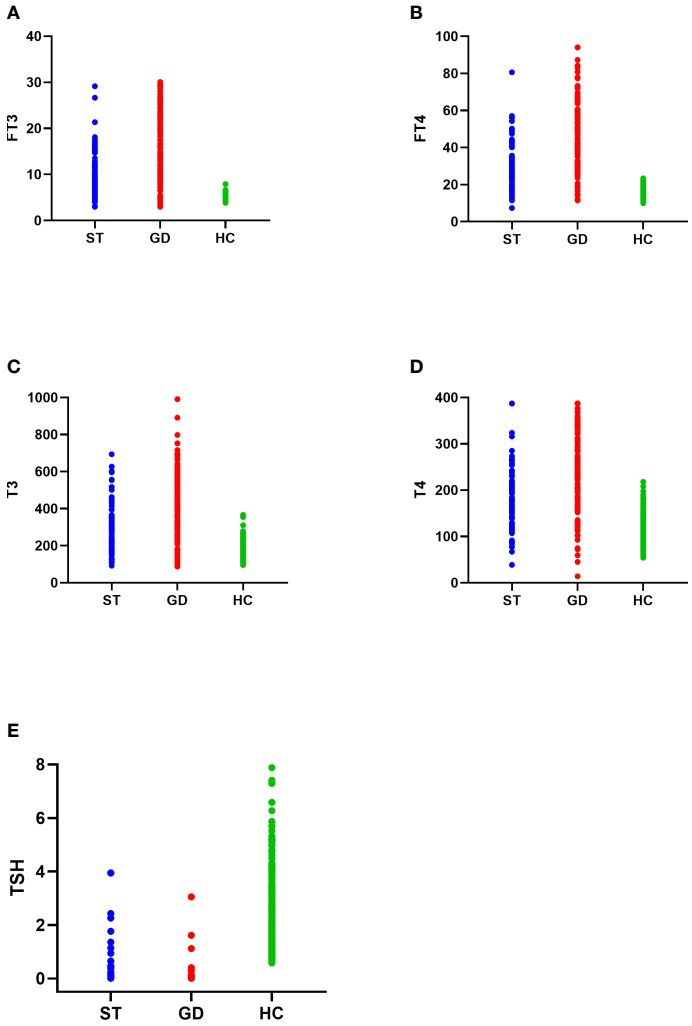

FIGURE 1
The distribution of FT3 **(A)**, FT4 **(B)**, T3 **(C)**, T4 **(D)**, and TSH **(E)** in the normal control group (HC), subacute thyroiditis group (ST) and Graves' disease group (GD), respectively.

This study demonstrated the value of FT4/TSH in the diagnosis of Graves' disease and subacute thyroiditis. The area under the ROC curve for the differential diagnosis of Graves' disease and subacute thyroiditis was 0.846. When the cut-off value for the differential diagnosis of Graves' disease and subacute thyroiditis was 5731.286 pmol/mIU, the sensitivity of the diagnosis was 71.52%, the specificity was 90.16%, the positive predictive value was 90.77%, the negative predictive value was 70.06%, and the diagnostic accuracy was 79.44%. In our study, ROC analysis of the T3/TSH ratio and T4/TSH ratio showed that their AUC were 0.843(95% confidence interval: 0.795-0.883) and 0.831(95% confidence interval: 0.782-0.872), which were lower than the AUCs of the TRAb level and FT4/TSH ratio (Table 2). The FT4/TSH ratio can be used as a reference indicator for the differential diagnosis of Graves' disease and has great advantages in reflecting thyroid function. On the one hand, the characteristics of FT4 are such that it is not influenced by changes in plasma protein concentration. On the other hand, FT4 is the first to show feedback regulation fluctuations in the presence of abnormal thyroid function. However, FT4 may fluctuate to a lesser extent and overlap with the normal range, so it is necessary to use TSH as a ratio to improve the diagnostic positivity. TSH is sensitive to changes in feedback from thyroid hormones. With the advances in detection techniques, we can find a significant difference between the degree of TSH decreases in Graves' disease and that in thyroiditis. Studies have shown a 2-fold change in FT4 and an approximately 100-fold change in TSH values (23). The increase of FT4 and its negative feedback inhibition of TSH can be used to comprehensively judge the changes in thyroid hormones. Overall, we use the FT4/TSH ratio as a new indicator to differentiate Graves' disease from subacute thyroiditis.

There were several limitations in this study, First, the sample size is small, and we need to enlarge the study considering a greater number of patients in order to provide further validation of the results. Second, GD and ST can be directly diagnosed in most cases, we should demonstrate the usefulness of the FT4/TSH ratio in the diagnosis of patients with GD without TSH receptor antibodies and

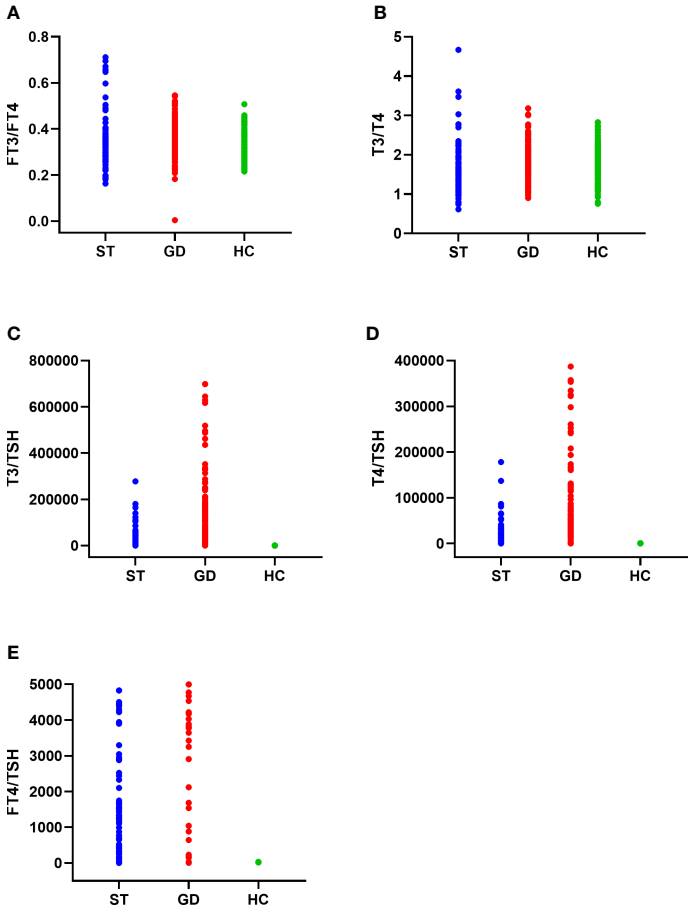

FIGURE 2
The distribution of FT3/FT4 ratio **(A)**, T3/T4 ratio **(B)**, T3/TSH ratio **(C)**, T4/TSH ratio **(D)**, and FT4/TSH ratio **(E)** in the normal control group (HC), subacute thyroiditis group (ST) and Graves' disease group (GD), respectively.

patients with ST with insignificant thyroid tenderness and/or weak systemic inflammatory symptoms in the future.

It should be noted that 20 samples were deleted according to the exclusion criteria. The samples were deleted because FT4 was greater than the upper limit (≥12 ng/dl, 2 samples) or TSH was below the lower limit of detection measurement (≤0.001 ulU/ml, 18 samples) and the ratio could not be calculated. According to our follow-up survey, these 20 samples were found to be finally diagnosed as patients with Graves' disease. Corollary to this, we suggest that Graves' disease is more likely to be diagnosed when FT4

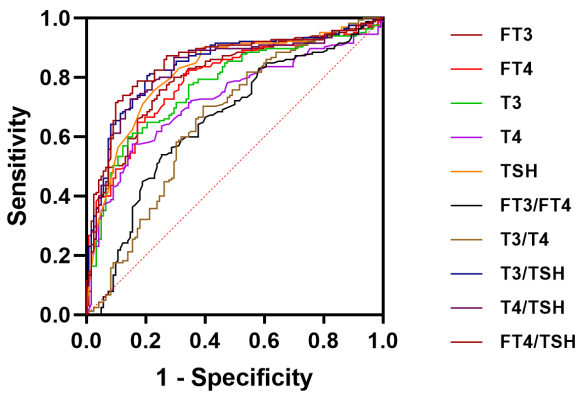

FIGURE 3
ROC curve analysis for FT3, FT4, T3, T4, TSH, FT3/FT4 ratio, T3/T4 ratio, T3/TSH ratio, T4/TSH ratio, and FT4/TSH ratio.

TABLE 2 Results of each parameter in the differential diagnosis of Graves' disease and subacute thyroiditis.

Variables	AUC	95%CI	cut-off value	Sensitivity (%)	Specificity (%)	Positive Predictive Value(%)	Negative Predictive Value(%)	Diagnostic Accuracy(%)
FT3	0.806*	0.756 - 0.850	>10.641	75.76	75.41	79.62	69.23	74.91
FT4	0.790*	0.738 - 0.836	>29.025	81.21	67.21	77.01	72.57	75.26
TSH	0.818*	0.768 - 0.861	≤0.008	75.76	77.05	81.70	70.15	76.31
T3	0.771*	0.718 - 0.818	>369.877	60.00	86.07	84.62	61.18	70.73
T4	0.727*	0.672 - 0.778	>220.59	57.58	84.43	83.33	59.54	68.99
FT_3/FT_4	0.654*	0.596 - 0.709	>0.353	53.94	74.59	72.95	53.94	62.02
T_3/T_4	0.658*	0.599 - 0.712	>1.593	70.30	60.66	70.73	60.16	66.20
T_3/TSH	0.843	0.795- 0.883	>36419.289	80.61	79.51	84.18	75.20	80.14
T_4/TSH	0.831	0.782- 0.872	>27520	72.73	85.25	86.96	69.80	78.05
FT_4/TSH	0.846	0.799 - 0.886	>5731.286	71.52	90.16	90.77	70.06	79.44

* Comparison with FT4/TSH ratio, the difference in area distribution under the ROC curve is considered statistically significant. $P<0.05$.

values are extremely high or TSH values are extremely low. The FT4/TSH ratio is more instructive only when FT4 is not yet reached the upper limit of detection and the TSH is not yet reached the lower limit of detection.

The FT4/TSH ratio can be used as a new indicator for the differential diagnosis of Graves' disease and subacute thyroiditis. The FT4/TSH ratio can provide a basis for the correct clinical diagnosis of the disease, especially if the patient is unable to perform radioactive tests such as the radioactive iodine uptake (RAIU) test or $^{99m}TcO_4$ thyroid scintigraphy, or the hospital is unable to perform these tests.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

YW, PX, and LW contributed to the conception and design of the study. JH and WW organized the database. MG, ZD, and XH performed the statistical analysis. ML and YZ wrote the first draft of the manuscript. XG, JG, YW, and PX wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Serum IL-27 levels increase in subjects with hypothyroidism and are negatively correlated with the occurrence of nonalcoholic fatty liver disease

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Background: The level of serum interleukin-27 (IL-27) was significantly decreased in the obesity group. After injection of IL-27, obese mice showed significant weight loss,reduced fat accumulation, improved insulin resistance and hepatic steatosis.IL-27 plays a key role in the regulation of metabolic processes, but there are scarce data on circulating IL-27 levels in hypothyroidism. The purpose of this study was to assess the serum levels of IL-27 in patients with hypothyroidism and its relationship with NAFLD.

Methods: 185 participants were included in this cross-sectional survey. According to thyroid function, the subjects were classified into three groups: euthyroidism (n = 55), subclinical hypothyroidism (n = 53), and hypothyroidism (n = 77). Serum IL-27 concentrations were measured by ELISA.

Results: Serum IL27 levels were significantly higher in subclinical hypothyroidism and hypothyroidism groups than in the euthyroidism group. Serum IL27 levels had a negative correlation with HOMA-IR,FBG,TG, subcutaneous fat,and visceral fat, and had a positive correlation with HDL-C ($P< 0.05$). Furthermore, logistic regression analysis indicated that IL-27 levels, HOMA-IR, and visceral fat showed significant associations with NAFLD after complete adjustment ($P< 0.05$). ROC curves showed that theoptimal cut-off value of serum IL-27 for discriminating NAFLD was 95.87pg/mL. The area under the ROC curve was 77.3% (95% CI = 0.694-0.851, $p < 0.001$).

Conclusions: Serum IL-27 levels demonstrated a compensatory increase in patients with subclinical hypothyroidism or hypothyroidism and showed an independent association with NAFLD. Circulating IL-27 levels could predict the occurrence of NAFLD in hypothyroidism. These results suggested that altering the circulating levels of IL-27 may be a potential therapeutic target for NAFLD.

KEYWORDS

hypothyroidism, IL-27, NAFLD, dyslipidemia, lipid metabolism

Introduction

Hypothyroidism has an association with an increased risk of developing metabolic syndromecomponents, including obesity, insulin resistance and nonalcoholic fatty liver disease, which is widely prevalent in the common population (1). Clinical and subclinical hypothyroidism is an independent risk factor for NAFLD (2). The underlying mechanisms between NAFLD and hypothyroidism include oxidative stress, insulin resistance, dyslipidemia,metabolic syndrome, and direct action of TSH on hepatocytes (2, 3). Thyroid hormones play a key role in hepatic lipid metabolism, stimulating hepatic lipogenesis and causing hepatic fat accumulation through the thyroid hormone β receptor (4). Certain adipocytokines, such as interleukin-1, tumor necrosis factor-α (TNF-α), visfatin and leptin, and increased oxidative stress that occurs in some cases of hypothyroidism, may coincide with the development of insulin resistance (5). However,the biological mechanisms underlying the development and progression of NAFLD in patients with hypothyroidism are not fully understood (6).

NAFLD is a continuous process from simple steatosis to NASH and eventually to cirrhosis, where there is no effective treatment other than lifestyle modification and regular physical activity (7, 8). In overweight/obese NAFLD, a 7–10% weight loss is the target of most lifestyle interventions, and results in improvement of liver enzymes and histology. Dietary recommendations should consider energy restriction and exclusion of NAFLD-promoting components, macronutrient composition should be adjusted according to the Mediterranean diet, Both aerobic exercise and resistance training effectively reduce liver fat (9). Therefore, early identification of patients at a high risk of NAFLD is extremely important.Liver biopsy is the gold standard for the diagnosis of NADLD, but it has not been widely accepted due to its aggressiveness and high cost (10). At present,non invasive testing is used as a risk assessment tool for steatosis and fibrosis,clinicians mainly use ultrasound to detect fatty liver. Nevertheless, it depends on the experience and skill level of the operator (11). In addition, magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) accurately determine the content of liver fat and the degree of fibrosis, but they have not been widely used in clinical practice due to the high cost (12). Therefore,noninvasive methods are urgently needed to identify NAFLD, monitor treatment effects, andtrack disease processes (13).

As a member of the IL-6/IL-12 cytokine family, IL-27 consists of two subunits, EBI3 and P28 (14, 15). IL-6 production has an association with decreased energy expenditure and increased body fat mass (16). The IL-27-IL-27Rα signaling pathway plays a key role in enhancing insulin resistance, promoting thermogenesis, and combating diet-induced obesity, which is a promising target for anti-obesity immunotherapy (17). IL-27 may be a novel target for the clinical treatment of metabolic diseases in the future. Currently, there are few inconsistent data on circulating IL-27 levels in patients with hypothyroidism. Hence, the purpose of this study was to assess the serum levels of IL-27 in patients with hypothyroidism and its relationship with NAFLD. Furthermore, the diagnostic accuracy of IL-27 was measured in the detection of NAFLD in hypothyroidism.

Materials and methods

Study group

A total of 185 patients (20-70 years) were included in this cross-sectional study. 77 patients were newly or previously diagnosed with clinical hypothyroidism, and 53 patients were newly or previously diagnosed with subclinical hypothyroidism, but had not received thyroid hormone replacement therapy within 3 months. 55 patients had normal thyroid functions. Exclusion criteria were: acute or chronic virus hepatitis, hepatolenticular degeneration, total parenteral nutrition, drug-induced liver disease, previous chronic heavy drinking, liver or kidney disease, heart failure,myocardial infarction breastfeeding or pregnancy, chronic inflammation, acute infection, and other specific diseases that cause fatty liver. Participants were recruited from the Endocrine Clinic at Beijing Chaoyang Hospital, Affiliated to Capital Medical University. All patients signed the informed consent, andthis study was approved by the Ethics Committee (2019-science-363) of Beijing Chaoyang Hospital, Capital Medical University.

Biochemical and anthropometric measurements

Body weight, height, and waist were measured by trained personnel. Subcutaneous fat and visceral fat were measured by bioelectrical impedance analysis (OMRON, HDS-2000 DUALSCAN). Blood samples were taken from all subjects the next morning after fasting. BMI was calculated as weight(kg)/height(m)^2. HDL-C, LDL-C, TG,TC, and FBG were tested using an autoanalyzer (Hitachi 747, Germany). Hemoglobin A1c (HbA1c) was detected using an HLC-723G7 analyzer (Tokyo, Japan) through high-performance liquid chromatography. Fasting insulin(FINS) was detected using chemiluminescence. Serum IL-27 levels were evaluated using an ELISA kit (R&D Systems, Minneapolis, MN, USA). Homeostasis model assessment–insulin resistance (HOMA-IR) was calculated as FINS (mIU/L) × FBG (mmol/L)/22.5 (18).

Definition

According to thyroid function, all participants were classified into three groups: euthyroidism, subclinical hypothyroidism,TSH (>4.78uIU/mL) levels with normal FT4 and FT3 levels, and hypothyroidism, TSH (>4.78uIU/mL) levels with decreased FT4 levels. Reference values for thyroid function tests were as follows: TT4, 4.5-10.9ug/dL;FT4, 0.89-1.76ng/dL; TSH, 0.55-4.78uIU/mL.NAFLD was defined as no history of drinking or drinking< 210g/week (<140g/week for women), and ultrasound imaging outcomes were consistent with diffuse fatty liver disease (19).

Statistical analysis

Data were analyzed with SPSS 26.0(IBM Corporation, New York) and GraphPad Prism9.0(Inc,CA,USA). The normal

distribution of data was represented by the mean ± standard. Non-normally distributed data were represented bythe median and interquartile range. One-way ANOVA or Kruskal-Wallis test and Bonferroni *post hoc* test were used for comparison between groups. In addition, covariance (ANCOVA) and Bonferroni *post hoc* tests were used to compare the potential confounders of the adjusted differences between groups. The association between serum IL-27 levels and NAFLD was investigated using Pearson analysis. To further explore the association between serum IL-27 levels and dyslipidemia, linear regression was conducted.ROC curves were used to define the cut-off value of IL-27. The significance was indicated as $P < 0.05$, and all tests were two-tailed.

Results

Characteristics of study groups

The clinical characteristics of all the participants are shown in Table 1. The subjects were classified into three groups. Among the three groups, sex, age, ALT, AST, GGT, TG, LDL-C, HOMA-IR, fasting glucose, and subcutaneous fat showed no significant differences. Compared with the euthyroidism group,patients with hypothyroidism tended to have higher levels of BMI, TC, and visceral fat ($P <0.05$). The cytokine level of IL-27showed a higher serum concentration in patients with hypothyroidism compared to the euthyroidism group at 119.13(16.78,131.35) versus 8.94 (4.65,32.07) pg/mL. Serum IL-27 levels were higher in the subclinical hypothyroidismgroup than in the euthyroidism group. There was no statistically significant difference in IL-27 between hypothyroidism and subclinical hypothyroidism patients (Figure 1).

Correlation between clinical parameters and serum IL-27 levels in hypothyroidism

Correlation analysis was used to investigate the association between serum IL-27 levels and metabolic parameters in patients with hypothyroidism (Figure 2). The concentration of circulating IL-27 was negatively related to TG(r=-0.192,P=0.028), FBG(r=-0.269, P=0.002), subcutaneous fatr=-0.391, P<0.001), visceral fat (r=-0.569, P<0.001), HOMA-IR(r=-0.411, P<0.001), and was positively related to HDL-C(r=0.39, P<0.001).

Independent relationship between serum IL-27 levels and NAFLD in hypothyroidism

According to the diagnosis of NAFLD, patients with hypothyroidism or subclinical hypothyroidism were classified into two groups.There was no significant difference in TC, LDL-C, sTSH, FT3, and FT4 between the two groups. Patients with NAFLD had higher age, BMI, AST, ALT, GGT, TG, FBG, HOMA-IR, subcutaneous fat, visceral fat and lower HDL-C,IL-27 compared to those without NAFLD(Table 2). To identify whether serum IL-27 levels had an independent correlation with NAFLD,

TABLE 1 Clinical and biochemical characteristics of all the subjects.

Variable	Clinical Hypothyroidism n=77	Subclinical hypothyroidism n=53	Euthyroidism n=55	*P*
Sex,male,n(%)	17 (22.1)	7 (13.2)	7 (12.7)	0.262
Age,years	45.49 ± 11.78	43.62 ± 12.43	41.29 ± 8.64*	0.105
BMI,kg/m2	25.09 ± 4.31	23.99 ± 4.03	23.36 ± 3.09*	**0.040**
AST,U/L	23.0 (18.0,29.0)	21.0 (18.0,26.0)	20 (17.75,23.67)	0.078
ALT,U/L	20.0 (13.0,32.0)	18.0 (13.0,29.0)	16.0 (12.0,24.0)	0.115
GGT,U/L	19.0 (12.0,25.6)	17.0 (11.6,24.5)	18.0 (12.0,25.5)	0.436
TG,mmol/L	1.48 (0.81,1.84)	1.42 (0.95,2.04)	1.08 (0.91,1.44)	0.162
TC,mmol/L	5.38 (4.78,6.30)	4.87 (4.22,6.00)*	4.92 (4.23,5.58)**	**0.013**
LDL-C,mmol/L	3.12 (2.69,4.30)	2.88 (2.49,4.3)	2.92 (2.47,3.57)	0.172
FBG,mmol/L	4.57 (4.29,5.14)	4.81 (4.26,5.13)	4.56 (4.24,4.83)	0.220
HOMA-IR	1.73 (1.21,2.16)	1.82 (1.39,2.64)	1.76 (1.38,2.19)	0.298
sTSH,uIU/ml	55.92 (9.4,105.2)	6.5 (5.5,10.5)***	2.3 (1.6,2.9)***,###	**<0.001**
Visceral fat,cm^2	75.0 (43.0,92.0)	66 (41.0,94.0)	62.0 (40.5,76.0)*	**0.017**
Subcutaneous fat,cm^2	186.0 (144.0,241.0)	192.0 (166.0,245.0)	184.5 (124.75.249.25)	0.190
IL-27,pg/mL	119.13 (16.78,131.35)	96.78 (13.87,132.00)	8.94 (4.65,32.07)***,###	**<0.001**

Data were presented as the mean ± SD or median (interquartile range). P values for categorical variables were calculated using Chi-square test, and P values for continuous variables were calculated using one-way ANOVA test or Kruskal–Wallis test with the Bonferroni post hoc test. Bold indicates P value < 0.05. *Compared with hypothyroidism. *P < 0.05; **P < 0.01; ***,### P < 0.001. BMI, body mass index; AST, aspartate tansaminase; ALT, alanine transaminase; GGT, gamma glufamyl transferase; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholestero; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment -insulin resistance.

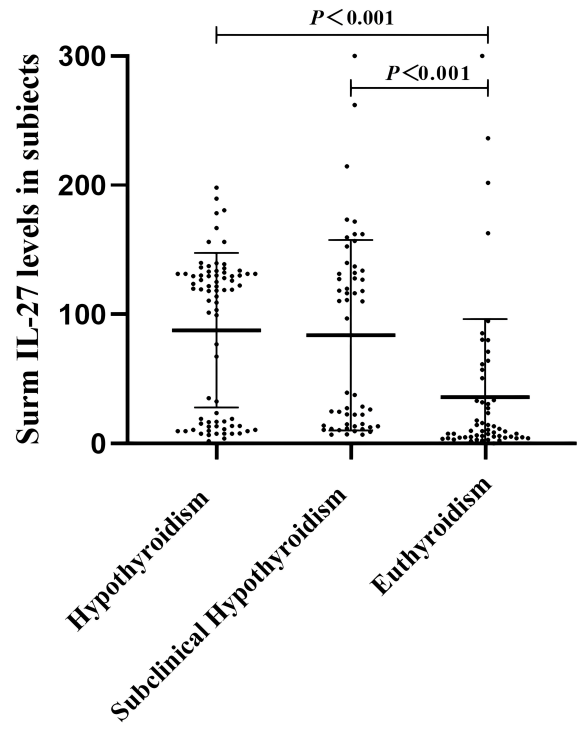

FIGURE 1
Comparison of IL-27 levels among the three groups.

logistic regression analysis was performed among all participants (Table 3). The results indicated that lower serum IL-27 levels had a significant association with NAFLD [OR (95%CI), 0.97 (0.96-0.99)] (P < 0.001). In addition,higher HOMA-IR[OR (95%CI), 16.85 (2.18,130.45)] and visceral fat[OR (95%CI), 1.10(1.01,1.19)](P < 0.05) also had an independent association with NAFLD.

Finally, the diagnostic value of IL-27 for NAFLD was analyzed by ROC curves (Figure 3). The optimal cut-off value of serum IL-27 for discrimination of NAFLD was 95.87pg/mL (AUC = 0.773, P < 0.001).

Discussion

In this study, a compensatory increase in the concentration of serum IL-27 was observed in patients with hypothyroidism or subclinical hypothyroidism. Serum IL-27 levels were negatively associated with FBG, HOMAIR, subcutaneous fat, visceral fat, and TG, and positively associated with HDL-C in patients with hypothyroidism. More notably, lower circulating IL-27 levels in patients with hypothyroidism were independently related to NAFLD. These results suggested that IL-27 could be a promising therapeutic target for NAFLD in patients with hypothyroidism.

Hypothyroidism is a common endocrine disorder characterized by increased sensitivity to cold and unexpected weight gain, suggesting a change in response to cold and energy expenditure (20). Thyroid hormones play an important role in the normal function and cold-induced thermogenesis of brown adipose tissues in rodents (21, 22). Wang et al. reported that IL-27Rα-deficient mice were cold-intolerant because of impaired adaptive thermogenesis, and IL-27 improved thermogenesis and directly targeted adipocytes to counteract obesity (17). Other studies have shown that IL-27 exerts its beneficial effects by the upregulation of adaptive thermogenesis in brown adipose tissues (23). Consistent with their findings, a compensatory increase in the concentration of serum IL-27 was observed in patients with hypothyroidism or subclinical hypothyroidism. IL-27 may combat hypothermia in patients with hypothyroidism through its febrile effects.

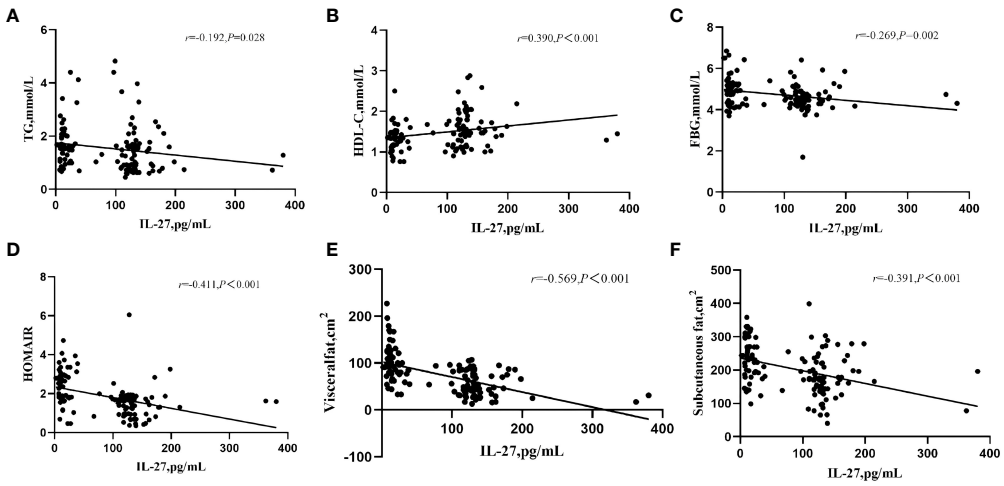

FIGURE 2
Correlation of serum IL-27 levels with TF **(A)** HDL-C **(B)**, FBG **(C)**, HOMIR **(D)**, visceral fat **(E)**, and subcutaneous fat **(F)** in Hypothyroidism. *P* value <0.05 were considered statistically significant.

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TABLE 2 Comparison of main metabolic related indexes between NAFLD and non-NAFLD patients with hypothyroidism.

Variable	NAFLD n=48	non-NAFLD n=82	*P*
Sex,male,n(%)	14 (29.16)	10 (12.19)	**0.016**
Age,years	49.06 ± 11.88	42.19 ± 11.45	**0.001**
BMI,kg/m^2	27.94 ± 3.97	22.70 ± 2.99	**<0.001**
AST,U/L	24.0 (20.2,28.0)	21.0 (17.0,26.5)	**0.060**
ALT,U/L	22.0 (16.0,32.5)	17.0 (12.0,28.0)	**0.028**
GGT,U/L	24.0 (16.5,34.5)	14.0 (11.0.19.0)	**<0.001**
TG,mmol/L	1.67 (1.32,2.27)	1.05 (0.74,1.62)	**<0.001**
TC,mmol/L	5.25 (4.22,6.46)	5.10 (4.54,6.21)	0.622
HDL-C,mmol/L	1.25 (1.03,1.44)	1.51 (1.30,1.79)	**<0.001**
LDL-C,mmol/L	3.59 ± 1.23	3.24 ± 1.06	0.106
FBG,mmol/L	5.05 ± 0.75	4.57 ± 0.63	**<0.001**
HOMA-IR	2.77 (1.82,3.18)	1.59 (0.93,1.81)	**<0.001**
Subcutaneous fat,cm^2	243.53 ± 59.22	178.93 ± 62.75	**<0.001**
Visceral fat,cm^2	96.0 (83.5,124.0)	48.0 (31.5,76.5)	**<0.001**
sTSH,uIU/mL	11.53 (6.34,63.28)	10.58 (6.11,72.71)	0.952
FT3,pg/mL	2.90 (2.35,3.38)	2.87 (2.23,3.26)	0.337
FT4,ng/dL	0.91 (0.60,1.24)	0.95 (0.57,1.15)	0.497
IL-27,pg/mL	13.74 (10.01,24.11)	126.82 (110.77,136.93)	**<0.001**

Data were presented as the mean ± SD or median (interquartile range). P values for categorical variables were calculated using Chi-square test, and P values for continuous variables were calculated using one-way ANOVA test or Kruskal–Wallis test with the Bonferroni post hoc test. Bold indicates P value < 0.05. BMI, body mass index; AST, aspartate tansaminase; ALT, alanine transaminase; GGT, gamma glufamyl transferase; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholestero; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment -insulin resistance.

NAFLD is a worldwide health problem and has an increased risk of diabetes, CVD and CKD (24). NAFLD is a complex and heterogeneous disease that is inaccurately diagnosed by liver biopsy (25). Current therapeutic strategies mainly focus on lifestyle interventions, and there are still limited appropriate drugs specifically available for NAFLD (26). Therefore, the discovery of non-invasive detection, novel therapeutic targets and strategies shall be required. Patients with hypothyroidism are at an increased risk of NAFLD. Hypothyroidism plays an important role in the development and progression of NAFLD (27).

Dyslipidemia related to hypothyroidism leads to intrahepatic fat accumulation, resulting in NAFLD and thus leading to the development of hepatic insulin resistance (1). Thyroid hormones increase the expression of HMG-CoA reductase in the liver to increase cholesterol synthesis (28). Plasma cholesteryl ester transfer proteins (CETPs) are decreased in a hypothyroid state, which shift cholesterol from HDL-C to LDL-C and VLDL (29). Consistently, data showed that NAFLD patients with hypothyroidism had higher BMI, LDL-C, FBG, HOMA-IR, subcutaneous fat, visceral fat, and lower HLD-C than non-NAFLD patients. It was found that IL-27 exerted a protective effect against diabetes by ameliorating STZ-induced hyperglycemia and islet inflammation. The hypertrophy of chronic inflammation and white adipocytes in white adipose tissues was blocked and HFD-induced liver steatosis was suppressed by Il-27 gene transfer (23). This study showed that serum IL-27 levels were negatively associated with FBG, HOMAIR, subcutaneous fat, visceral fat, and TG, and positively associated with HDL-C. Therefore, the findings support previous observations that IL-27 could improve insulin resistance and reduce fat accumulation. Meanwhile, data suggested that IL-27 could be involved in lipid metabolism. Thus, it was hypothesized that IL-27 may improve fatty liver by improving insulin resistance and reducing blood lipids. Besides, NAFLD patients with hypothyroidism had lower IL-27 compared to those without NAFLD. It can be speculated that the increase of IL-27 content may improve NAFLD. IL-27 levels may be a potential therapeutic target for dyslipidemia and NAFLD. The role of circulating IL-27 in improving NAFLD in hypothyroidism needs further investigation.

Multiple studies have demonstrated variables for predicting NAFLD (30). The noninvasive screening mode facilitates the timely identification and intervention of NAFLD and its complications in high-risk patients (31). Baseline HOMA-IR and weight gain were used as predictors of NAFLD incidence in a 7-year prospective study (32). Logistic regression analysis displayed that circulating IL-27 levels,HOMA-IR, and visceral fat were independently related to an increased risk of NAFLD. It was demonstrated that IL-27 could also predict the occurrence of

TABLE 3 Correlation analysis between serum IL27 concentration and non-alcoholic fatty liver disease in hypothyroidism.

Variable	Single-factor analysis		multivariate analysis	
	OR (95%CI)	*P*	OR (95%CI)	*P*
Age,years BMI,kg/m^2	1.02 (0.96,1.07) 1.08 (1.04,1.13)	0.604 **0.001**	0.91 (0.62,1.33)	0.617
AST,U/L	1.02 (0.99,1.07)	0.166		
ALT,U/L	1.04 (1.00,1.08)	**0.031**	1.03 (0.96,1.12)	0.352
GGT,U/L	1.01 (0.99,1.02)	0.348		
TG,mmol/L	2.13 (1.16,3.93)	**0.015**	2.34 (0.81,6.79)	0.116
TC,mmol/L	1.32 (0.89,1.96)	0.166		
HDL-C,mmol/L	0.15 (0.02,0.87)	**0.035**	3.15 (0.16,61.03)	0.448
LDL-C,mmol/L	1.41 (0.85,2.31)	0.181		
HOMA-IR	8.37 (2.67,26.22)	**<0.001**	16.85 (2.18,130.45)	**0.007**
Subcutaneous fat,cm^2	1.01 (0.99,1.02)	**0.012**	1.01 (0.99,1.03)	0.248
Visceral fat,cm^2	1.08 (1.00,1.02)	**<0.001**	1.10 (1.01,1.19)	**0.027**
sTSH,uIU/mL	1.01 (0.99,1.02)	0.350		
FT3,pg/mL	1.02 (0.53,1.94)	0.961		
FT4,ng/dL	0.82 (0.23,2.93)	0.756		
IL-27,pg/mL	0.95 (0.93,0.96)	**<0.001**	0.97 (0.96,0.99)	**0.001**

Bold indicates P value < 0.05.

NAFLD. In this study,IL-27 could predict NAFLD with an AUC of 0.897 (95% CI 0.757-0.859). In conclusion, the exact mechanism by which IL-27 regulates NAFLD metabolism needs to be further studied.

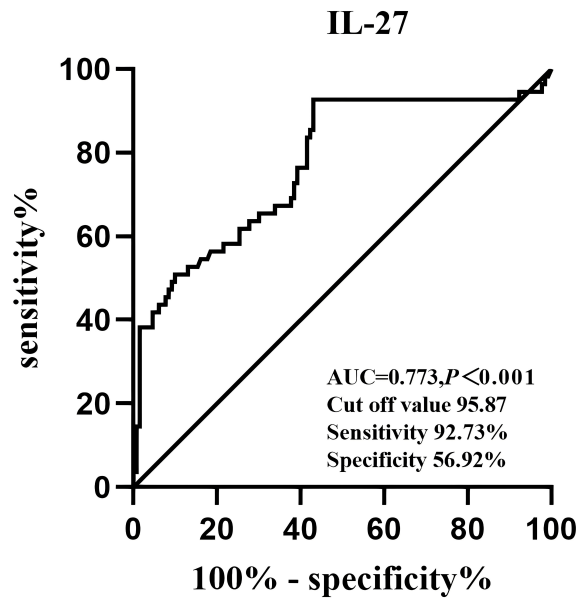

FIGURE 3
Receiver operative characteristics curves and cut off values of serum IL-27 levels for NAFLD.

This study had a number of limitations.First, the sample size was small and the cross-sectional study failed to construct a causal relationship between IL-27 and disease. Second, other potential confounders were not eliminated, particularly cold exposure and exercise. Finally, only the Chinese population was included in this study, thus making the generalizability of the findings an issue.

In summary, serum IL-27 levels had a compensatory increase in patients with hypothyroidism or subclinical hypothyroidism and had an independent association with NAFLD. This study manifests that altering circulating IL-27 levels could predict the occurrence of NAFLD in hypothyroidism.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Beijing Chaoyang Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by YW, HZ, XG, NY, and ZC. The first draft of the manuscript was written by YW. The paper was revised by JL and GW. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1173826/full#supplementary-material

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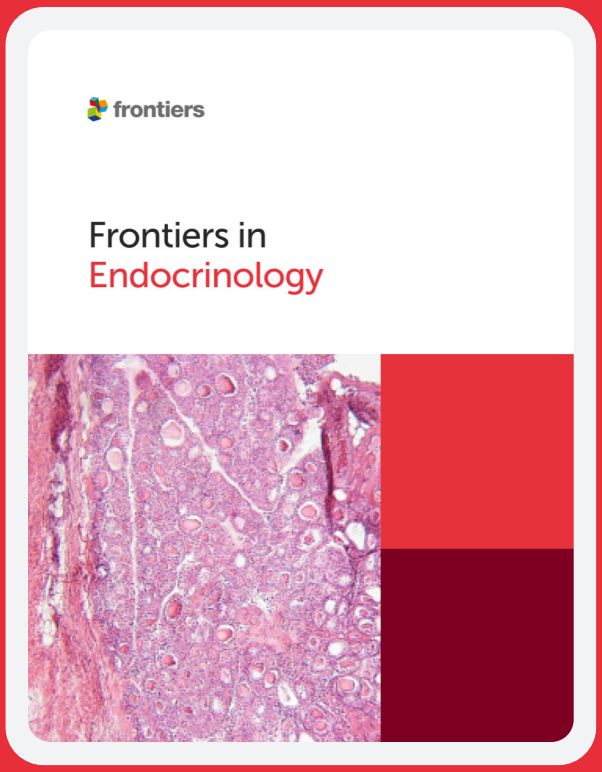

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