THE LEGACY OF DR. LEONARD D. KOHN TO THYROID PATHOPHYSIOLOGY

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THE LEGACY OF DR. LEONARD D. KOHN TO THYROID PATHOPHYSIOLOGY

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Table of Contents

frontiers | Frontiers in Endocrinology

EDITORIAL
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Editorial: The Legacy of Dr. Leonard D. Kohn to Thyroid Pathophysiology

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Keywords: Leonard D Kohn, thyroid cancer, thyroid autoimmunity, FRTL-5 cells, thyroid biology

Editorial on the Research Topic

The Legacy of Dr. Leonard D. Kohn to Thyroid Pathophysiology

This Research Topic honors the memory of Dr. Leonard D. Kohn (Len) (1935-2012), a prominent scientist particularly in the field of thyroid biology and thyroid autoimmunity. Len was Section Chief in the Laboratory of Biochemistry and Metabolism at the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the National Institutes of Health (NIH) in Bethesda MD, USA for 25 years. He then moved to Ohio University as a Distinguished Senior Research Scientist at the Edison Biotechnology Institute. Several generations of young investigators from all over the world were trained in his laboratories and Len had an enormous impact on his trainees' research career. The present topic collects a series of 10 articles (8 original research and 2 updated reviews) authored by Len's former trainees. These articles cover various topics of thyroid pathophysiology and some of them (Chen et al.; Giuliani et al; Napolitano et al.) constitute the development of research projects initiated in Len's laboratories several years ago.

In this Research Topic 4 articles (Chen et al.; Giuliani et al; Napolitano et al.; Bucci et al.) deal with thyroid autoimmunity.

The study of Chen et al. used the thyroid cell line FRTL-5 as a model to investigate the effects of Prunella Vulgaris (PV), a plant used in Chinese medicine, on innate immunity. The study shows that PV inhibits the activation of the innate immune response induced in the FRTL-5 cells by the transfection of ds-DNA and ds-RNA. It should be emphasized that the ability of ds-polynucleotides to activate, in thyroid cells, genes and pathways involved in the immune response was observed by Suzuki in Len's laboratory at NIH (1). In their work Chen et al. show that PV treatment causes a decreased activation of nuclear factor-kB (NF-kB) and Interferon regulatory factor 3 (IRF3) and abolishes the expression of several genes involved in the immune response including the Major Histocompatibility Complex (MHC) class I.

Giuliani et al. show, using FRTL-5 cells, that the "tissue-specific" region of the MHC class I promoter has a dominant role in the regulation of the gene expression and that different hormones and factors act on it by modifying the binding of two distinct members of the transcription factors families: activator protein-1 (AP-1) and NF-kB, c-jun and p65 respectively. The study is the continuation of a research project, started 30 years ago in Len's laboratory (2, 3), aimed at evaluating the role of MHC class I expression in thyroid diseases.

Napolitano et al. review the role of the TSH receptor antibodies (TSHrAb) in chronic autoimmune thyroiditis by analyzing the conditions under which the biological assay for TSHrAb detection can be clinically useful. It should be noted that Len's laboratories played a pivotal role in the development of bioassays for TSHrAb (4).

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Bucci et al. performed an updated review on the relationship between thyroid autoimmunity (TAI) and female infertility. In particular, the authors evaluate the role of euthyroid TAI in infertility and pregnancy outcome, also considering the role of TAI in assisted reproductive technology. This is a very interesting and controversial issue, and the authors provide a timely review of the literature.

Two articles (Ulianich et al. and Lee et al.) mainly deal with aspects of thyroid biology.

In their article Ulianich et al. show the effects of endoplasmic reticulum (ER) stress in the thyroid cell lines PCCl3 and FRT. In detail, the induction of ER stress by thapsigargin and tunicamycin results in cells dedifferentiation, loss of epithelial organization, shift towards a mesenchymal phenotype, and activation of the antioxidant response. These data show a new molecular mechanism of cell response following ER stress that can lead to a loss of thyroid function.

Lee et al. investigate the role of the primary cilium of thyrocytes in thyroglobulin (Tg) endocytosis using *"in vivo"* murine models. They demonstrated that the Lrp2/megalin complex, involved in Tg uptake, is localized in the primary cilium of thyroid follicular cells. It is interesting to note that the interaction between Tg and thyroid cell membrane was an old interest of Len (5), this interest was subsequently directed to the role of Tg in the regulation of thyroid growth and function (6, 7).

Three original research articles, from Korean researchers, deal with some clinical features of thyroid cancer.

The study by Park et al. evaluate the incidence of childhood thyroid cancer in Korea between 1999 and 2017. They found that, unlike adults, the incidence of thyroid cancer in children continues to increase. The authors also collected epidemiological data on radiation exposure, iodine intake, prevalence of obesity, and behavior habits that provide some explanation for this finding.

Park et al. conducted a retrospective study in patients with medullary thyroid carcinoma to determine the role of preoperative serum calcitonin levels in the prognosis of this disease. They define a preoperative serum calcitonin cut-off value to predict structural recurrence. This cut-off value is also associated with the clinical outcome.

In their work Lee et al. show the adverse effect of TSH-suppressive therapy in patients over 60 years old thyroidectomized for differentiated thyroid cancer. They found that low serum TSH concentrations are associated with a lower grip strength particularly in patients between the age of 60 and 70. These findings are important given the association between low grip strength and cardiovascular morbidity, as well as all-cause mortality (8).

Finally, a clinical study by Sohn et al. focuses on hypothyroidism. The authors address all-cause mortality in hypothyroid patients undergoing treatment with levothyroxine. They report that the mortality rate is higher in the hypothyroid patients compared to the control, with the highest risk within 1 year of treatment. Possible explanations of these results are discussed.

In conclusion, this Research Topic constitutes a tribute to the memory of Len by some of his former research fellows from the Countries that were most represented in Len's laboratories: Japan, Italy and Korea. The Research Topic collects both original research and updated reviews on thyroid biology and clinic. The variety of the issues covered in this Research Topic reflects Len's scientific broad-mindedness.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

1. Suzuki K, Mori A, Ishii KJ, Saito J, Singer DS, Klinman DM, et al. Activation of Target-Tissue Immune-Recognition Molecules by Double-Stranded Polynucleotides. *Proc Natl Acad Sci USA* (1999) 96:2285–90. doi: 10.1073/pnas.96.5.2285
2. Saji M, Moriarty J, Ban T, Singer DS, Kohn LD. Major Histocompatibility Complex Class I Gene Expression in Rat Thyroid Cells Is Regulated by Hormones, Methimazole, and Iodide as Well as Interferon. *J Clin Endocrinol Metab* (1992) 75:871–8. doi: 10.1210/jcem.75.3.1381373
3. Giuliani C, Saji M, Napolitano G, Palmer LA, Taniguchi SI, Shong M, et al. Hormonal Modulation of Major Histocompatibility Complex Class I Gene Expression Involves an Enhancer A-Binding Complex Consisting of Fra-2 and the P50 Subunit of NF-Kappa B. *J Biol Chem* (1995) 270:11453–62. doi: 10.1074/jbc.270.19.11453
4. Giuliani C, Saji M, Bucci I, Napolitano G. Bioassays for TSH Receptor Autoantibodies, from FRTL-5 Cells to TSH Receptor-LH/CG Receptor Chimeras: The Contribution of Leonard D. Kohn. *Front Endocrinol* (2016) 7:103. doi: 10.3389/fendo.2016.00103
5. Consiglio E, Salvatore G, Rall JE, Kohn LD. Thyroglobulin Interactions With Thyroid Plasma Membranes. The Existence of Specific Receptors and Their Potential Role. *J Biol Chem* (1979) 254:5065–76.
6. Suzuki K, Nakazato M, Ulianich L, Mori-Aoki A, Moriyama E, Chung HK, et al. Thyroglobulin Autoregulation of Thyroid-Specific Gene Expression and Follicular Function. *Rev Endocr Metab Disord* (2000) 1:217–24. doi: 10.1023/a:1010035200212
7. Noguchi Y, Harii N, Giuliani C, Tatsuno I, Suzuki K, Kohn LD. Thyroglobulin (Tg) Induces Thyroid Cell Growth in a Concentration-Specific Manner by a Mechanism Other Than Thyrotropin/cAMP Stimulation. *Biochem Biophys Res Commun* (2010) 391:890–4. doi: 10.1016/j.bbrc.2009.11.158
8. Leong DP, Teo KK, Rangarajan S, Lopez-Jaramillo P, Avezum AJr, Orlandini A, et al. Prospective Urban Rural Epidemiology (PURE) Study Investigators. Prognostic Value of Grip Strength: Findings From the Prospective Urban Rural Epidemiology (PURE) Study. *Lancet* (2015) 386:266–73. doi: 10.1016/S0140-6736(14)62000-6

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ORIGINAL RESEARCH
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The Pervasive Effects of ER Stress on a Typical Endocrine Cell: Dedifferentiation, Mesenchymal Shift and Antioxidant Response in the Thyrocyte

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The endoplasmic reticulum stress and the unfolded protein response are triggered following an imbalance between protein load and protein folding. Until recently, two possible outcomes of the unfolded protein response have been considered: life or death. We sought to substantiate a third alternative, dedifferentiation, mesenchymal shift, and activation of the antioxidant response by using typical endocrine cells, i.e. thyroid cells. The thyroid is a unique system both of endoplasmic reticulum stress (a single protein, thyroglobulin represents the majority of proteins synthesized in the endoplasmic reticulum by the thyrocyte) and of polarized epithelium (the single layer of thyrocytes delimiting the follicle). Following endoplasmic reticulum stress, in thyroid cells the folding of thyroglobulin was disrupted. The mRNAs of unfolded protein response were induced or spliced (X-box binding protein-1). Differentiation was inhibited: mRNA levels of thyroid specific genes, and of thyroid transcription factors were dramatically downregulated, at least in part, transcriptionally. The dedifferentiating response was accompanied by an upregulation of mRNAs of antioxidant genes. Moreover, cadherin-1, and the thyroid (and kidney)-specific cadherin-16 mRNAs were downregulated, vimentin, and SNAI1 mRNAs were upregulated. In addition, loss of cortical actin and stress fibers formation were observed. Together, these data indicate that ER stress in thyroid cells induces dedifferentiation, loss of epithelial organization, shift towards a mesenchymal phenotype, and activation of the antioxidant response, highlighting, at the same time, a new and wide strategy to achieve survival following ER stress, and, as a sort of the other side of the coin, a possible new molecular mechanism of decline/loss of function leading to a deficit of thyroid hormones formation.

Keywords: ER stress, thyroid, dedifferentiation, mesenchymal phenotype, antioxidant response

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INTRODUCTION

The endoplasmic reticulum (ER) is the cellular organelle where newly synthesized secretory and transmembrane (cargo) proteins are cotranslationally translocated and folded. Only correctly folded proteins can move on along the secretory pathway, while misfolded proteins are retained in the ER and eventually degraded trough endoplasmic reticulum-associated degradation (ERAD) (1). Protein misfolding may arise when the ER environment is perturbed by, among others, alteration of calcium homeostasis or redox status, increased cargo protein synthesis, or/and altered glycosylation, placing a condition of stress on the ER.

When ER stress ensues, an adaptive mechanism, the unfolded protein response (UPR) is triggered. The UPR involves transcriptional induction of genes that enhance ER protein folding capacity and promote ERAD (1). Translation of mRNAs is also initially inhibited, together with cotranslational degradation of secretory proteins and degradation of ER-localized mRNAs (1). However, when ER stress is excessive or prolonged and recovery fails, the UPR activates an apoptotic program (1). Indeed, much attention has been devoted to the understanding of the life/death switch mechanism (2–4). However, recent reports have contributed to the idea that adaptation does not necessarily mean full recovery of the pre-existing function. Indeed, reprogramming gene expression to a less differentiated state after ER stress has been shown in a number of systems (5–11).

We sought to extend the concept of regression of differentiation to tissue organization, particularly on endocrine cells. We reasoned that the thyroid may represent an ideal system to this aim, since the thyrocyte is a typical endocrine cell. Thus, the thyrocyte must synthesize much more of a single protein [thyroglobulin (Tg), which accounts for about 50% of newly synthesized cargo proteins of the thyrocyte] than any other protein (12–19) such like, for example, pancreatic β-cells (20), and, indeed, both cell type are particularly susceptible to ER stress (14, 20). In addition, endocrine function is often related to a complex tissue structural organization. Thus, thyroid function is based on the follicle, a single layer of polarized thyrocytes delimiting a central cavity of the follicle (19), and, for example, the function of the pancreatic β-cell, is based on the complex structural organization of the pancreatic islet (21). To test these two different aspects of regression to a less differentiated state, we decided to use two thyroid cell lines, the fully differentiated thyroid cell line PCCl3 (22), and the highly polarized FRT thyroid cell line (23).

Abbreviations: ATF4, activating transcription factor-4; ATF6, activating transcription factor-6; CDH1, cadherin-1; CDH16, thyroid (and kidney)-specific cadherin-16; eIF2α, eukaryotic translation initiation factor 2 alpha; EMT, epithelial-mesenchymal transition; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; GRP78, glucose-regulated protein 78; HO1, heme oxigenase 1; NIS, sodium-iodide symporter; Pax-8, paired box gene 8; SNAI1, snail 1; SNAI2, snail 2; SOD1, superoxide dismutase 1; Tg, thyroglobulin; Th/Tn, thapsigargin and tunicamycin; TTF-1, thyroid transcription factor 1; TPO, thyroperoxidase; TXNRD1, thioredoxin reductase 1; UPR, unfolded protein response; XBP-1s, spliced active form of X-box binding protein-1.

MATERIALS AND METHODS

Cell Culture and Th/Tn Treatments

PCCl3 and FRT cells were cultured as previously reported (12, 22, 23). In brief, these cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and a mixture of six hormones and growth factors, i.e. insulin (1 μg/ml), TSH (1 mIU/ml), cortisone (10 nM), human transferrin (5 pg/ml), somatostatin (10 ng/ml), and glycyl-histidyl-L-lysine (10 ng/ml) (referred as complete medium). Thapsigargin (Th) and tunicamycin (Tn) (Calbiochem Merck) were added to the medium for 30 min, followed by 24 h in fresh complete medium without Th/Tn.

Plasmids and Antibodies

The luciferase reporter plasmid paired box gene 8 (Pax8LUC) was provided by Dr. P.A. Kopp. Antibodies were directed towards: Tg (12), β-actin, tubulin, SNAI1, vinculin (Santa Cruz Biotechnology), cadherin-1 (CDH1) (Cell Signaling Technology Inc.), thyroid (and kidney)-specific cadherin-16 (CDH16) (provided by Dr. G. Calì), activating transcription factor-4 (ATF4) (Cell Signaling, Danvers, MA, USA), phospho-eukaryotic translation initiation factor 2 alpha (p-eIF2α) (Abnova, Taipei, Taiwan). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham Biosciences.

Cell Viability Assay

The conversion of MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenol tetrazolium bromide) by PC Cl3 cells was used as an indicator of cell number as described by Mosmann (24). PC Cl3 cells were grown in 35 mm diameter plates for 48 h in complete medium. Th/Tn were added to the medium for 30 min, followed by 24 h in fresh complete medium without Th/Tn. MTT (0.5 mg/ml) was added to the cells for a 4-h incubation and cells were lysed in acidified isopropanol/HCl 0.04N. The lysates were subsequently read on a spectrophotometer at 550 nm (Bio-rad, Richmond, CA, USA) after a 1:2 dilution with water. The results were expressed as percent viability compared to control.

RNA Isolation and Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted with the TRIzol reagent, according to the manufacturer's protocol. Reverse transcription of 1 μg of total RNA was performed using SuperScript III, following the manufacturer's instructions. Quantitative real-time RT-PCR analysis was performed as previously described (25). Briefly, reactions were performed in triplicate by using iQ SYBR Green Supermix on iCycler real time detection system (Bio-Rad). Relative quantification of gene expression was calculated by the $\Delta\Delta Ct$ method. Each *Ct* value was first normalized to the respective *Glyceraldehyde-3-Phosphate Dehydrogenase*

(GAPDH) Ct value of a sample to account for variability in the concentration of RNA and in the conversion efficiency of the RT reaction. GAPDH was not affected by Th/Tn treatments. The primers used are listed in **Supplementary Materials** (**Table S1**).

Immunofluorescence

1.5×10^5 cells were plated on 12 mm diameter glass coverslips. Forty-eight hours later, cells were vehicle-treated or treated plus 0.5 μg/ml Tn or 0.5 μM Th for 30 min. The medium was then replaced with medium without Th/Tn and cells incubated for 24 h. Immunofluorescence was performed as previously reported (26). Briefly, cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed twice in 50 mm NH_4Cl in PBS, and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Nuclei were stained with HOECHST 33258. Immunofluorescence analysis was performed at a confocal laser scanning microscope LSM 510 Meta (Zeiss, Gottingen, Germany). The λ of diode UV laser was 405, the argon ion laser was set at 488 nm. Fluorescence emission was revealed by 420–480 band pass filter for Hoechst and by 505–530 band pass filter for Alexa Fluor 488. Double staining IF images were acquired separately in the green, and blue channels at a resolution of 1,024 × 1,024 pixels, with the confocal pinhole set to one Airy unit and then saved in TIFF format.

Transient Expression Analysis

Cells were plated in six-well plates to about 80% confluence 24 h before transfection. Cells were washed with serum-free medium before addition of 1 ml of plasmid/Lipofectamine mixture. The plasmid/Lipofectamine mixture was made by incubating 2.5 μg of luciferase reporter plasmid and 0.5 μg of pRL-TK vector (Promega) with 5 μl Lipofectamine 2000 (Invitrogen) and 200 μl of serum-free medium for 30 min at room temperature, before dilution with 800 μl serum-free medium. Cells were incubated for 5 h at 37°C before addition of 1 ml medium supplemented with 20% serum. After 24 h, cells were treated with 0.5 and 1.0 μg/ml of Tn for 30 min, 1 h, and 2 h. The medium was then replaced with medium without Tn. Twenty-four hours later, firefly and renilla activities were determined in cell lysates using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Orion I, Berthold Detection Systems) according to the manufacturer's instructions. Results were expressed as the ratio of firefly to renilla activity.

Western Blots Analysis

Western blots were carried out as previously reported (16). Briefly, cells were treated or mock treated with Th or Tn in medium for 30 min, followed by 24 h in medium without Th/Tn. After evaluation of protein content, 30 μg of cell extract was analyzed by SDS-PAGE and electrotransferred to polyvinylidene difluoride. Blocking was for 15 h at 4°C with Tris-buffered saline-Tween 20 (TBST) buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20) containing 10% nonfat dry milk, followed by incubation in TBST buffer for 2 h at room temperature with a 1:2,000 dilution of anti-Tg, 1:500 anti-p-eIF2α, 1:1,000 anti-ATF4/antiCDH1/antiCDH16/antiSNAI1/anti vinculin, 1:2,000 anti-β-actin/anti-tubulin. After being washed with TBST, the blot was incubated for 1 h at room temperature with antirabbit horseradish peroxidase-conjugated antibodies diluted 1:3,000 in TBST. Band detection was by enhanced chemiluminescence. The molecular mass markers were from Euroclone.

Statistical Procedures

Data are presented as means ± SD of at least three independent experiments, each performed in triplicate. The difference between groups was evaluated using Student's t test. $p < 0.05$ was considered significant. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Th/Tn Cause Retention of Tg in the ER and Activate the UPR

The widely used ER stress inducing agents Th and Tn induce in thyroid cells misfolding of Tg, its retention in the ER, and activation of the UPR (13–16). As shown in **Figure 1A**, PCCl3 cells treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn, increased glucose-regulated protein 78 (GRP78), ATF4, activating transcription factor-6 (ATF6), and spliced active form of X-box binding protein-1 (XBP-1s) mRNA, even at the lowest concentration investigated (**Figure 1A**). The activation of the UPR was confirmed at the protein level, by increased ATF4 and phospho-eIF2α (**Figure 1B**, and **Supplementary Figures S1, S2, S3, S4, S5, S9**). Of note, our specific treatment protocol (30 min treatment with relatively low doses of Th/Tn, 0.5 μM and 0.5 μg/ml, respectively, followed by removal of the drug and incubation in complete medium) allowed us to substantially avoid cell death, as shown by cell viability assay and light microscopy imaging (**Figures 1C, D**). Instead, cell death occurred with doses of Th/Tn twenty-fold greater (**Figures 1C, D**).

ER Stress Results in Decreased Thyroid-Specific Gene Expression and Activation of an Antioxidant Response in PCCl3 Cells

To study if and how the expression of thyroid-specific genes was affected by an ER stress, we treated PCCl3 cells with Th/Tn following the same protocol of **Figure 1**. Th/Tn, even at the lowest doses, dramatically decreased mRNAs of thyroid-specific markers, Tg, sodium-iodide symporter (NIS), and thyroperoxidase (TPO) (**Figure 2A**). Transcription of Tg, TPO and NIS genes is directed by a combination of thyroid-specific transcription factors, mostly thyroid transcription factor 1 (TTF-1) and Pax-8, with Pax-8 playing a critical role (27). Th/Tn decreased the mRNAs levels of TTF-1 and Pax-8 (**Figure 2A**). Extending these results, Tg protein levels, in total extracts from Th/Tn-treated PC Cl3 cells, exhibited a dramatic decrease (**Figure 1B**). These results suggested that decreased Pax-8 transcriptionally caused a downregulation of Tg, TPO, and NIS genes. However, a more subtle question was as to whether the downregulation of the transcription factor itself had a transcriptional component. This was, in fact, the case, as

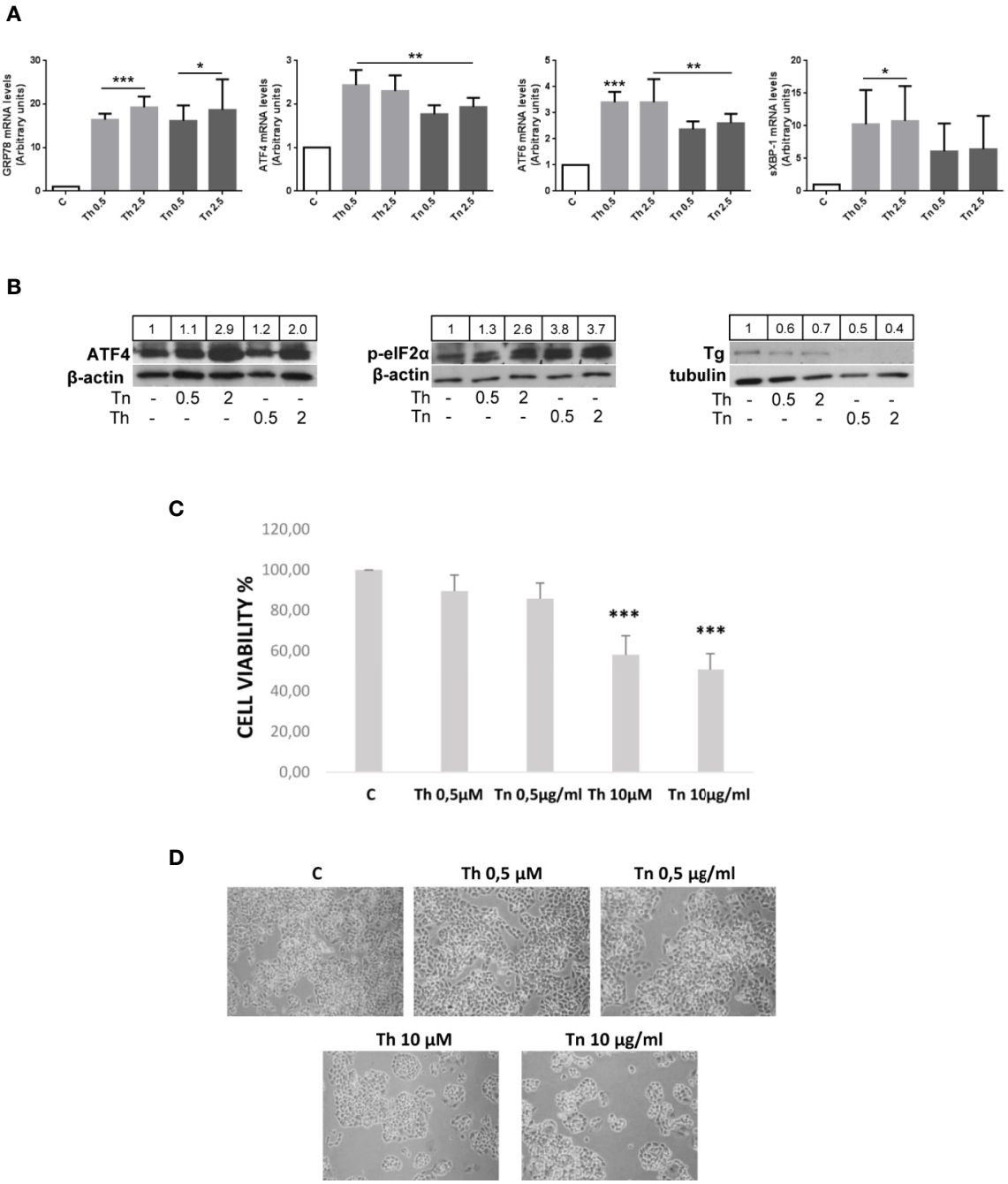

FIGURE 1 | Th/Tn induce ER stress and UPR activation in PCCl3 cells without appreciably affecting viability. **(A)** Cells were plated in 100 mm diameter plates to about 80% confluence 24 h before treatments. Cells were treated or mock treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn. Total RNA was extracted with the TRIzol reagent, according to the manufacturer's protocol. Quantitative real-time RT-PCR analysis was performed as described in *Materials and Methods*. PCCl3 cells vehicle-treated (C) or treated with increasing concentrations of Th/Tn. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, of each group respect to control. **(B)** Cells were plated in 60 mm diameter plates to about 80% confluence 24 h before treatments. Cells were treated or mock treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn. Western blots of total protein extracts from PCCl3 cells vehicle-treated or treated with increasing concentrations of Th/Tn. The ratio of the densitometric values ATF4/β-actin, p-eIF2-α/β-actin, and Tg/tubulin is reported. **(C, D)** Cells were plated in 35 mm diameter plates to about 50% confluence 48 h before treatments. Cells were treated or mock treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn. Cells were photographed by a Nikon Eclipse TS100 inverted microscope. Successively, MTT (0.5 μg/ml) was added to the cells for a 4-h incubation and cells were lysed in acidified isopropanol/HCl 0.04N. The lysates were subsequently read on a spectrophotometer at 550 nm (Bio-rad, Richmond, CA, USA) after a 1:2 dilution with water. The results were expressed as percent viability compared to controls. ***$p < 0.001$, of each group respect to control.

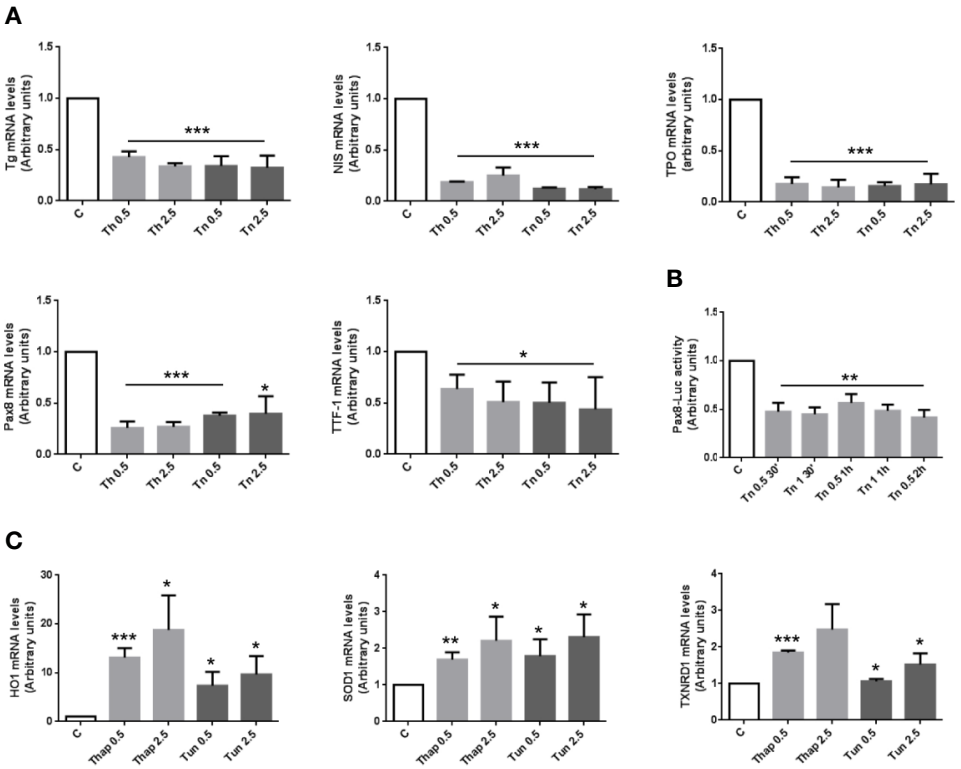

FIGURE 2 | ER stress induces an inhibition of differentiation with a mechanism, at least in part, transcriptional, and an antioxidant response in PCCl3 cells. **(A)** Cells were plated in 100 mm diameter plates to about 80% confluence 24 h before treatments. Cells were treated or mock treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn. Total RNA was extracted with the TRIzol reagent, according to the manufacturer's protocol. Quantitative real-time RT-PCR analysis was performed as described in *Materials and Methods*. PCCl3 cells vehicle-treated (C) or treated with increasing concentrations of Th/Tn. *p < 0.05, ***p < 0.001, of each group respect to control. **(B)** Cells were plated in six-well plates to about 80% confluence 24 h before transfection. PCCl3 cells transfected with 2.5 μg of luciferase reporter plasmid and 0.5 μg of pRL-TK vector with 5 μl Lipofectamine 2000, as reported in *Materials and Methods*. Twenty-four hours after transfection cells were vehicle-treated or treated with 0.5 or 1.0 μg/ml Tn for 30, 60, and 120 min and harvested after 24 h in medium without Tn. Firefly and renilla activities were determined in cell lysates using the Dual-Luciferase Reporter Assay System and a luminometer. Results were expressed as the ratio of firefly to renilla activity. **p < 0.01, of each group respect to control. **(C)** Cells were plated in 100 mm diameter plates to about 80% confluence 24 h before treatments. Cells were treated or mock treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn. Total RNA was extracted with the TRIzol reagent, according to the manufacturer's protocol. Quantitative real-time RT-PCR analysis was performed as described in *Materials and Methods*. PCCl3 cells vehicle-treated (C) or treated with increasing concentrations of Th/Tn. *p < 0.05, **p < 0.01, ***p < 0.001, of each group respect to control.

shown by Pax8 promoter-luciferase assay (**Figure 2B**). These data indicate that ER stress induced by Th/Tn inhibits thyroid-specific gene expression, at least in part, transcriptionally in PCCl3 cells.

Next, we reasoned that the dedifferentiating response may not be the only one executed by thyroid cells in light of an adaptive effort to ER stress. Thus, ER stress activated also an antioxidant response, as shown by the increase in mRNA levels of heme oxigenase 1 (HO1), superoxide dismutase 1 (SOD1), and thioredoxin reductase 1 (TXNRD1) (**Figure 2C**).

ER Stress Induces a Shift Towards a Mesenchymal Phenotype in Thyroid Cells

To investigate if the dedifferentiation effect of ER stress also involved alterations in the organization of the polarized epithelial monolayer, we analyzed CDH1 expression and distribution in PCCl3 cells.

By real time RT-PCR and Western Blot, CDH1 was profoundly downregulated (**Figures 3A, B**, and **Supplementary Figures S6, S7, S9**). Next, we analyzed by immunofluorescence the cellular distribution of CDH1. In control conditions, CDH1 was mainly localized at cell-cell borders (**Figure 3C*i***). Following a treatment with Th/Tn, cells dramatically lost cell-cell contacts with residual CDH1 localized at the remaining contacts (arrowheads, **Figures 3C*ii*, C*iii***). Next, we studied the actin cytoskeleton organization and compared it with the distribution of a differentiation marker (Tg). In control cells, the Tg signal showed a distribution characteristic of ER, where it is co-translationally imported and folded (**Figure 3D*i***). The F-actin distribution was mainly cortical (**Figure 3D*ii***), as evidenced by Phalloidin staining, with the result that the signals of Tg and actin minimally overlapped (**Figure 3D*iii***). Following a Tn treatment, as expected, Tg abundance dramatically decreased, but a few cells still express small amounts of

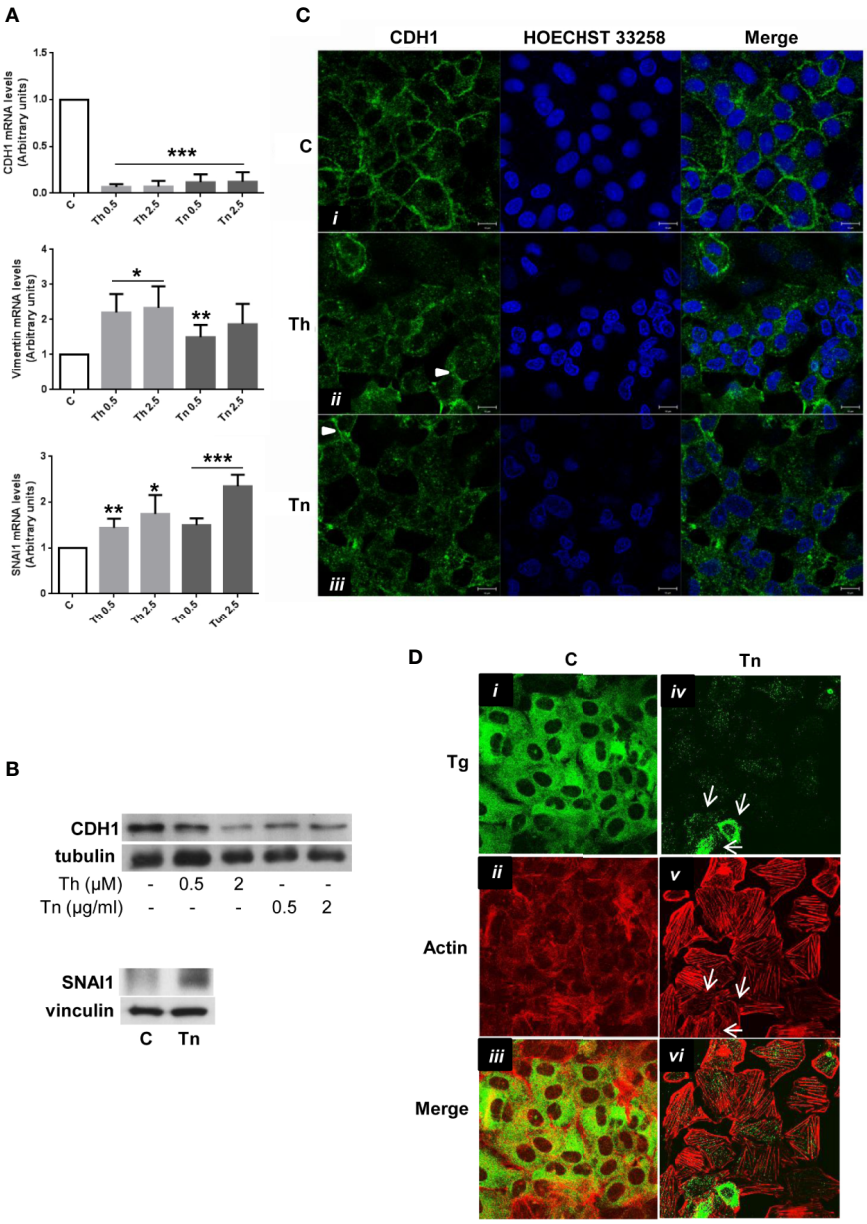

FIGURE 3 | ER stress induces a shift towards a mesenchymal phenotype in PCCl3 cells. **(A)** Cells were plated in 100 mm diameter plates to about 80% confluence 24 h before treatments. Cells were treated or mock treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn. Total RNA was extracted with the TRIzol reagent, according to the manufacturer's protocol. Quantitative real-time RT-PCR analysis was performed as described in *Materials and Methods*. PCCl3 cells vehicle-treated (C) or treated with increasing concentrations of Th/Tn. *p < 0.05, **p < 0.01, ***p < 0.001, of each group respect to control. **(B)** Cells were plated in 60 mm diameter plates to about 80% confluence 24 h before treatments. Cells were treated or mock treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn. Western blots of total protein extracts from PCCl3 cells vehicle-treated or treated with increasing concentrations of Th/Tn (CDH1) or with 0.5 μg/ml Tn (SNAI1). **(C)** PCCl3 cells were grown on glass coverslips for 48 h, then were vehicle-treated (*i*) or treated for 30 min with 0.5 μM Th or 0.5 μg/ml Tn (*ii*, *iii*, respectively). The medium was then replaced with medium without Th/Tn and cells incubated for 24 h. Cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed twice in 50 mm NH_4Cl in PBS, and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Cells were double stained with anti-CDH1 antibodies and HOECHST 33258. Following Th/Tn treatments, the signal for CDH1 decreased. Arrowheads in (*ii*, *iii*) indicate residual CDH1 localized at the remaining cell-cell contacts. Bars, 10 μm. **(D)** PCCl3 cells were grown on glass coverslips for 48 h, then were vehicle-treated (*i*, *ii*, *iii*) or treated for 30 min with 0.5 μg/ml Tn (*iv*, *v*, *vi*). The medium was then replaced with medium without Tn and cells incubated for 24 h. Cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed twice in 50 mm NH_4Cl in PBS, and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Cells were double-stained with anti-Tg antibodies and rhodamine-conjugated phalloidin. In control cells, rhodamine-conjugated phalloidin staining is mainly at the level of cortical actin. Following Tn treatment, the signal for Tg decreased and stress fibers were formed. Arrows indicate: few cells expressing various amounts of residual Tg (*iv*), the correlation between residual Tg expression and partially, not fully, formed stress fibers (*v*), and, consequently, the lack of overlap between Tg and actin signals (*vi*).

residual Tg, although they may be in the process to lose it (**Figure 3D*iv***, arrows). F-actin distribution profoundly changed with loss of cortical actin and formation of stress fibers (**Figure 3D*v***). In addition, in cells showing residual Tg expression there was also a decreased appearance of stress fibers (**Figure 3D*v***, arrows). Thus, Tg and actin signals remained distinct (**Figure 3D*vi***). These changes suggested a shift towards a mesenchymal phenotype, and therefore, we investigated the expression of mesenchymal markers.

Following Th/Tn treatments, vimentin mRNA increased (**Figure 3A**). Several transcription factors [snail1 (SNAI1) and snail2 (SNAI2), among others] downregulate transcriptionally CDH1 (28, 29). Thus, we found an increase of the mRNA and protein levels of SNAI1 following Th/Tn treatments (**Figures 3A, B**, and **Supplementary Figure S10**).

Since PCCl3 cells express thyroid markers but display low level of cell polarity, we sought to extend our results to FRT cells that are well polarized both morphologically and functionally, although they are poorly differentiated (23). Remarkably, FRT cells, at variance with PCCl3 cells, express CDH16, the kidney-specific cadherin, also expressed in thyroid (26). CDH16 has been implicated in the differentiation of the kidney (30) and, recently, of the thyroid follicle (31). CDH16 was markedly downregulated after Th/Tn treatments (**Figure 4A**, and **Supplementary Figure S8, S9**). As for CDH1 in PCCl3 cells, we studied CDH16 cellular distribution in FRT cells. Under normal conditions, FRT cells showed very well-organized cell-cell junctions, with a strong CDH16 staining (**Figure 4B*i***). Yet, following Th/Tn treatments, CDH16 staining decreased and became intermittent and jagged, indicating, as for PCCl3 cells, dramatic alteration of cell-cell junctions (**Figures 4B*ii*, B*iii***). Thus, ER stress induced by Th/Tn caused, in both PCCl3 and FRT cells, similar detrimental changes in the cell-cell junction organization.

DISCUSSION

The accumulation of unfolded proteins in the lumen of ER induces a coordinated adaptive program called UPR. In metazoans, among other responses, the UPR upregulates transcriptionally genes that enhance the ER folding capacity and promote ERAD. If the adaptive response fails, cells execute apoptosis. While much attention has been devoted to the study of the survival/death switch (2–4), a new response to ER stress has emerged, which consists in an inhibition of differentiation (5–11).

In this study, we sought to extend the concept of regression to a less differentiated state following ER stress to tissue organization, focusing on an endocrine system. Thus, cellular dedifferentiation and shift towards a mesenchymal phenotype may be both present and part of a wide program of reshaping gene expression. To study these two different and perhaps complementary aspects, we decided to use the thyroid system, in which a highly cellular differentiation is coupled with a complex tissue organization, the thyroid follicle (19). Notably,

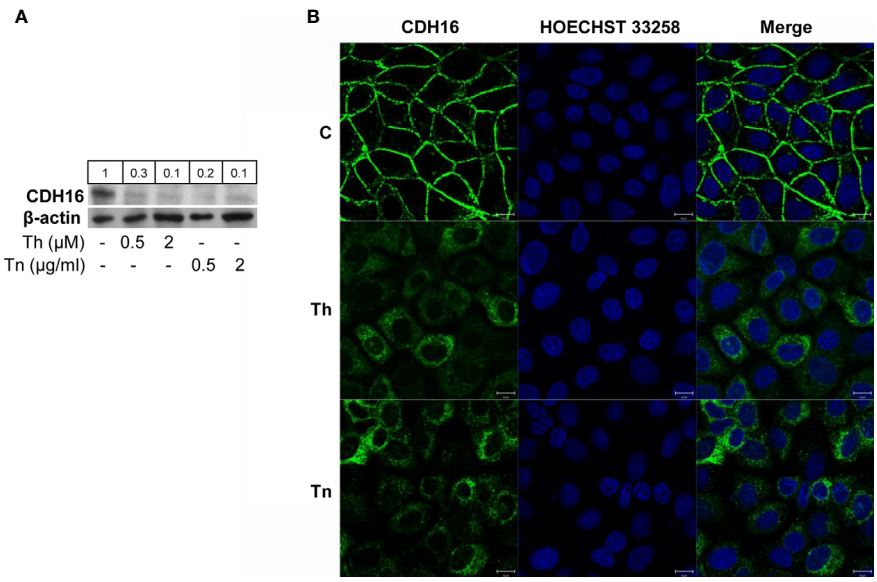

FIGURE 4 | ER stress induces CDH16 downregulation in FRT cells. **(A)** Cells were plated in 60 mm diameter plates to about 80% confluence 24 h before treatments. Cells were treated or mock treated for 30 min with various concentrations of Th/Tn, followed by 24 h in complete medium without Th/Tn. Western blots of total protein extracts from FRT cells vehicle-treated or treated with increasing concentrations of Th/Tn. **(B)** FRT cells were grown on glass coverslips for 48 h, then were vehicle-treated (*i*) or treated for 30 min with 0.5 μM Th or 0.5 μg/ml Tn (*ii*, *iii*, respectively). The medium was then replaced with medium without Th/Tn and cells incubated for 24 h. Then, cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed twice in 50 mm NH_4Cl in PBS, and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Cells were double stained with anti-CDH16 antibodies and HOECHST 33258. Following Th/Tn treatments, the signal for CDH16 dramatically decreased, and cell-cell contacts were lost. Bars, 10 μm.

in thyroid the differentiation genes (Tg, TPO, NIS) encode cargo proteins [like in pancreatic β-cell (insulin)] and in thyroid, genes involved in the organization of the follicle epithelium monolayer (cadherins) also encode cargo proteins (31, 32) [like in the structure of the pancreatic islet (33)]. Thus, in thyroid their down-regulation by ER stress would doubly impact on ER-specific protein load. Moreover, we decided to investigate another cytoprotective response to cellular stress, the induction of antioxidant enzymes (34, 35).

We used two thyroid cell lines, PCCl3 cells, in which both protein folding/misfolding (12–19) and differentiation (27) have been deeply studied at the molecular level, and FRT cells, which are highly polarized both at the structural and the functional level (23). Th/Tn disrupt the folding of Tg (13–19). Thus, Tg accumulates in the lumen of the ER, and the UPR is activated (14, and this study, **Figure 1**). The prevalent concept of the UPR outcome is dichotomous: cell survival, if the response restores a new equilibrium, or death, if the stress is severe and/or chronic and homeostasis cannot be restored (36). However, in recent years, another possibility has been described, inhibition of the dedifferentiated state. Thus, dedifferentiation has been shown in primary and immortalized chondrocytes following ER stress induced by Th/Tn (5), in hypertrophic chondrocytes of transgenic mice expressing a deletion mutant of collagen X (6), in lens fiber cells expressing mutant collagen IV (7), in hypertrophic chondrocytes of transgenic mice ectopically expressing a mutant Tg (cog Tg) driven by the collagen X promoter (37), and in a different line of thyroid cells, FRTL-5 cells (11). Interestingly, the misfolding of the same protein (Tg) in different cell type [(11), this study, and (37)], and, conversely, the misfolding of different proteins (Tg and collagen X, 37 and 6, respectively) in the same cell type, causes an analogous outcome, dedifferentiation, which therefore appears to be neither protein- nor cell-specific.

However, following an ER stress, thyroid cells not only dedifferentiate, but also activate an antioxidant response (**Figure 2C**). Indeed, ER and oxidative stress are widely interconnected. ROS are produced while proteins fold in the lumen of the ER (38) but are overproduced in the presence of protein misfolding (39). Thus, ER stress (in our case produced by Th/Tn) causes oxidative stress (39). In turn, oxidative stress exacerbates ER stress, since ROS inactivate SERCA 3 and 2b pumps (40), causing a Ca2+ loss from the ER lumen and protein misfolding. Both SERCAs are expressed in thyroid, with a prevalence of SERCA 2b (41). Given this vicious cycle, a cellular response, to be effectively cytoprotective in the short term, has to counteract both protein misfolding and ROS accumulation. It is what we have observed in PCCl3 cells, with the upregulation of both, molecular chaperones and antioxidant enzymes following an ER stress.

Furthermore, we report ER stress negatively impact on epithelial tissue organization. Indeed, we show that expression and localization of CDH1 and CDH16 is dramatically altered following ER stress in PCCl3 and FRT cells, respectively. In PCCl3 cells expression of vimentin increases, while the actin cytoskeleton is reorganized with formation of stress fibers. These results may be explained, at least in part, by the induction of SNAI1, known to repress CDH1 transcription (28), to induce vimentin expression (42), to cause disappearance of cortical actin and formation of stress fibers (43) (see **Figure 3D**), and, more in general, to induce EMT (42–44).

Strikingly, disappearance of cortical actin and formation of stress fibers co-exist with downregulation of thyroid markers in the same cell (**Figure 3D**). Even more strikingly, in cells where the loss of Tg expression was marginal, the actin re-organization (disappearance of cortical actin and formation of stress fibers) was less evident (**Figure 3D**, arrows), suggesting a possible causal link between these two phenomena. That a link between dedifferentiation and mesenchymal shift may exist is suggested also by two studies (5, 45). Yang et al. (5) reported that ER stress induces downregulation of mRNAs of differentiation markers of prehypertrophic chondrocytes, while Seki et al. (45) reported that SNAI1 inhibits transcription of these markers by binding to promoter E-boxes, during the chondrocyte prehypertrophic to the hypertrophic passage. Thus, the prehypertrophic-hypertrophic passage may impose ER stress on chondrocytes (in a way similar to lymphocyte to plasma cell transition) (46), and the resulting upregulation of SNAI1 links dedifferentiation to EMT. A similar mechanism may be present in thyroid. SNAI1, upregulated by ER stress, may inhibit thyroid differentiation repressing transcription of thyroid transcription factors. These results confirm the conclusions of **Figures 1**, **2**, **4** of the paper by the same authors that were object of concerns causing the retraction of the paper (47). These results also extend previous conclusions, by showing that also the thyroid (and kidney)-specific cadherin-16 was downregulated and antioxidant genes were upregulated following ER stress. The new finding concerning CDH16 is of particular interest in light of the recent demonstration that this thyroid-specific cadherin controls apical-basal follicular polarization and follicle formation (31).

In conclusion, our results describe a new strategy, besides survival or death, in the cell response to ER stress. Thus, following ER stress, thyroid cells execute an antioxidant response and regress to a less differentiated state, not only involving tissue-specific proteins, but also epithelial tissue differentiation and organization, shifting towards a mesenchymal phenotype. These results highlight, at the same time, a new and wide strategy to achieve survival following ER stress, but also, in a sort of the other side of the coin, a possible new molecular mechanism of decline/loss of function leading to a deficit of thyroid hormones formation.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

BJ conceived the biological problem underlying the manuscript. LU, PM, CG, GC, and BJ designed the experiments and analyzed

the results. LU, PM, CG, GC, DC, AT, AM, DP, and GR performed the experiments. LU, PM, CG, GC, CM, FB, EC, and BJ discussed during the course of the experimental work. BJ wrote the paper. All authors contributed to the article and approved the submitted version.

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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.2020.588685/full#supplementary-material

REFERENCES

1. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* (2011) 334:1081–6. doi: 10.1126/science.1209038
2. Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, et al. Adaptation to ER stress is mediated by differential stabilities of prosurvival and pro-apoptotic mRNAs and proteins. *PloS Biol* (2006) 4:11. doi: 10.1371/journal.pbio.0040374
3. Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B, et al. IRE1 signaling affects cell fate during the unfolded protein response. *Science* (2007) 318:944–9. doi: 10.1126/science.1146361
4. Han D, Lerner AG, Vande Walle L, Upton JP, Xu W, Hagen A, et al. IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. *Cell* (2009) 138:562–75. doi: 10.1016/j.cell.2009.07.017
5. Yang L, Carlson SG, McBurney D, Horton WEJr. Multiple signals induce endoplasmic reticulum stress in both primary and immortalized chondrocytes resulting in loss of differentiation, impaired cell growth and apoptosis. *J Biol Chem* (2005) 280:31156–65. doi: 10.1074/jbc.M501069200
6. Tsang KY, Chan D, Cheslett D, Chan WC, So CL, Melhado IG, et al. Surviving endoplasmic reticulum stress is coupled to altered chondrocyte differentiation and function. *PloS Biol* (2007) 5:44. doi: 10.1371/journal.pbio.0050044
7. Firtina Z, Danysh BP, Bai X, Gould DB, Kobayashi T, Duncan MK. Abnormal expression of collagen IV in lens activates the unfolded protein response resulting in cataract. *J Biol Chem* (2009) 284:35872–84. doi: 10.1074/jbc.M109.060384
8. Tsang KY, Chan D, Bateman JF, Cheah KSE. In vivo cellular adaptation to ER stress: survival strategies with double-edged consequences. *J Cell Sci* (2010) 123:2145–54. doi: 10.1242/jcs.068833
9. Chan WCW, Tsang KY, Cheng YW, Ng VCW, Chik H, Tan ZJ, et al. Activating the unfolded protein response in osteocytes causes hyperostosis consistent with craniodiaphyseal dysplasia. *Hum Mol Genet* (2017) 26:4572–87. doi: 10.1093/hmg/ddx339
10. Treglia AS, Turco S, Ulianich L, Ausiello P, Lofrumento DD, Nicolardi G, et al. Cell fate following ER stress: just a matter of "quo ante" recovery or death? *Histol Histopathol* (2012) 27:1–12 doi: 10.14670/HH-27.1
11. Wen G, Ringseis R, Eder K. Endoplasmic reticulum stress inhibits expression of genes involved in thyroid hormone synthesis and their key transcriptional regulators in FRTL-5 thyrocytes. *PloS One* (2017) 12:11. doi: 10.1371/journal.pone.018756112
12. Di Jeso B, Liguoro D, Ferranti P, Marinaccio M, Acquaviva R, Formisano S, et al. Modulation of the carbohydrate moiety of thyroglobulin by thyrotropin and calcium in Fisher rat thyroid line-5 cells. *J Biol Chem* (1992) 267:1938–44.
13. Di Jeso B, Pereira R, Consiglio E, Formisano S, Satrustegui J, Sandoval IV. Demonstration of a Ca2+ requirement for thyroglobulin dimerization and export to the Golgi complex. *Eur J Biochem* (1998) 252:583–90. doi: 10.1046/j.1432-1327.1998.2520583.x
14. Leonardi A, Vito P, Mauro C, Pacifico F, Ulianich L, Consiglio E, et al. Endoplasmic reticulum stress causes thyroglobulin retention in this organelle and triggers activation of nuclear factor-kappa B via tumor necrosis factor receptor-associated factor 2. *Endocrinology* (2002) 143:2169–77. doi: 10.1210/endo.143.6.8825
15. Di Jeso B, Ulianich L, Pacifico F, Leonardi A, Vito P, Consiglio E, et al. Folding of thyroglobulin in the calnexin/calreticulin pathway and its alteration by loss of Ca2+ from the endoplasmic reticulum. *Biochem J* (2003) 370:449–58. doi: 10.1042/bj20021257
16. Di Jeso B, Park YN, Ulianich L, Treglia AS, Urbanas ML, High S, et al. Mixed-disulfide folding intermediates between thyroglobulin and endoplasmic reticulum resident oxidoreductases ERp57 and protein disulfide isomerase. *Mol Cell Biol* (2005) 25:9793–805. doi: 10.1128/MCB.25.22.9793-9805.2005
17. Lee J, Di Jeso B, Arvan P. The cholinesterase-like domain of thyroglobulin functions as an intramolecular chaperonel. *J Clin Invest* (2008) 118:2950–8. doi: 10.1172/JCI35164
18. Di Jeso B, Morishita Y, Treglia AS, Lofrumento DD, Nicolardi G, Beguinot F, et al. Transient covalent interactions of newly synthesized thyroglobulin with oxidoreductases of the endoplasmic reticulum. *J Biol Chem* (2014) 289:11488–96. doi: 10.1074/jbc.M113.520767
19. Di Jeso B, Arvan P. Thyroglobulin from Molecular and Cellular Biology to Clinical Endocrinology. *Endocrine Rev* (2016) 37:2–36. doi: 10.1210/er.2015-1090
20. Lombardi A, Ulianich L, Treglia AS, Nigro C, Parrillo L, Lofrumento DD, et al. Increased hexosamine biosynthetic pathway flux dedifferentiates INS-1E cells and murine islets by an extracellular signal-regulated kinase (ERK)1/2-mediated signal transmission pathway. *Diabetologia* (2012) 55:141–53. doi: 10.1007/s00125-011-2315-1
21. Halban PA, Wollheim CB, Blondel B, Meda P, Niesor EN, Mintz DH. The possible importance of contact between pancreatic-islet cells for the control of insulin release. *Endocrinology* (1982) 111:86–94. doi: 10.1210/endo-111-1-86
22. Fusco A, Berlingieri MT, Di Fiore PP, Portella G, Grieco M, Vecchio G. One- and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes. *Mol Cell Biol* (1987) 7:3365–70. doi: 10.1128/MCB.7.9.3365
23. Ambesi-Impiombato FS, Coon HG. Thyroid cells in culture. *Int Rev Cytol* (1979) Supplement 10:163–72. doi: 10.1016/S0074-7696(08)60619-1
24. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* (1983) 65:55–63. doi: 10.1016/0022-1759(83)90303-4

25. Longo M, Spinelli R, D'Esposito V, Zatterale F, Fiory F, Nigro C, et al. Pathologic endoplasmic reticulum stress induced by glucotoxic insults inhibits adipocyte differentiation and induces an inflammatory phenotype. *Biochim Biophys Acta* (2016) 1863:1146–56. doi: 10.1016/j.bbamcr.2016.02.019
26. Calì G, Gentile F, Mogavero S, Pallante P, Nitsch R, Ciancia G, et al. CDH16/Ksp-Cadherin Is Expressed in the Developing Thyroid Gland and Is Strongly Down-Regulated in Thyroid Carcinomas. *Endocrinology* (2012) 153:522–34. doi: 10.1210/en.2011-1572
27. Damante G, Tell G, Di Lauro R. A unique combination of transcription factors controls differentiation of thyroid cells. *Prog Nucleic Acid Res Mol Biol* (2001) 66:307–56. doi: 10.1016/S0079-6603(00)66033-6
28. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, et al. The transcription factor SNAI1/snail is a repressor of Ecadherin gene expression in epithelial tumour cells. *Nat Cell Biol* (2000) 2:84–9. doi: 10.1038/35000034
29. Hajra KM, Chen DY, Fearon ER. The slug zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res* (2002) 62:1613–8.
30. Thedieck C, Kuczyk M, Klingel K, Steiert I, Muller CA, Klein G. Expression of Ksp-cadherin during kidney development and in renal cell carcinoma. *Br J Cancer* (2005) 92:2010–7. doi: 10.1038/sj.bjc.6602597
31. Koumarianou P, Gomez-Lopez G, Santisteban P. Pax8 controls thyroid follicular polarity through cadherin-16. *J Cell Sci* (2017) 130:219–31. doi: 10.1242/jcs.184291
32. Yap AS, Stevenson BR, Keast JR, Manley SW. Cadherin-mediated adhesion and apical membrane assembly define distinct steps during thyroid epithelial polarization and lumen formation. *Endocrinology* (1995) 136:4672–80. doi: 10.1210/endo.136.10.7664688
33. Jain R, Lammert E. Cell-cell interactions in the endocrine pancreas. *Diabetes Obes Metab* (2009) 11:159–67. doi: 10.1111/j.1463-1326.2009.01102.x
34. Katsuoka F, Motohashi H, Ishii T, Aburatani H, Engel JD, Yamamoto M. Genetic evidence that small Maf proteins are essential for the activation of antioxidant response element-dependent genes. *Mol Cell Biol* (2005) 25:8044–51. doi: 10.1128/MCB.25.18.8044-8051.2005
35. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, et al. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J Clin Invest* (2004) 114:1248–59. doi: 10.1172/JCI200421146
36. Doultsinos D, Avril T, Lhomond S, Dejeans N, Guédat P, Chevet E. Control of the Unfolded Protein Response in Health and Disease. *SLAS Discovery* (2017) 22:787–800. doi: 10.1177/2472555217701685
37. Rajpar MH, McDermott B, Kung L, Eardley R, Knowles L, Heeran M, et al. Targeted induction of endoplasmic reticulum stress induces cartilage pathology. *PloS Genet* (2009) 5:e1000691. doi: 10.1371/journal.pgen.1000691
38. Pollard MG, Travers KJ, Weissman JS. Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. *Mol Cell* (1998) 1:171–82. doi: 10.1016/S1097-2765(00)80018-0
39. Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* (2007) 9:2277–93. doi: 10.1089/ars.2007.1782
40. Barnes KA, Samson SE, Grover AK. Sarco/endoplasmic reticulum Ca2+-pump isoform SERCA3a is more resistant to superoxide damage than SERCA2b. *Mol Cell Biochem* (2000) 203:17–21 doi: 10.1023/a:1007053802481
41. Pacifico F, Ulianich L, De Micheli S, Treglia S, Leonardi A, Vito P, et al. The expression of the sarco/endoplasmic reticulum Ca2+-ATPases in thyroid and its down-regulation following neoplastic transformation. *J Mol Endocrinol* (2003) 30:399–409. doi: 10.1677/jme.0.0300399
42. Kaufhold S, Bonavida B. Central role of Snail1 in the regulation of EMT and resistance in cancer: a target for therapeutic intervention. *J Exp Clin Cancer Res* (2014) 33:62. doi: 10.1186/s13046-014-0062-0
43. De Craene B, Gilbert B, Stove C, Bruyneel E, van Roy F, Berx G. The transcription factor SNAI1/snail induces tumor cell invasion through modulation of the epithelial cell differentiation program. *Cancer Res* (2005) 65:6237–44. doi: 10.1158/0008-5472.CAN-04-3545
44. Yang SW, Zhang ZG, Hao YX, Zhao YL, Qian F, Shi Y, et al. HIF-1a induces the epithelial-mesenchymal transition in gastric cancer stem cells through the Snail pathway. *Oncotarget* (2017) 8:9535–45. doi: 10.18632/oncotarget.14484
45. Seki K, Fujimori T, Savagner P, Hata A, Aikawa T, Ogata N, et al. Mouse Snail family transcription repressors regulate chondrocyte, extracellular matrix, type II collagen, and aggrecan. *J Biol Chem* (2003) 278:41862–70. doi: 10.1074/jbc.M308336200
46. Gass JN, Gunn KE, Sriburi R, Brewer JW. Stressed-out B cells? Plasma-cell differentiation and the unfolded protein response. *Trends Immunol* (2004) 25:17–24. doi: 10.1016/j.it.2003.11.004
47. Ulianich L, Garbi C, Treglia AS, Punzi D, Miele C, Raciti GA, et al. Retraction: ER stress is associated with dedifferentiation and an epithelial-to-mesenchymal transition-like phenotype in PC Cl3 thyroid cells. *J Cell Sci* (2016) 129:3518. doi: 10.1242/jcs.196584

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Innate Immune-Modulatory Activity of *Prunella vulgaris* in Thyrocytes Functions as a Potential Mechanism for Treating Hashimoto's Thyroiditis

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Prunella vulgaris (PV), a perennial herb, has been used to treat thyroid diseases in China for over 2,000 years. In particular, its therapeutic effect has been described for Hashimoto's thyroiditis, including reducing titers autoantibodies against thyroid peroxidase and thyroglobulin of and T helper 17 (Th17) cells. However, the underlying mechanism for how PV exerts such effects has not been investigated. We examined the effects of PV on innate immune activation, which is thought to be one of the triggers for the development of autoimmune diseases, including Hashimoto's thyroiditis. In cultured thyrocytes, PV reduced mRNA levels of inflammatory cytokines that were originally induced as a result of innate immune activation initiated by transfection of double-stranded DNA (dsDNA) or dsRNA. PV suppressed activation of nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF3), and suppressed corresponding promoter activation, which were initially activated by dsDNA or dsRNA. PV also suppressed the mRNA levels of molecules responsible for antigen processing and presentation, and PV protected thyrocytes from apoptosis induced by dsDNA and dsRNA. Additionally, PV suppressed the expression of genes involved in iodide uptake and oxidation. Taken together, these results suggest that PV exerts its protective effect on thyrocytes by suppressing both innate and adaptive immune responses and cell death. PV may also protect cells from iodide-associated oxidative injury. This report is among the first to identify the mechanisms to explain PV's beneficial effects in Hashimoto's thyroiditis.

Keywords: ***Prunella vulgaris*, innate immune response, autoimmune thyroid diseases, dsDNA, dsRNA**

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INTRODUCTION

Prunella vulgaris (PV) is an herbaceous plant in the genus *Prunella*. The young leaves and stems can be eaten raw in salads, and the spikes are dried, powdered and brewed for use in beverages or as herbal medicine. PV's anti-inflammatory and immunomodulatory effects have been recognized during the long-term practice of traditional Chinese medicine (1). It is prescribed to treat headache,

vertigo, mastitis, hyperplasia of mammary glands, lymphadenopathy, hyperthyroidism and thyroid goiters, in forms of topical ointments, oral liquid and capsules (2, 3). Additionally, PV in liquid or in capsules, in combination with Western medicines (e.g. levothyroxine, indomethacin or prednisone), has been used to treat patients with Hashimoto's thyroiditis. It has been shown that PV significantly improved titers of TPO-Ab and TG-Ab, and the proportion of T helper 17 (Th17) cells among $CD4^+$ T cell populations, compared with Western medicines alone (4–8). However, the underlying mechanisms for its therapeutic effects are poorly understood.

Hashimoto's thyroiditis is an autoimmune disease characterized by chronic thyroiditis with severe parenchymal infiltration of lymphocytes. Although the precise pathologic mechanisms are not fully understood, it is generally believed that the disease manifests through a combination of genetic susceptibility and environmental risk factors (9). Recent studies suggest that innate immune responses in thyrocytes triggered by pathogen-associated molecular patterns (PAMPs; characteristic of harmful foreign bacteria, virus or fungi) and/or danger-associated molecular patterns (DAMPs; typically, ectopic exposure of tissue injury-derived self-nucleotides, proteins or free oxygen radicals) are initiating events for Hashimoto's thyroiditis (10, 11).

Recognition of PAMPs or DAMPs by thyrocytes causes the activation of innate immune responses, which is characterized by the production of an array of inflammatory mediators, such as tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and interferon-β (IFN-β) (10, 11). These immune mediators recruit lymphocytes to the inflamed site, which putatively increases the likelihood of breaking self-tolerance, particularly in genetically susceptible individuals. Moreover, the inflamed thyrocytes are functionally suppressed or even undergo cell death, which may directly precipitate thyroid destruction and hypothyroidism (12, 13). Therefore, agents that interfere with innate immune activation in thyrocytes may be able to improve thyroiditis.

In the current study, we investigated potential immunomodulatory effects of PV on innate immune response in PAMP/DAMP-stimulated rat thyroid FRTL-5 cells. We used double-stranded DNA (dsDNA) as a model of both PAMPs (DNA viruses and bacteria) (14–16) and DAMPs (self-DNA fragments from injured cells) (10, 12, 14), and dsRNA as a model of PAMPs (RNA viruses). We also studied the effect of PV on the thyroid-specific functional gene expressions in FRTL-5 cells.

MATERIALS AND METHODS

Preparation of Aqueous Extraction of PV

Aqueous extraction of PV was prepared and used as previously reported (17–19). In brief, a fine powder of 40 g of PV was mixed with 400 mL of H_2O and boiled for 2 h in a glass beaker. The boiled extracts were centrifuged at 20,000 rpm for 10 min to remove debris and the supernatant was further powdered in a rotary vacuum evaporator under 10 mbar at 70°C for 5 h. The powdered PV extract was weighed and dissolved in H_2O to a stock concentration of 50 mg/mL, then filtrated through 0.22 μm PES membrane (Merck Millipore, Darmstadt, Germany) and stored at −80°C for future use.

Cell Culture and Treatment

FRTL-5 rat thyroid cells were grown in Coon's modified Ham's F-12 medium containing 5% heat-treated bovine serum (Invitrogen, Carlsbad, CA) and a mixture of six hormones, including bovine TSH (1 mU/mL), insulin (10 μg/mL), hydrocortisone (0.36 ng/mL), transferrin (5 μg/mL), Gly-His-Lys-acetate (2 ng/mL), and somatostatin (10 ng/mL) as described (20). All reagents were purchased from Sigma-Aldrich (St. Louis, MO). PV was used at concentrations of 0, 31.25, 62.5, 125, 250, 500 μg/mL in culture medium.

Transfection of Nucleic Acids

One microgram of synthetic polynucleotides, i.e. poly (dA:dT) and poly(I:C) (GE Healthcare, Little Chalfont, UK), was mixed with 3 μL of Fugene HD transfection reagent (Roche Diagnostics, Basel, Switzerland) and 100 μL of serum-free cell culture medium, and then incubated for 15 min at room temperature. The solution was added to the cells and incubated for 6 h at 37°C in a CO_2 incubator after which the medium was replaced with regular culture medium containing 5% bovine serum.

RNA Purification and Real-Time PCR

Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Waltham, MA) as described previously (20). Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. A total of 20 ng of cDNA mixed with 20 μL of FastStart Universal SYBR Green Master (Roche Diagnostics) was amplified by incubating for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C, and one cycle of 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C. The mRNA levels were normalized against that of *Gapdh* levels using the ΔΔCt method as described (20). The primers used were as follows: *Gapdh* forward, 5′-ACA GCAACAGGGTGGTGGAC-3′; *Gapdh* reverse, 5′-TTTGAGG GTGCAGCGAACTT-3′; *Tpo* forward, 5′-CACGGCTTACCA GGCTACAA-3′; *Tpo* reverse, 5′-GCCTCCCAACCAGAC ATCAA-3′; *Duox2* forward, 5′-CAGCGCTACGACGGCTGGT TTA-3′; *Duox2* reverse, 5′-CCCAAGCACTGTGCGGTTGT-3′; *Duoxa2* forward, 5′-TCAGCGTACCGCTGCTCATCGT-3′; *Duoxa2* reverse, 5′-ACCAACCAGAACCAGCGCGAGT-3′; *Slc5a5* forward, 5′-CTACCGTGGGTGGTATGAAGG-3′; *Slc5a5* reverse, 5′-TGCCACCCACTATGAAAGTCC-3′; *Tnfa* forward, 5′-ATGGGCTCCCTCTCATCAGT-3′; *Tnfa* reverse, 5′-GCTTGGTGGTTTGCTACGAC-3′; *Il6* forward, 5′-AGCG ATGATGCACTGTCAGA-3′; *Il6* reverse, 5′-GGAACTCCAG AAGACCAGAGC-3′; *Ifnb* forward, 5′-CTTGGGTGACAT CCACGACT-3′; *Ifnb* reverse, 5′-AAGACTTCTGCTCG GACCAC-3′.

Transient Transfection of Plasmids and Reporter Gene Assays

FRTL-5 cells ($1x10^5$) plated on poly-D-lysine coated 24-well plates (Greiner Bio One, FL) were transfected with 0.2 μg of either NF-κB-dependent luciferase reporter plasmid (p5NF-κB-luc) or rat IFN-β-dependent promoter luciferase reporter plasmid (pGL3-IFNβ) using Fugene HD transfection reagent (Roche Diagnostics) in serum-free medium. The pGL3-basic plasmid was used as a control. After transfection for 6 h, the medium was replaced with basal medium, and cells were treated with PV extract for 24 h. Cells were then stimulated with either dsDNA or dsRNA for 6 h, and a reporter gene assay was performed using the Bright-Glo Luciferase assay system (Promega, Madison,WI). Luciferase activities were measured using FLUO star galaxy (BMG Labtech, Offenburg, Germany), and were normalized to corresponding protein concentrations that were determined using Bio-Rad DC Protein Assay Kits (Bio-Rad Laboratories, Hercules, CA). Data was expressed relative to the activity of the control group.

Protein Preparation and Western Blot Analysis

Cells were lysed in a buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0 for 1 h. The supernatant was collected after centrifugation, and 6 μg of protein was used for Western blotting. Briefly, the proteins were separated on NuPage 4–12% Bis-Tris gels (Invitrogen) by electrophoresis and transferred to nitrocellulose i-Blot gel transfer stacks (Invitrogen). The membrane was washed with PBS with 0.1% Tween 20 (PBST), placed in blocking buffer (PBST containing 5% nonfat milk) for 1 h. Then the membrane was incubated with primary antibodies at 4°C overnight. The primary antibodies used were rabbit anti-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α (IκB-α) (Cell Signaling Technology, Danvers, MA; 1:1,000), phosphorylation interferon regulatory factor 3 (pIRF3) (Cell Signaling Technology; 1:1,000), and mouse monoclonal anti-β-actin (Sigma; 1:5,000) as an internal control. After washing with PBST, membranes were incubated with a horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Cell Signaling Technology; 1:5,000) or goat anti-rabbit IgG (Cell Signaling Technology; 1:1,000) as secondary antibodies. Specific proteins were visualized using Immunostar LD reagent (Wako Pure Chemical, Osaka, Japan), and the chemiluminescence was detected using the C-DiGit blot scanner (LI-COR).

Fluorescence Staining

FRTL-5 cells were cultured on glass-bottom dishes (Matsunami Glass, Osaka, Japan) in the presence or absence of 350 μg/mL of PV for 24 h. Cells were then stimulated with 1 μg/mL dsDNA or dsRNA for 24 h and stained with Hoechst 33342. Fluorescence was visualized and the images were captured on an FV10i confocal laser scanning microscope (Olympus, Tokyo, Japan).

Statistical Analysis

All experiments were repeated at least three times using different batches of cells, and the mean ± SD was calculated. The significance of the differences between experimental values was determined by an unpaired two-tailed t-test, with $p < 0.05$ considered to be significant.

RESULTS

PV Abolishes mRNA Expression of Inflammatory Cytokines Induced by dsDNA or dsRNA

Inflammatory cytokines including IL-6, TNF-α and IFN-β have been found extensively in both thyroids and sera of patients with Hashimoto's thyroiditis (21, 22). These molecules recruit lymphocytes into the affected sites to precipitate inflammation while interfering with thyroid hormone synthesis (23), thus affecting the balance between the maintenance of self-tolerance and the initiation of autoimmunity. In addition to the infiltrating T cells, we previously showed that the thyrocytes themselves can produce various cytokines in response to dsDNA or dsRNA (10, 12, 14), thus playing an active role in thyroid inflammation and the development of autoimmunity.

In order to investigate the possible effects of PV on innate immune responses induced in thyrocytes, FRTL-5 cells were stimulated with dsDNA or dsRNA in the presence or absence of PV. The concentrations of PV used in this study is approximately equal to 15-230-fold dilution of its daily dosage for a patient. No cytotoxic effect of PV was noticed within this range, as determined by trypan blue exclusion test of cell viability (**Supplementary Figure 1**). Real-time PCR analysis showed that mRNA levels of *Ifnb*, *Tnfa* and *Il6* were significantly upregulated 24 h after stimulation with dsDNA or dsRNA (**Figure 1**). However, the increase in the expression of inflammatory cytokines was remarkably reversed by PV in a dose-dependent manner (**Figure 1**), indicating that PV exerts a powerful suppressive effect on innate immune activation in thyrocytes. mRNA levels of *Gapdh* were not affected by PV (data not shown).

PV Suppresses the Activation of Nuclear Factor κB (NF-κB) and Interferon Regulatory Factor 3 (IRF3), With Suppression of Their Downstream Corresponding Promoters

NF-κB and IFN-β are essential inducers of series of inflammatory cytokines upon activation of innate immunity (24, 25). To determine if PV affects cytokine transcription, we performed luciferase reporter gene assays using NF-κB- and IFN-β-dependent promoter constructs. Stimulating the cells with dsRNA induced NF-κB-dependent promoter activation, as well as IFN-β promoter activation (**Figure 2**). However, PV significantly suppressed the promoter activation of NF-κB- and IFN-β-dependent promoter constructs in both dsDNA- and dsRNA-stimulated cells (**Figure 2**).

It is known in unstimulated cells that NF-κB is sequestered in an inactive state in the cytoplasm by binding with a family of

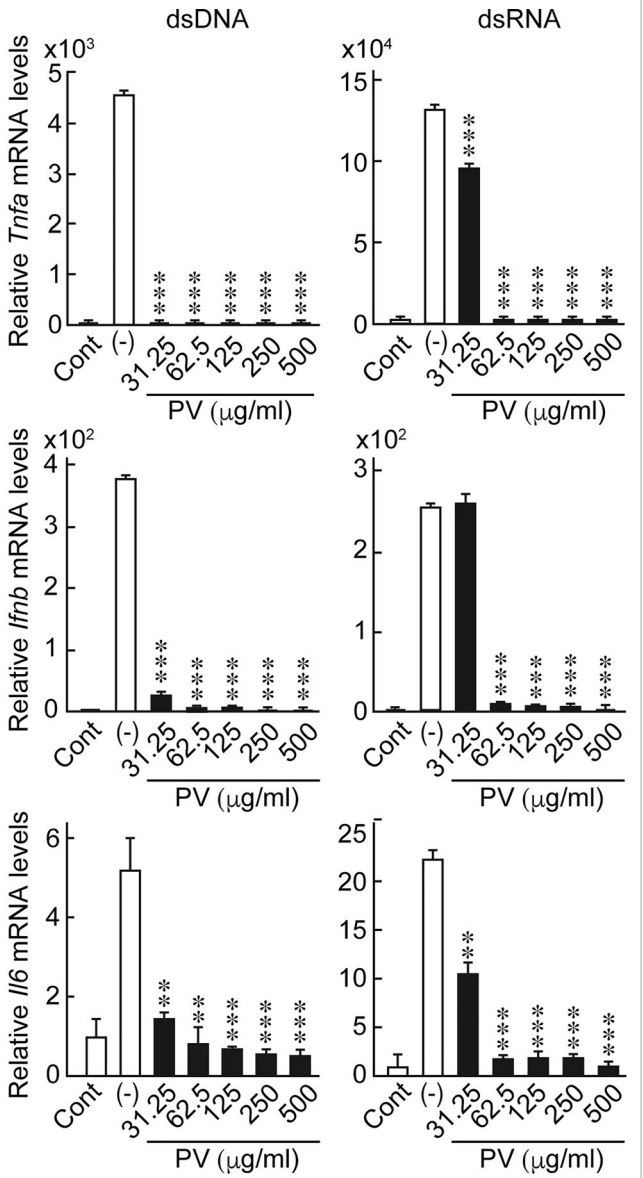

FIGURE 1 | PV suppresses the mRNA expression of inflammatory cytokines induced by dsDNA or dsRNA. FRTL-5 cells were pretreated with PV at increasing concentrations (0, 31.25, 62.5, 125, 250, 500 μg/mL) for 24 h. Cells were then stimulated with 1 μg of dsDNA or dsRNA for 24 h in the presence of absence of PV. Total RNA was purified and subjected to real-time PCR analysis to determine the relative mRNA expression levels of *Tnfa, Ifnb and Il6.* mRNA levels were normalized against *Gapdh* levels and expressed as fold-change relative to the control. Data are presented as mean ± SD (n = 3) relative to the mRNA levels of non-treated control cells. $^{**}p < 0.01$ and $^{***}p < 0.001$, compared to the PV (–) value.

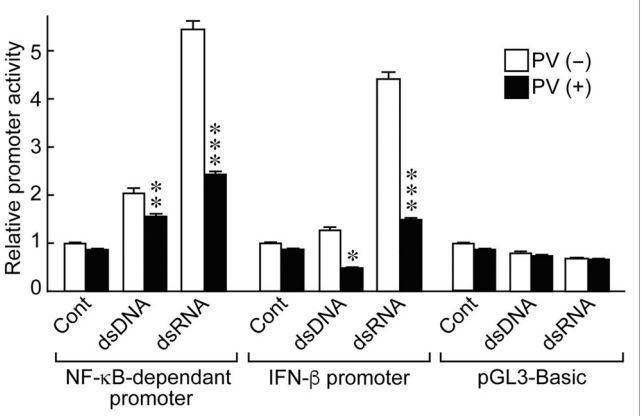

FIGURE 2 | PV suppresses NF-κB-dependent and IFN-β-dependent promoter activation stimulated by dsDNA or dsRNA. FRTL-5 cells were transfected with luciferase chimeric plasmids containing an NF-κB-dependent promoter (p5NF-κB-luc), an IFN-β promoter (pGL3-IFN-β), or a basic vector (pGL3-basic) for 6 h. Cells were then treated with 350 μg/mL of PV for 24 h, and stimulated with 1 μg of dsDNA or dsRNA for another 24 h. Luciferase reporter gene assays were performed as described in the Materials and Methods. Data are the results from three different experiments, each performed in triplicate, and expressed as the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, compared to PV (–) value.

inhibitors, termed inhibitor of κB (IκB), and activation of NF-κB is initiated by signal-induced degradation of IκB proteins (25). IRF3 is known as another major regulator that plays an important role in innate immune response (24). In unstimulated cells, IRF3 is found in an inactive cytoplasmic form, and upon serine/threonine phosphorylation, it translocates to the nucleus and activates the transcription of type I IFNs as well as other IFN-inducible genes (24). Therefore, we examined protein levels of IκB-α, the major subtype of IκB, and phosphorylated IRF3 (pIRF3) by Western blot analysis. When FRTL-5 cells were stimulated by dsDNA or dsRNA, the levels of IκB-α protein were decreased at 12 h and 18 h, whereas pIRF3 was rather induced in the absence of PV (**Figure 3**). However, PV reversed the effects of dsDNA and dsRNA to keep IκB-α and pIRF3 levels almost to the levels seen prior to stimulation with dsDNA or dsRNA (**Figure 3**). These results together suggest that PV interferes with NF-κB and IRF3 signaling pathways, which were initiated by dsDNA or dsRNA in thyrocytes, indicating at least two molecular pathways underlying the protective innate immune response-suppressive effect of PV.

PV Inhibits Genes Related to Antigen Presentation Pathways Induced by dsDNA or dsRNA

Innate immune activation and inflammatory cytokine production theoretically activates the adaptive immune system, which increases the chance of breaking tolerance to immunogenic self-antigens, eventually leading to the development of thyroid autoimmunity. Moreover, the induction of genes related to antigen presentation pathways in thyrocytes is putatively another important mechanism by which the innate immune system mobilizes the adaptive immune system (10, 12, 14–16). In FRTL-5 cells, dsRNA stimulation significantly induced the gene expression of major histocompatibility complex class I (*Mhc1*) and low molecular

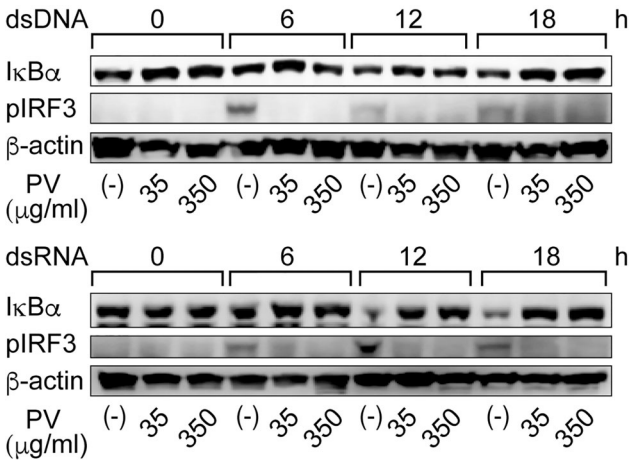

FIGURE 3 | PV suppresses degradation of IκB-α and phosphorylation of IRF3 induced by dsDNA or dsRNA. FRTL-5 cells were pretreated with PV at 35 μg/mL or 350 μg/mL for 24 h, then stimulated with 1 μg of dsDNA or dsRNA for the indicated period of time. Whole cell proteins were extracted, and Western blot analysis was performed to determine the protein expression levels of IκB-α, pIRF3 and β-actin.

weight protein 2 (*Lmp2*), which are involved in the MHC class I antigen processing pathway. Therefore, we evaluated the effect of PV on mRNA levels of *Mhc1* and *Lmp2* induced by dsRNA. We found that PV inhibited dsRNA-induced *Mhc1* and *Lmp2* mRNA levels in a dose-dependent manner in FRTL-5 cells (**Figure 4**), revealing another aspect of the versatile immunomodulatory effects of PV that may hinder autoantigen presentation.

PV Exerts a Protective Effect on Inflammation-Associated Cell Death Induced by dsDNA or dsRNA in Thyrocytes

Inflammatory responses induce apoptosis, which is also seen in Hashimoto's thyroiditis. After exposure to dsDNA or dsRNA, a majority of FRTL-5 thyroid cells exhibited condensed, fragmented nuclei with a much brighter color, indicative of cellular apoptosis, as revealed by Hoechst 33342 staining [**Figure 5**, **PV** (–)]. However, PV completely prevented such apoptotic changes [**Figure 5**, **PV** (+)], indicating that PV is a powerful protective agent against inflammation-related cell death in thyrocytes.

PV Suppresses Gene Expression Involved in Iodide Uptake and Oxidation

In order to evaluate potential effects of PV on thyroid function, we examined the gene expression of the thyroid functional genes responsible for each step of thyroid hormone biosynthesis. When FRTL-5 cells were treated with PV, mRNA levels of *Slc5a5*, *Tpo*, *Duox2*, and *Duoxa2*, the functional genes involved in the transport and organification of iodide, were significantly suppressed in a dose-dependent manner (**Figure 6**). mRNA levels of *Gapdh* were not affected by PV (data not shown). These results suggest that PV may suppress active iodide uptake and oxidation.

DISCUSSION

As the precise pathogenesis of Hashimoto's thyroiditis remains unclear, there is no established treatment that can effectively

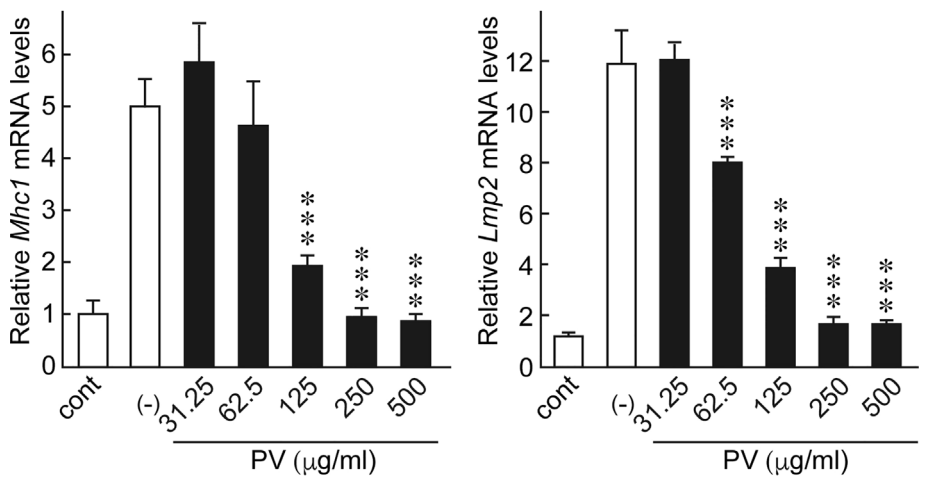

FIGURE 4 | PV inhibited genes involved in antigen presentation pathways induced by dsRNA. FRTL-5 cells were pretreated with PV at increasing concentrations (0, 31.25, 62.5, 125, 250, 500 μg/mL) for 24 h. Cells were then stimulated with 1 μg of dsRNA for 24 h in the presence of PV. Total RNA was purified from the cells and subjected to real-time PCR analysis to determine the relative mRNA expression levels of *Mhc1* and *Lmp2*. mRNA levels were normalized against *Gapdh* levels and expressed as fold-change relative to the control. Data are presented as mean ± SD (n = 3) relative to the mRNA levels of non-treated control levels.
***$p < 0.001$, compared to PV (–) value.

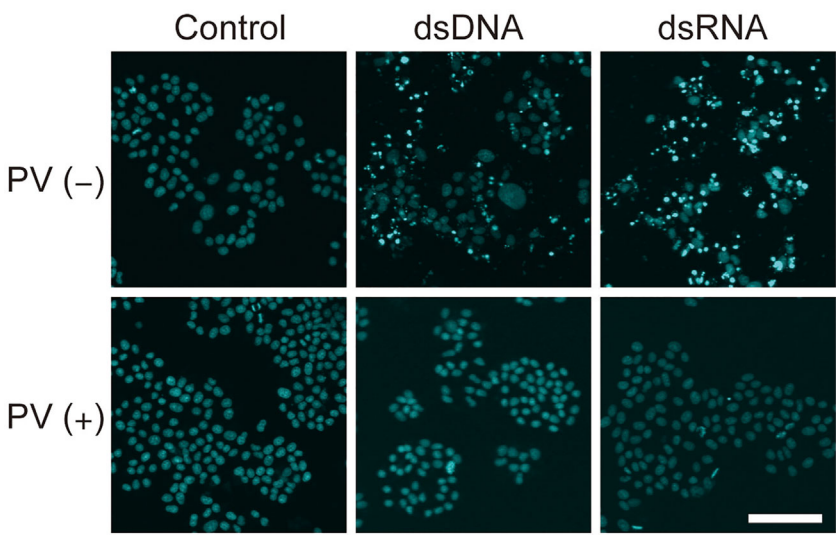

FIGURE 5 | PV exerts a protective effect on cell death in FRTL-5 cells induced by dsDNA or dsRNA. FRTL-5 cells were cultured with or without PV at 350 μg/mL for 24 h. Cells were then stimulated with 1 μg/mL dsDNA or dsRNA for 24 h, followed by Hoechst 33342 staining. Fluorescence was observed using a confocal laser scanning microscope. Scale bar, 100 μm.

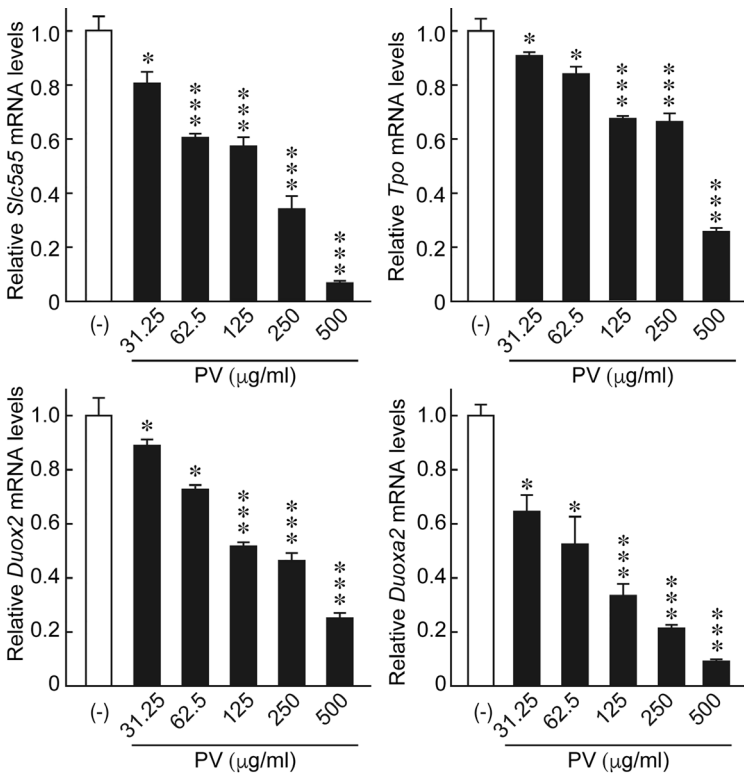

FIGURE 6 | PV suppresses the mRNA expression of genes essential for thyroid hormone biosynthesis. FRTL-5 cells were treated with PV at increasing concentrations (0, 31.25, 62.5, 125, 250, 500 μg/mL) for 24 h. Total RNA was purified from the cells and subjected to real-time PCR analysis to determine the relative mRNA expression levels of *Slc5a5*, *Tpo*, *Duox2* and *Duoxa2*. mRNA levels were normalized against *Gapdh* levels and expressed as fold-change relative to the control. Data are presented as mean ± SD relative to the levels of PV (-) cells (n = 3). $^{*}p < 0.05$ and $^{***}p < 0.001$, compared to PV (–) value.

ameliorate the destructive thyroid autoimmune response or delay the progression of Hashimoto's thyroiditis. The only available therapy for Hashimoto's thyroiditis is to manage hormone levels with thyroid hormone replacement, such as levothyroxine, which, in most cases, needs to be taken for the rest of the patient's life. Although the underlying mechanisms are unknown, PV has been empirically used to treat thyroid disorders, including Hashimoto's thyroiditis, in traditional Chinese medicine for thousands of years. Only recently have modern clinical trials begun to gather evidence that supports the efficacy of PV to reduce the titers of TPO-Ab, TG-Ab, and Th 17 cells, a subset of pro-inflammatory T helper cells implicated in autoimmune and inflammatory disorders (2–8). In addition, *in vitro* studies have revealed an anti-inflammatory effect of PV by targeting NF-κB in stimulated macrophages (26, 27). In the current study, we further elucidated a novel anti-inflammatory activity of PV in thyrocytes that likely contributes to its therapeutic effect on Hashimoto's thyroiditis.

Studies have suggested that the innate immune response in thyrocytes facilitates autosensitization that may eventually lead to thyroid autoimmunity (10, 12). PAMPs and DAMPs are the two main patterns of stimuli that cells encounter *in vivo* indicative of harmful microbial infection or cellular damage. The former refers to the molecules associated with pathogens such as lipopolysaccharides (LPS), peptidoglycans (PGN), and viral dsRNA that initiate and perpetuate infectious pathogen-induced inflammatory responses (28). The latter, in contrast, are host biomolecules derived from dying cells such as self dsDNA, heat-shock proteins, purine metabolites, and hyaluronan fragments that initiate and perpetuate non-infectious inflammatory responses. The innate immune responses triggered by PAMPs or DAMPs rapidly wall off the potentially dangerous events (i.e. infection or non-physiological cell death) before they are out of control. At the same time, the adaptive immune system is ready to be alerted if immunogenic antigens (either foreign or self) are present. However, inflammatory responses are double-edged swords, as they are indiscriminate and can damage healthy tissues. In the thyroids of predisposed individuals, inflammatory responses triggered by PAMPs or DAMPs might facilitate adaptive immune responses to autoantigens by attracting lymphocytes into the periphery, activating antigen presentation processes, and interfering with hormone synthesis (12). The inflammatory mediators can orchestrate together with the infiltrating lymphocytes to induce *de novo* formation of lymph follicles, and consequently convert the usually self-tolerant peripheral environment into an organ prone to autoimmunity. One reason that the thyroid is particularly prone to autoimmunity may be stemmed from thyrocytes' vulnerability towards stimulations with PAMPs or DAMPs (12).

As we demonstrated in the current study, the gene expression of inflammatory mediators such as IFN-β, TNF-α and IL-6 surged in FRTL-5 cells stimulated by dsDNA or dsRNA, in parallel with activation of both NF-κB and IRF3 signaling pathways and nuclear changes indicative of apoptosis. In addition, molecules associated with antigen presentation pathways, such as MHC class I and LMP-2 were also significantly induced in thyrocytes after stimulation with dsDNA or dsRNA. This inflammatory phenotype, characterized by the activation of both NF-κB and IRF3 signaling pathways, induced production of pro-inflammatory cytokines, chemokines and type I interferons, cell-surface expression of molecule involved in antigen presentation process, has been repeatedly noticed after stimulation with both artificial nucleid acids and cytosol self-genomic DNA released from injured cells (10, 12, 14). These observations together emphasize that in response to PAMPs or DAMPs, thyrocytes would launch intense inflammatory and pro-immunogenic reactions.

Strikingly, PV annulled such inflammatory responses induced by dsDNA or dsRNA in thyrocytes, and provided an exceptional protective effect in the immune-activated thyrocytes. Such anti-inflammatory action of PV in thyrocytes, in addition to its previously documented immunomodulatory effects in macrophages (26, 27, 29), may help to explain the observed therapeutic efficacy of PV for Hashimoto's thyroiditis. In turn, our new findings highlight that innate immune responses in thyrocytes triggered by dsDNA or dsRNA is likely a critical factor for precipitating thyroid autoimmunity, and herbal/compound agents (i.e. PV) that interfere with such innate immune responses in thyrocytes may serve as novel therapies for Hashimoto's thyroiditis. So far the structures of the main chemical compounds in PV, identified on the basis of spectral analysis, include polygalacerebroside, ursolic acid, β-amyrin, quercetin, quercetin-3-O-β-D-galactoside, α-spinasterol, stigmasterol, β-sitosterol, and daucosterol (30). We did have tested a few of these purified chemicals, which showed similar effects as did the PV crude extract in the inflamed thyrocytes, however to a much-weakened extent. Thus, the overall effects of PV unlikely come from a single component but rather a natural combination. It will take a large-scale study to exhaust the combinations of the known chemicals in PV to elucidate its "core recipe".

In addition to thyrocytes, innate immune response has been noticed in a variety of cell types upon stimulation with dsDNA or dsRNA (31–33). And we recently reported that self dsDNA-inducible inflammation in primary human keratinocytes played a role in the pathogenesis of psoriasis which is also an autoimmune disease (34). And given that PV has been used to treat all kinds of immune disorders and inflammation from viral infections, allergies, Crohn's disease, diabetes, ulcerative colitis, gastroenteritis, to atherosclerosis, headache and cancers, the immune-modulating effects of PV may be true in many cells types (35–39). We next will confirm the effects of PV on normal human primary thyrocytes.

In addition, we found that PV significantly suppressed the mRNA levels of genes essential for iodide transport and organification, such as *Slc5a5, Tpo, Duox2 and Duoxa2*, in a dose-dependent manner in thyrocytes. Slc5a5 (or NIS) is known as the major plasma symporter responsible for iodine influx into thyrocytes (40). Oxidation of iodide further requires TPO and H_2O_2 generated by DUOX2 and DUOXA2 (41). Excessive iodide uptake and oxidation is generally believed to be a risk factor for thyroid autoimmunity due to harmful oxidative stress, and

should be avoided in Hashimoto's thyroiditis (42). Thus, PV may also function as an antioxidant in the thyroid by checking on excessive iodide uptake and oxidation. Further functional studies are needed to clarify how PV impacts iodide uptake and hormone production in thyrocytes. Before that, physicians should carefully monitor the thyroid hormone and TSH levels of patients receiving PV.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. KS and YL designed and drafted the manuscript. The experimental procedures and data analysis were performed by FC, AK, YL, and MK. KS and AK gave experimental guidance.

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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.2020.579648/full#supplementary-material

REFERENCES

1. Board of Pharmacopoeia of P.R. China. *Pharmacopoeia of the People"s Republic of China*. Beijing: China Medico-Pharmaceutical Science & Technology Puhlishig House (2015).
2. Chen Y, Zhang X, Guo Q, Cao L, Qin Q, Li C, et al. Plant morphology, physiological characteristics, accumulation of secondary metabolites and antioxidant activities of *Prunella vulgaris* L. under UV solar exclusion. *Biol Res* (2019) 52(1):17. doi: 10.1186/s40659-019-0225-8
3. Wang SJ, Wang XH, Dai YY, Ma MH, Rahman K, Nian H, et al. Prunella vulgaris: a comprehensive review of chemical constituents, pharmacological effects and clinical applications. *Curr Pharm Des* (2019) 25(3):359–69. doi: 10.2174/1381612825666190313121608
4. Liu JR, Wang Q. Effects of euthyrox combined with *Prunella vulgaris* capsules on autoantibodies and Th17 cells in patients with Hashimoto's thyroiditis [Article in Chinese]. *Chin J Gerontol* (2012) 24:5413–5. doi: 10.3969/j.issn.1005-9202.2012.24.023
5. Yang K, Guo KQ, Wu HY. Clinical effect of *Prunellae* oral liquid on goiter with different thyroid function [Article in Chinese]. *Zhongguo Zhong Xi Yi Jie He Za Zhi* (2007) 27:37–9. doi: 10.3321/j.issn:1003-5370.2007.01.009
6. Cao Y, Chen JF. *Prunella vulgaris* combined with indomethacin in the treatment of 23 cases of subacute thyroiditis [Article in Chinese]. *Chin J Surg Intergrated Trational Western Med* (2009) 15(3):288–90. doi: 10.3969/j.issn.1007-6948.2009.03.036
7. Ren JM, Wu MH. Clinical observation of *Prunella vulgaris* oral solution assisted treatment of hypothyroidism in Hashimoto's disease [Article in Chinese]. *J China-Japan Friendship Hosp* (2006) 20(5):315. doi: 10.3969/j.issn.1001-0025.2006.05.020
8. Yang K, Liao YQ, Guo KQ, Ye LX, Ruan HL. *Prunella vulgaris* oral solution assisted with low-dose prednisone in the treatment of subacute thyroiditis [Article in Chinese]. *J Yunyang Med Coll* (2008) 27(1):64–5. doi: 10.3321/j.issn:1003-5370.2007.01.009
9. Hayter SM, Cook MC. Updated assessment of the prevalence, spectrum and case definition of autoimmune disease. *Autoimmun Rev* (2012) 11(10):754–65.
10. Kawashima A, Yamazaki K, Hara T, Akama T, Yoshihara A, Sue M, et al. Demonstration of innate immune responses in the thyroid gland: potential to sense danger and a possible trigger for autoimmune reactions. *Thyroid* (2013) 23(4):477–87. doi: 10.1089/thy.2011.0480
11. Morohoshi K, Takahashi Y, Mori K. Viral infection and innate pattern recognition receptors in induction of Hashimoto's thyroiditis. *Discov Med* (2011) 12(67):505–11.
12. Kawashima A, Tanigawa K, Akama T, Wu H, Sue M, Yoshihara A, et al. Fragments of genomic DNA released by injured cells activate innate immunity and suppress endocrine function in the thyroid. *Endocrinology* (2011) 152 (4):1702–12. doi: 10.1210/en.2010-1132
13. Mikos H, Mikos M, Obara-Moszynska M, Niedziela M. The role of the immune system and cytokines involved in the pathogenesis of autoimmune thyroid disease (AITD). *Endokrynol Pol* (2014) 65(2):150–5. doi: 10.5603/EP.2014.0021
14. Suzuki K, Mori A, Ishii KJ, Saito J, Singer DS, Klinman DM, et al. Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc Natl Acad Sci U S A* (1999) 96(5):2285–90. doi: 10.1073/pnas.96.5.2285
15. Ishii KJ, Suzuki K, Coban C, Takeshita F, Itoh Y, Matoba H, et al. Genomic DNA released by dying cells induces the maturation of APCs. *J Immunol* (2001) 167(5):2602–7. doi: 10.4049/jimmunol.167.5.2602
16. Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, et al. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol* (2006) 7(1):40–8. doi: 10.1038/ni1282
17. Han EH, Choi JH, Hwang YP, Park HJ, Choi CY, Chung YC, et al. Immunostimulatory activity of aqueous extract isolated from *Prunella vulgaris*. *Food Chem Toxicol* (2009) 47(1):62–9. doi: 10.1016/j.fct.2008.10.010
18. Hwang SM, Lee YJ, Lee YP, Yoon JJ, Lee SM, Cha JD, et al. Anti-proliferative effect of an aqueous extract of *Prunella vulgaris* in vascular smooth muscle cells. *Evid Based Complement Alternat Med* (2013) 2013:936463. doi: 10.1155/2013/936463
19. Psotova J, Svobodova A, Kolarova H, Walterova D. Photoprotective properties of *Prunella vulgaris* and rosmarinic acid on human keratinocytes. *J Photochem Photobiol B* (2006) 84(3):167–74. doi: 10.1016/j.jphotobiol.2006.02.012
20. Ishido Y, Luo Y, Yoshihara A, Hayashi M, Yoshida A, Hisatome I, et al. Follicular thyroglobulin enhances gene expression necessary for thyroid hormone secretion. *Endocr J* (2015) 62(11):1007–15. doi: 10.1507/endocrj.EJ15-0263
21. Drugarin D, Negru S, Koreck A, Zosin I, Cristea C. The pattern of a T(H)1 cytokine in autoimmune thyroiditis. *Immunol Lett* (2000) 71(2):73–7. doi: 10.1016/S0165-2478(99)00156-X
22. Figueroa-Vega N, Alfonso-Perez M, Benedicto I, Sanchez-Madrid F, Gonzalez-Amaro R, Marazuela M. Increased circulating pro-inflammatory cytokines and Th17 lymphocytes in Hashimoto's thyroiditis. *J Clin Endocrinol Metab* (2010) 95(2):953–62. doi: 10.1210/jc.2009-1719
23. Ajjan RA, Weetman AP. Cytokines in thyroid autoimmunity. *Autoimmunity* (2003) 36(6-7):351–9. doi: 10.1080/08916930310001603046

24. Jefferies CA. Regulating IRFs in IFN Driven Disease. *Front Immunol* (2019) 10:325. doi: 10.3389/fimmu.2019.00325
25. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* (2009) 1(6):a001651. doi: 10.1101/cshperspect.a001651
26. Hwang YJ, Lee EJ, Kim HR, Hwang KA. NF-kappaB-targeted anti-inflammatory activity of *Prunella vulgaris* var. *lilacina* in macrophages RAW 264.7. *Int J Mol Sci* (2013) 14(11):21489–503. doi: 10.3390/ijms141121489
27. Li C, Huang Q, Fu X, Yue XJ, Liu RH, You LJ. Characterization, antioxidant and immunomodulatory activities of polysaccharides from *Prunella vulgaris* Linn. *Int J Biol Macromol* (2015) 75:298–305. doi: 10.1016/j.ijbiomac.2015.01.010
28. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* (2010) 140(6):805–20. doi: 10.1016/j.cell.2010.01.022
29. Cho IH, Jang EH, Hong D, Jung B, Park MJ, Kim JH. Suppression of LPS-induced epithelial-mesenchymal transition by aqueous extracts of *Prunella vulgaris* through inhibition of the NF-kappaB/Snail signaling pathway and regulation of EMT-related protein expression. *Oncol Rep* (2015) 34(5):2445–50. doi: 10.3892/or.2015.4218
30. Gu X, Li Y, Mu J, Zhang Y. Chemical constituents of Prunella vulgaris. *J Environ Sci (China)* (2013) 25 Suppl 1:S161–3. doi: 10.1016/S1001-0742(14)60648-3
31. Patel SJ, Jindal R, King KR, Tilles AW, Yarmush ML. The inflammatory response to double stranded DNA in endothelial cells is mediated by NFkappaB and TNFalpha. *PLoS One* (2011) 6(5):e19910. doi: 10.1371/journal.pone.0019910
32. Luff JA, Yuan H, Suter MM, Muller EJ, Schlegel R, Moore PF. Canine keratinocytes upregulate type I interferons and proinflammatory cytokines in response to poly(dA:dT) but not to canine papillomavirus. *Vet Immunol Immunopathol* (2013) 153(3-4):177–86. doi: 10.1016/j.vetimm.2013.02.001
33. Kawane K, Ohtani M, Miwa K, Kizawa T, Kanbara Y, Yoshioka Y, et al. Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature* (2006) 443(7114):998–1002. doi: 10.1038/nature05245
34. Luo Y, Hara T, Kawashima A, Ishido Y, Suzuki S, Ishii N, et al. Pathological role of excessive DNA as a trigger of keratinocyte proliferation in psoriasis. *Clin Exp Immunol* (2020) 202(1):1–10. doi: 10.1111/cei.13455
35. Oh C, Price J, Brindley MA, Widrlechner MP, Qu L, McCoy JA, et al. Inhibition of HIV-1 infection by aqueous extracts of Prunella vulgaris L. *Virol J* (2011) 8:188. doi: 10.1186/1743-422X-8-188
36. Zheng J, He J, Ji B, Li Y, Zhang X. Antihyperglycemic activity of Prunella vulgaris L. in streptozotocin-induced diabetic mice. *Asia Pac J Clin Nutr* (2007) 16(Suppl 1):427–31.
37. Park SH, Koo HJ, Sung YY, Kim HK. The protective effect of Prunella vulgaris ethanol extract against vascular inflammation in TNF-alpha-stimulated human aortic smooth muscle cells. *BMB Rep* (2013) 46(7):352–7. doi: 10.5483/BMBRep.2013.46.7.214
38. Feng L, Jia X, Zhu M, Chen Y, Shi F. Chemoprevention by Prunella vulgaris L. extract of non-small cell lung cancer *via* promoting apoptosis and regulating the cell cycle. *Asian Pac J Cancer Prev* (2010) 11(5):1355–8.
39. Roh KB, Park D, Jung E. Inhibitory effects of Prunella vulgaris L. extract on 11beta-HSD1 in human skin cells. *Evid Based Complement Alternat Med* (2018) 2018:1762478. doi: 10.1155/2018/1762478
40. Portulano C, Paroder-Belenitsky M, Carrasco N. The Na+/I- symporter (NIS): mechanism and medical impact. *Endocr Rev* (2014) 35(1):106–49. doi: 10.1210/er.2012-1036
41. Morand S, Ueyama T, Tsujibe S, Saito N, Korzeniowska A, Leto TL. Duox maturation factors form cell surface complexes with Duox affecting the specificity of reactive oxygen species generation. *FASEB J* (2009) 23(4):1205–18. doi: 10.1096/fj.08-120006
42. Luo Y, Kawashima A, Ishido Y, Yoshihara A, Oda K, Hiroi N, et al. Iodine excess as an environmental risk factor for autoimmune thyroid disease. *Int J Mol Sci* (2014) 15(7):12895–912. doi: 10.3390/ijms150712895

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Risk of All-Cause Mortality in Levothyroxine-Treated Hypothyroid Patients: A Nationwide Korean Cohort Study

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Background: Although hypothyroidism is associated with various comorbidities, its relationship with increased all-cause mortality remains controversial. The aim of this nationwide retrospective cohort study was to investigate whether hypothyroid patients treated with levothyroxine had increased mortality compared to controls.

Methods: Hypothyroid subjects were identified through the Korean National Health Insurance Service Claims database between 2008 and 2017. Hypothyroidism in this study was defined as overt hypothyroidism treated with long-term prescription of levothyroxine (>6 months). After 1:3 age-, sex- and index year-matching, 501,882 patients with newly diagnosed hypothyroidism and 1,505,646 controls without hypothyroidism were included.

Results: During a mean follow-up of 6 years, 25,954 (5.2%) hypothyroid patients and 59,105 (3.9%) controls died. Hypothyroidism was significantly associated with increased all-cause mortality (adjusted hazard ratio [HR], 1.14; 95% confidence interval [CI] 1.12–1.16) even with levothyroxine treatment. When stratified by age, sex, and cardiovascular disease risk, independent associations between hypothyroidism and mortality remained significant in all subgroups. The risk of mortality was higher in the < 65 age group (HR: 1.25, 95% CI: 1.22–1.29), men (HR: 1.28, 95% CI: 1.25–1.31), and the high cardiovascular disease risk group (HR: 1.31, 95% CI: 1.29–1.34). The mortality rate of hypothyroid patients was highest within 1 year of treatment and decreased with time.

Conclusion: This nationwide, population-based cohort study showed that all-cause mortality was significantly higher in levothyroxine-treated hypothyroid patients than in non-hypothyroid controls. This association remained significant regardless of age, sex, and cardiovascular disease risk.

Keywords: hypothyroidism, mortality, levothyroxine, cohort study, cardiovascular disease

INTRODUCTION

Hypothyroidism is a common endocrine disease, and its incidence and prevalence vary depending on the population being studied (1–3). In Korea, the reported incidence and prevalence of hypothyroidism, when defined as overt hypothyroidism with thyroid hormone prescription of > 60 days, were 2.26 and 14.28 per 1,000 individuals, respectively (2).

The etiology of hypothyroidism includes various conditions. Chronic autoimmune thyroiditis is the major subtype of hypothyroidism in iodine-sufficient areas of the world (4). However, non-autoimmune hypothyroidism has been reported as the most common cause of hypothyroidism in Korea (5). Regardless of cause, hypothyroidism is associated with a number of well-characterized metabolic changes, such as hyperlipidemia, hypertension, and coagulopathy, as well as endothelial dysfunction and cardiovascular disorders (6–10), all of which could theoretically lead to increased mortality. However, studies of hypothyroidism have yielded considerable variation in mortality data (11–24). Some studies have found an increased risk of mortality in hypothyroid patients (12–14, 16, 17, 19, 21–24), but others have not (11, 15, 18, 20). Possible reasons for such inconsistencies may be heterogeneity between studies in terms of definition and severity of hypothyroidism, characteristics of participants, selection of the control population, and controls for comorbidities.

In most patients, hypothyroidism is permanent and requires lifelong treatment. Standard treatment involves the administration of thyroid hormones to maintain serum thyroid-stimulating hormone (TSH) levels within the reference range (25). Most patients become clinically euthyroid once TSH levels return to normal. However, recovery of TSH levels might not reflect the normalization of hypothyroidism-mediated metabolic changes (26, 27), suggesting that mortality risk may persist in hypothyroid patients even with treatment.

To date, a number of cohort studies and meta-analyses have investigated the relationship between hypothyroidism and mortality (11–24, 28–38). However, most have been restricted to subjects with subclinical hypothyroidism (17, 20, 21, 28, 29, 33, 34, 38) and some studies have defined hypothyroidism based on only one TSH level measurement (13, 15, 22, 28, 30, 37). Therefore, these studies may not represent patients with hypothyroidism who require long-term thyroid hormone replacement.

In this nationwide retrospective cohort study, we investigated all-cause mortality risks of hypothyroid patients undergoing long-term levothyroxine therapy compared to age- and sex-matched control subjects.

METHODS

Data Source

This nationwide retrospective cohort study was based on the Korean National Health Insurance (NHI) database provided by the Health Insurance Review and Assessment Service (HIRA). The NHI is the only public medical insurance system operated by the Korean government and membership is compulsory. To claim payments for patient care, all clinics and hospitals in Korea are required to submit data including personal identification number, diagnosis, and prescription information to the HIRA. Therefore, the HIRA database includes claims for the entire South Korean population and comprises patient demographics, diagnosis information based on the International Classification of Diseases, 10th Revision (ICD-10) codes, all inpatient and outpatient claims data, interventions, and prescriptions. HIRA de-identifies patient data in accordance with the Act on the Protection of Personal Information Maintained by Public Agencies.

Study Population

Hypothyroidism cohort. In this study, claims data for levothyroxine prescriptions from 2008 to 2017 were evaluated to define the incidence of hypothyroidism. Hypothyroidism in this study was defined as overt hypothyroidism with long-term prescription of levothyroxine (>6 months). Only subjects aged 18–90 years at the time of prescription were included. A one-year washout period was applied to newly diagnosed cases of hypothyroidism. When a thyroid hormone is prescribed, it is usually repeated within one year in clinical practice. Therefore, we only included patients with a minimum washout period of 1 year. The washout period was defined as the period between January 2007 and the date of first prescription (the "index date"). The exclusion criteria were as follows: (i) a diagnosis of thyroid cancer at any time or a history of thyroidectomy or radioactive iodine treatment to exclude any effects of iatrogenic thyrotoxicosis; (ii) short-term prescription of thyroid hormones (<180 days) to avoid inclusion of transient hypothyroidism; and (iii) death within 6 months from the index date. Selection of the hypothyroidism cohort is illustrated in **Figure 1**.

Control cohort. Data for non-hypothyroid subjects (the control cohort) were also retrieved from the HIRA database. Individuals who received thyroid hormones or had a diagnosis of thyroid dysfunction-related diseases (ICD-10 code: E03.8, E03.9, E06.3) during the study period were excluded. In addition, the same exclusion criteria as in the hypothyroidism cohort were applied before matching the hypothyroidism cohort. Each hypothyroidism case was then matched to three non-hypothyroidism control individuals (1:3 matching) based on age, sex, and index date.

Comorbidity

Comorbidities were mainly selected based on metabolic abnormalities related to hypothyroidism. Hypothyroidism is closely associated with cardiovascular risk factors that lead to cardiovascular events (6–10). Decreased hemodynamics in the circulatory system also lead to declines in glomerular filtration rate (GFR) in severe hypothyroidism (39). As cancer is the most common cause of death in Korea, we adjusted for the presence of malignancy because the primary endpoint was all-cause mortality (40).

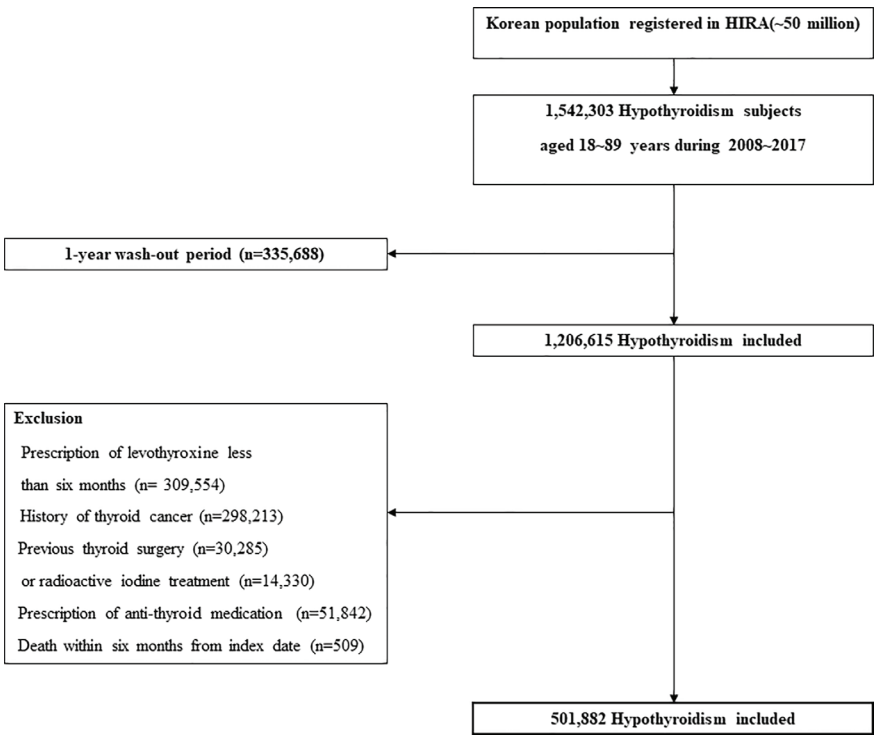

FIGURE 1 | Selection of the hypothyroidism cohort.

Baseline comorbidities were identified according to their respective ICD-10 codes: diabetes mellitus (E10-E14), hypertension (I10), congestive heart failure (I50), myocardial infarction (MI) (I21, I22, I25.2), stroke (I60-I64, I69), chronic kidney disease (N17-N19), and malignancy (C code). A pre-existing comorbidity was defined as a disease diagnosed in at least three outpatient visits in the 1-year period preceding the index date. In cases of MI, stroke, and heart failure, inpatient hospitalization with records of any of their corresponding ICD codes as primary diagnosis was also defined as comorbidities.

Mortality

The primary outcome of this study was all-cause mortality. Mortality was recorded in the NHI cohort based on the database of the Ministry of Public Administration and Security, which compulsorily receives all reports on deaths through official death notices. Cases and times of death from inception to December 31, 2018, were identified for all subjects.

Statistical Analysis

The chi-square test was used to compare proportions and categorical variables between the hypothyroid and control cohorts. Student's *t* test was used for comparisons of mean age between two groups. Crude incidence rates for mortality were calculated as the number of deaths per 1000 person-years. The incidence rates were further stratified by age (<65 and ≥65 years), sex, and cardiovascular disease (CVD) risk (high CVD vs. low CVD risk). High CVD risk was defined as the presence of hypertension, diabetes mellitus, or prevalent CVD (MI, stroke, and heart failure). Kaplan–Meier curves were used to describe and compare cumulative survival rates between the hypothyroid and control cohorts. The follow-up period started on the index date (date of first prescription of thyroid hormone) and was censored on the date of death or at the end of the study (December 31, 2018).

The Cox proportional hazard model was used to explore independent associations between hypothyroidism and mortality risk, and hazard ratios (HRs) were computed with 95% confidence intervals (CI). We conducted multivariable adjustments for age, sex, and comorbidities that affect mortality. Sensitivity analyses were performed in three groups according to the follow-up duration (<1 year, ≤1 to <3 years, and >3 years) from initial treatment with levothyroxine. All statistical analyses were conducted using R (version 4.0.2).

RESULTS

Baseline Characteristics of the Cohort

A total of 501,882 patients with hypothyroidism and 1,505,646 controls treated between January 2008 and December 2017 were included. Baseline characteristics of the subjects are presented in **Table 1**. The mean age was 50.6 years, and women were

TABLE 1 | Baseline characteristics of the study populations.

	Hypothyroidism cohort	Control cohort	*P*-value
Total	501,882	1,505,646	
Male	87,233 (17.4%)	261,699 (17.4%)	0.99
Female	414,649 (82.6%)	1,243,947 (82.6%)	
Age			
mean ± SD	50.6 ± 15.0	50.6 ± 15.0	
<65 years	404,230 (80.5%)	1,212,690 (80.5%)	0.99
≥65 years	97,652 (19.5%)	292,956 (19.5%)	
Days of prescription			
<2 years	191,185 (38%)		
≤2 to <4 years	111,937 (22%)		
≤4 to <6 years	78,332 (16%)		
≤6 years	120,428 (24%)		
Comorbidities			
Hypertension	119,420 (23.8%)	299,126 (19.9%)	<0.001
Diabetes	62,441 (12.4%)	123,070 (8.2%)	<0.001
Myocardial infarction	3,303 (0.7%)	5,818 (0.4%)	<0.001
Stroke	18,186 (3.6%)	42,165 (2.8%)	<0.001
Heart failure	8,901 (1.8%)	12,737 (0.8%)	<0.001
Renal failure	8,370 (1.7%)	5,998 (0.4%)	<0.001
Malignancy	20,997 (4.2%)	33,485 (2.2%)	<0.001

SD, standard deviation.

predominant (82.6%). Hypothyroid patients had a significantly higher prevalence of all selected comorbidities than the control cohort. The mean follow-up durations were 71.6 [standard deviation (SD), 35.6] months and 72.1 (SD, 35.4) months for the hypothyroid and control cohorts, respectively.

Impact of Hypothyroidism on the All-Cause Mortality

Cumulative mortality risk is illustrated in **Figure 2**. The mortality rate of hypothyroid patients was higher than that of the control cohort (**Table 2**). During the follow-up period, 25,954 (5.2%) hypothyroid patients died, compared with 59,105 (3.9%) controls. The crude mortality rate among hypothyroid patients was 8.66 per 1,000 person-years, compared with 6.53 per 1,000 person-years in the control cohort. When stratified by age, sex, and CVD, the crude mortality rate was consistently higher in all subgroups of the hypothyroid cohort.

Cox proportional hazards analyses revealed that the mortality risk in hypothyroid subjects was higher than that in controls. The crude HR was 1.33 (95% CI: 1.31–1.35) and adjusted HR was 1.14 (95% CI: 1.12–1.16) (**Table 2**). When stratified by age, sex, and CVD risk, an independent association between hypothyroidism and mortality remained significant in all subgroups. The risk of mortality was higher in the <65 age group (HR: 1.25, 95% CI: 1.22–1.29), men (HR: 1.28, 95% CI: 1.25–1.31), and the high CVD risk group (HR: 1.31, 95% CI: 1.29–1.34) than in subgroups ≥65 years (HR: 1.08, 95% CI: 1.06–1.10), women

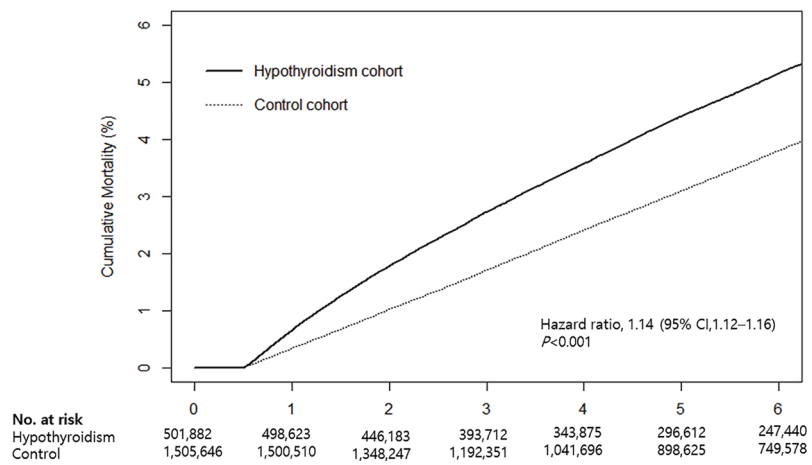

FIGURE 2 | Kaplan-Meier curves for cumulative mortality in hypothyroid patients and the control cohort.

TABLE 2 | Incidence rates and hazard ratios for mortality in the hypothyroidism cohort and the control cohort.

Subgroup	Hypothyroidism cohort		Control cohort		Hazard ratio for mortality	
	Number of deaths	Mortality rate [CI] (per 1,000 person-years)	Number of deaths	Mortality rate [CI] (per 1,000 person-years)	Crude HR* [CI]	Adjusted HR** [CI]
All	25,954	8.66 [8.33–9.00]	59,105	6.53 [6.37–6.70]	1.33 [1.31–1.35]	1.14 [1.12–1.16]
Male	11,470	24.44 [23.04–25.90]	23,211	16.03 [15.38–16.69]	1.53 [1.49–1.56]	1.28 [1.25–1.31]
Female	14,484	5.73 [5.44–6.04]	35,894	4.72 [4.57–4.88]	1.21 [1.19–1.24]	1.06 [1.04–1.09]
Age groups						
<65 years	8,649	3.49 [3.26–3.73]	16,275	2.18 [2.07–2.29]	1.60 [1.56–1.64]	1.25 [1.22–1.29]
≥65 years	17,305	33.69 [32.12–35.32]	42,830	27.25 [26.44–28.07]	1.24 [1.22–1.26]	1.08 [1.06–1.10]
Comorbidities						
Low CVD risk group	8,111	3.64 [3.40–3.90]	25,157	3.52 [3.39–3.66]	1.03 [1.01–1.06]	1.10 [1.08–1.13]
High CVD*** risk group	17,843	23.19 [22.13–24.30]	33,948	17.81 [17.22–18.42]	1.30 [1.28–1.33]	1.31 [1.29–1.34]

CI, confidence interval; CVD, cardiovascular disease.

**Reference group is the control cohort.*

***Adjusted for age, sex, and comorbidities in subgroup analysis. Sex subgroup is adjusted for age and comorbidities. Age subgroup is adjusted for sex and comorbidities. CVD risk subgroup is adjusted for age and sex.*

****High CVD risk group is defined as the presence of hypertension, diabetes mellitus, or prevalent CVD (MI, stroke, and heart failure).*

(HR: 1.06, 95% CI: 1.04–1.09), and low CVD risk (HR: 1.10, 95% CI: 1.08–1.13).

In sensitivity analyses according to follow-up duration, mortality risk increased in the hypothyroid cohort at <1 year, ≤1–3 years, and ≥3 years after initial treatment with levothyroxine, but this association tended to decrease as the follow-up time increased (**Table 3**). The mortality rate in hypothyroid patients was the highest within 1 year of treatment and decreased with time.

DISCUSSION

In this nationwide, retrospective population-based cohort study, we found that hypothyroidism was associated with an increased risk of all-cause mortality. This association remained significant regardless of age, sex, and CVD risk.

Many previous studies have investigated the link between hypothyroidism and mortality risk, but their results have been inconsistent (11–24, 28–34). Possible reasons for such discrepancies include considerable variations in the definition and severity of hypothyroidism, characteristics of study populations, and adjustments for comorbidities. Many studies have defined hypothyroidism based on a single measurement of TSH level and have used different reference ranges (13, 15, 22, 28, 30, 37), which carries the possibility of phenotype misclassification, as spontaneous normalization of an abnormally elevated TSH may occur in a substantial proportion of individuals (41). With regard to study populations, studies based on hospitalized patients have indicated a positive correlation between serum TSH concentration

TABLE 3 | Hazard ratios for all-cause mortality stratified by follow-up duration after initial treatment.

Subgroup	Adjusted HR* [CI]			
	Total	<1 year	≤1–<3 year	≥3 years
All	1.14 [1.12–1.16]	1.50 [1.44–1.57]	1.27 [1.24–1.30]	1.01 [0.99–1.03]
Male	1.28 [1.25–1.31]	1.82 [1.71–1.95]	1.43 [1.38–1.49]	1.08 [1.04–1.11]
Female	1.06 [1.04–1.09]	1.28 [1.20–1.37]	1.17 [1.13–1.21]	0.98 [0.96–1.01]
Age				
<65 years	1.25 [1.22–1.29]	1.81 [1.66–1.97]	1.53 [1.46–1.60]	1.05 [1.01–1.09]
≥65 years	1.08 [1.06–1.10]	1.37 [1.30–1.44]	1.16 [1.12–1.19]	0.98 [0.96–1.01]
Comorbidities				
Low CVD risk group	1.10 [1.08–1.13]	1.57 [1.44–1.70]	1.30 [1.24–1.36]	0.96 [0.93–0.99]
High CVD** risk group	1.31 [1.29–1.34]	1.72 [1.63–1.81]	1.45 [1.40–1.49]	1.15 [1.12–1.18]

CI, confidence interval; CVD, cardiovascular disease.

**Adjusted for age, sex, and comorbidities in subgroup analysis. Sex subgroup is adjusted for age and comorbidities. Age subgroup is adjusted for sex and comorbidities. CVD risk subgroup is adjusted for age and sex.*

and mortality risk (13, 42), whereas no increased mortality risk has been reported in hypothyroid subjects in primary care settings compared with the euthyroid population (11, 15, 20). In addition, some studies did not adequately adjust for comorbid conditions (11, 15, 24, 42). Our definition of hypothyroidism, as individuals treated with levothyroxine for > 6 months, may solely represent overt hypothyroidism in real clinical practice. After adjustment for various comorbidities that affect mortality, hypothyroidism was still associated with 14% excess mortality when compared with controls. In line with our findings, Huang et al. also reported increased mortality in older (>65 years) hypothyroid subjects taking thyroid hormones when compared with non-hypothyroid individuals (12). Our results are also in agreement with those of recent meta-analyses (14, 19). A meta-analysis of 55 cohort studies, with no restrictions on age or degree of hypothyroidism, showed that hypothyroidism was associated with a higher risk of all-cause mortality than the euthyroid state (relative risk, 1.25; 95% CI: 1.13–1.39) (14). Another meta-analysis of 27 cohort studies focusing on the elderly population observed a significant association with increased all-cause mortality in patients with overt hypothyroidism, but not in those with subclinical hypothyroidism (19).

There are several plausible explanations for the increased risk of mortality in levothyroxine-treated hypothyroid patients in this study. First, it is possible that patients with severe hypothyroidism who were untreated for a long time have been included, although the medical records or thyroid function tests of enrolled patients in this study are not known. Second, treatment with levothyroxine may not fully reverse CVD risk factors that lead to increased mortality. Third, it is possible that hypothyroidism has either been over- or under-treated.

In the present study, patients with hypothyroidism had significantly higher prevalence of comorbidities than the control group. First, the higher prevalence of comorbidities in hypothyroid patients can plausibly be explained by the biological action of hypothyroidism itself. Hypothyroidism is known to induce many effects on the cardiovascular system, such as systolic and diastolic dysfunction, endothelial dysfunction, atherogenic lipid profiles, hypertension, and insulin resistance (6–10). A number of population studies have reported higher prevalence of atherosclerotic cardiovascular events and heart failure in hypothyroid patients (13–15, 28, 33). In addition, decreased hemodynamics in the circulatory system lead to declines in GFR in severe hypothyroidism (39). Second, it may be explained by the surveillance effect. Patients with comorbidities are more likely to visit clinics and undergo thyroid function tests than the subjects without comorbidities. Therefore, hypothyroidism is more likely to be detected in individuals with underlying disease.

Previous studies have suggested that the association between hypothyroidism and mortality is dependent on underlying comorbidities, especially CVD (13, 16). Studies in populations with high underlying CVD risk have shown that hypothyroidism is associated with higher all-cause and cardiovascular mortality (13, 23, 36, 37, 43). In a meta-analysis by Rodondi et al., the association between subclinical hypothyroidism and mortality did not differ according to pre-existing CVD (31). In the present study, we found that hypothyroidism is associated with higher all-cause mortality, with or without underlying CVD, although the association seemed to be more significant in the presence of CVD. Considering the impact of hypothyroidism on atherosclerosis, cardiac contractility, and arrhythmia, its association with higher mortality is plausible, particularly in populations with underlying CVD.

The risk of mortality in hypothyroidism may differ by age and sex (18, 20, 24, 28, 32, 34, 38). Previous studies have suggested that the association between hypothyroidism and mortality is less evident in individuals aged > 65 years (20, 32, 34, 44). Recently, Peng et al. found that the use of thyroid hormone replacement was not associated with all-cause mortality in patients aged > 65 years with subclinical hypothyroidism (38). The Leiden 85+ study, which enrolled individuals aged 85 years and older, indicated a decreased risk of cardiovascular and all-cause mortality with higher TSH levels (18), but these findings have not been replicated. Some studies have found increased risk of mortality only in male subjects with subclinical hypothyroidism (24, 28) while others have not (30). In the present study, the increased risk of mortality in hypothyroidism was consistently significant in age- and sex-stratified analyses, with the HR for mortality being higher in the younger age group and in men, which partly corroborated the results of previous studies.

In the present study, the mortality rate of hypothyroid patients increased regardless of age, sex, and CVD risk, but the HR for mortality decreased as the duration of thyroid hormone treatment increased. There are several plausible explanations for the higher mortality risk observed during initial treatment for hypothyroidism. First, the index date when hypothyroid patients were enrolled was defined as the date of first prescription of levothyroxine. Given that some hypothyroid patients may have been untreated for long periods of time before initiating thyroid hormone, there is a possibility that a large number of patients with hypothyroidism died during the early periods of therapy due to comorbidities associated with hypothyroidism. Second, mortality might increase during the early period of treatment due to under- or over-treatment. Thyroid function is more likely to be stable over a longer period of treatment; therefore, the mortality risk of hypothyroidism may be similar to that of the general population if euthyroid state is maintained. Thayakaran et al. found that hypothyroidism did not affect mortality when TSH concentrations were within the recommended normal limits (45). However, this finding was not confirmed in the present study owing to the absence of individual thyroid function tests.

The main strengths of our study include the large hypothyroid cohort of 501,882 individuals, the use of real-world data from primary care settings, and access to complete follow-up data. However, this study had several limitations. The diagnoses of hypothyroidism used in the study were based on administrative claims data, which may be less accurate than diagnoses based on biochemical data, as in other registry-based studies. It is possible that the hypothyroidism cohort includes patients with subclinical hypothyroidism who were asymptomatic but received a levothyroxine prescription after undergoing thyroid function tests. However, since the hypothyroidism cohort only targeted patients

who took levothyroxine for more than 6 months, patients who took levothyroxine temporarily were likely to have been excluded. It is also possible that some patients with subclinical hypothyroidism were classified in the control cohort because the prevalence of subclinical hypothyroidism is about 3% in the general Korean population (46). Therefore, misclassification affects both the hypothyroidism cohort and the control cohort, and thus most likely does not affect our mortality risk estimates. It was impossible to obtain precise information concerning the timing of onset or duration of hypothyroidism, as was included in previous registry-based studies. Although thyroid function tests and the medical records of patients enrolled in the present study were unknown, patients with severe hypothyroidism who were untreated for long periods of time may have been included. Whether levothyroxine treated patients recovered to euthyroid state is unknown. However, we deduce that mortality tends to decrease in levothyroxine treated hypothyroid patients, as thyroid function is likely to be stable over time, because hazard ratios for mortality decreased as the duration of thyroid hormone treatment increased in our study. Lastly, although we adjusted for various underlying comorbidities, data for other potential confounders such as smoking status, body mass index, and alcohol consumption were not available.

In conclusion, in this nationwide, population-based cohort study we found that all-cause mortality was significantly higher in levothyroxine-treated hypothyroid patients than in non-hypothyroid controls. This association remained significant regardless of the age, sex, and cardiovascular disease risk. Further prospective cohort studies on the effects of hypothyroidism and levothyroxine treatment on mortality are warranted.

DATA AVAILABILITY STATEMENT

Data are available through the Health Insurance Review and Assessment Service in Korea 279 (HIRA). Researchers who wish to access the data can apply at (https://www.hira.or.kr).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health Insurance Review and Assessment Service in Korea (Approval no. 2020-069). The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

SS and GS designed the study and wrote the manuscript. GS had access to all the data. SS and GS analyzed the data. JC was responsible for the decision to submit the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Hollowell JG, Staehling NW, Flanders WD, Hannon WH, Gunter EW, Spencer CA, et al. Serum TSH, T(4), and Thyroid Antibodies in the United States Population (1988 to 1994): National Health and Nutrition Examination Survey (Nhanes III). *J Clin Endocrinol Metab* (2002) 87:489–99. doi: 10.1210/jcem.87.2.8182
2. Seo GH, Chung JH. Incidence and Prevalence of Overt Hypothyroidism and Causative Diseases in Korea as Determined Using Claims Data Provided by the Health Insurance Review and Assessment Service. *Endocrinol Metab (Seoul)* (2015) 30:288–96. doi: 10.3803/EnM.2015.30.3.288
3. Canaris GJ, Manowitz NR, Mayor G, Ridgway EC. The Colorado Thyroid Disease Prevalence Study. *Arch Intern Med* (2000) 160:526–34. doi: 10.1001/archinte.160.4.526
4. Carlé A, Laurberg P, Pedersen IB, Knudsen N, Perrild H, Ovesen L, et al. Epidemiology of Subtypes of Hypothyroidism in Denmark. *Eur J Endocrinol* (2006) 154:21–8. doi: 10.1530/eje.1.02068
5. Kim HI, Oh HK, Park SY, Jang HW, Shin MH, Han JM, et al. Non-Immune-Related Hypothyroidism and its Relationship With Excess Iodine. *Eur J Nutr* (2019) 58:2851–8. doi: 10.1007/s00394-018-1837-4
6. Cai Y, Ren Y, Shi J. Blood Pressure Levels in Patients With Subclinical Thyroid Dysfunction: A Meta-Analysis of Cross-Sectional Data. *Hypertens Res* (2011) 34:1098–105. doi: 10.1038/hr.2011.91
7. Erem C. Thyroid Disorders and Hypercoagulability. *Semin Thromb Hemost* (2011) 37:17–26. doi: 10.1055/s-0030-1270067
8. Lekakis J, Papamichael C, Alevizaki M, Piperingos G, Marafelia P, Mantzos J, et al. Flow-Mediated, Endothelium-Dependent Vasodilation is Impaired in Subjects With Hypothyroidism, Borderline Hypothyroidism, and High-Normal Serum Thyrotropin (TSH) Values. *Thyroid* (1997) 7:411–4. doi: 10.1089/thy.1997.7.411
9. Klein I, Danzi S. Thyroid Disease and the Heart. *Circulation* (2007) 116:1725–35. doi: 10.1161/CIRCULATIONAHA.106.678326
10. O'Brien T, Dinneen SF, O'Brien PC, Palumbo PJ. Hyperlipidemia in Patients With Primary and Secondary Hypothyroidism. *Mayo Clin Proc* (1993) 68:860–6. doi: 10.1016/s0025-6196(12)60694-6
11. Flynn RW, Macdonald TM, Jung RT, Morris AD, Leese GP. Mortality and Vascular Outcomes in Patients Treated for Thyroid Dysfunction. *J Clin Endocrinol Metab* (2006) 91:2159–64. doi: 10.1210/jc.2005-1833
12. Huang HK, Wang JH, Kao SL. Association of Hypothyroidism With All-Cause Mortality: A Cohort Study in an Older Adult Population. *J Clin Endocrinol Metab* (2018) 103:3310–8. doi: 10.1210/jc.2018-00408
13. McQuade C, Skugor M, Brennan DM, Hoar B, Stevenson C, Hoogwerf BJ. Hypothyroidism and Moderate Subclinical Hypothyroidism are Associated With Increased All-Cause Mortality Independent of Coronary Heart Disease Risk Factors: A PreCIS Database Study. *Thyroid* (2011) 21:837–43. doi: 10.1089/thy.2010.0298
14. Ning Y, Cheng YJ, Liu LJ, Sara JD, Cao ZY, Zheng WP, et al. What is the Association of Hypothyroidism With Risks of Cardiovascular Events and Mortality? A Meta-Analysis of 55 Cohort Studies Involving 1,898,314 Participants. *BMC Med* (2017) 15:21. doi: 10.1186/s12916-017-0777-9
15. Selmer C, Olesen JB, Hansen ML, von Kappelgaard LM, Madsen JC, Hansen PR, et al. Subclinical and Overt Thyroid Dysfunction and Risk of All-Cause Mortality and Cardiovascular Events: A Large Population Study. *J Clin Endocrinol Metab* (2014) 99:2372–82. doi: 10.1210/jc.2013-4184
16. Thvilum M, Brandt F, Almind D, Christensen K, Hegedus L, Brix TH. Excess Mortality in Patients Diagnosed With Hypothyroidism: A Nationwide Cohort

Study of Singletons and Twins. *J Clin Endocrinol Metab* (2013) 98:1069–75. doi: 10.1210/jc.2012-3375
17. Grossman A, Weiss A, Koren-Morag N, Shimon I, Beloosesky Y, Meyerovitch J. Subclinical Thyroid Disease and Mortality in the Elderly: A Retrospective Cohort Study. *Am J Med* (2016) 129:423–30. doi: 10.1016/j.amjmed.2015.11.027
18. Gussekloo J, van Exel E, de Craen AJ, Meinders AE, Frölich M, Westendorp RG. Thyroid Status, Disability and Cognitive Function, and Survival in Old Age. *Jama* (2004) 292:2591–9. doi: 10.1001/jama.292.21.2591
19. Tsai TY, Tu YK, Munir KM, Lin SM, Chang RH, Kao SL, et al. Association of Hypothyroidism and Mortality in the Elderly Population: A Systematic Review and Meta-Analysis. *J Clin Endocrinol Metab* (2020) 105:2068–80. doi: 10.1210/clinem/dgz186
20. Waring AC, Harrison S, Samuels MH, Ensrud KE, Le BES, Hoffman AR, et al. Thyroid Function and Mortality in Older Men: A Prospective Study. *J Clin Endocrinol Metab* (2012) 97:862–70. doi: 10.1210/jc.2011-2684
21. Moon S, Kim MJ, Yu JM, Yoo HJ, Park YJ. Subclinical Hypothyroidism and the Risk of Cardiovascular Disease and All-Cause Mortality: A Meta-Analysis of Prospective Cohort Studies. *Thyroid* (2018) 28:1101–10. doi: 10.1089/thy.2017.0414
22. Laulund AS, Nybo M, Brix TH, Abrahamsen B, Jorgensen HL, Hegedus L. Duration of Thyroid Dysfunction Correlates With All-Cause Mortality. The OPENTHYRO Register Cohort. *PloS One* (2014) 9:e110437. doi: 10.1371/journal.pone.0110437
23. Iervasi G, Molinaro S, Landi P, Taddei MC, Galli E, Mariani F, et al. Association Between Increased Mortality and Mild Thyroid Dysfunction in Cardiac Patients. *Arch Intern Med* (2007) 167:1526–32. doi: 10.1001/archinte.167.14.1526
24. Kovar FM, Fang IF, Perkmann T, Haslacher H, Slavka G, Födinger M, et al. Subclinical Hypothyroidism and Mortality in a Large Austrian Cohort: A Possible Impact on Treatment? *Wien Klin Wochenschr* (2015) 127:924–30. doi: 10.1007/s00508-015-0846-z
25. Garber JR, Cobin RH, Gharib H, Hennessey JV, Klein I, Mechanick JI, et al. Clinical Practice Guidelines for Hypothyroidism in Adults: Cosponsored by the American Association of Clinical Endocrinologists and the American Thyroid Association. *Thyroid* (2012) 22:1200–35. doi: 10.1089/thy.2012.0205
26. Peterson SJ, McAninch EA, Bianco AC. Is a Normal Tsh Synonymous With "Euthyroidism" in Levothyroxine Monotherapy? *J Clin Endocrinol Metab* (2016) 101:4964–73. doi: 10.1210/jc.2016-2660
27. Samuels MH, Kolobova I, Smeraglio A, Peters D, Purnell JQ, Schuff KG. Effects of Levothyroxine Replacement or Suppressive Therapy on Energy Expenditure and Body Composition. *Thyroid* (2016) 26:347–55. doi: 10.1089/thy.2015.0345
28. Imaizumi M, Akahoshi M, Ichimaru S, Nakashima E, Hida A, Soda M, et al. Risk for Ischemic Heart Disease and All-Cause Mortality in Subclinical Hypothyroidism. *J Clin Endocrinol Metab* (2004) 89:3365–70. doi: 10.1210/jc.2003-031089
29. Razvi S, Weaver JU, Butler TJ, Pearce SH. Levothyroxine Treatment of Subclinical Hypothyroidism, Fatal and Nonfatal Cardiovascular Events, and Mortality. *Arch Intern Med* (2012) 172:811–7. doi: 10.1001/archinternmed.2012.1159
30. Cappola AR, Fried LP, Arnold AM, Danese MD, Kuller LH, Burke GL, et al. Thyroid Status, Cardiovascular Risk, and Mortality in Older Adults. *Jama* (2006) 295:1033–41. doi: 10.1001/jama.295.9.1033
31. Rodondi N, den Elzen WP, Bauer DC, Cappola AR, Razvi S, Walsh JP, et al. Subclinical Hypothyroidism and the Risk of Coronary Heart Disease and Mortality. *Jama* (2010) 304:1365–74. doi: 10.1001/jama.2010.1361
32. Ceresini G, Ceda GP, Lauretani F, Maggio M, Usberti E, Marina M, et al. Thyroid Status and 6-Year Mortality in Elderly People Living in a Mildly Iodine-Deficient Area: The Aging in the Chianti Area Study. *J Am Geriatr Soc* (2013) 61:868–74. doi: 10.1111/jgs.12267
33. Tseng FY, Lin WY, Lin CC, Lee LT, Li TC, Sung PK, et al. Subclinical Hypothyroidism is Associated With Increased Risk for All-Cause and Cardiovascular Mortality in Adults. *J Am Coll Cardiol* (2012) 60:730–7. doi: 10.1016/j.jacc.2012.03.047
34. Razvi S, Shakoor A, Vanderpump M, Weaver JU, Pearce SH. The Influence of Age on the Relationship Between Subclinical Hypothyroidism and Ischemic Heart Disease: A Metaanalysis. *J Clin Endocrinol Metab* (2008) 93:2998–3007. doi: 10.1210/jc.2008-0167
35. Akirov A, Gimbel H, Grossman A, Shochat T, Shimon I. Elevated TSH in Adults Treated for Hypothyroidism is Associated With Increased Mortality. *Eur J Endocrinol* (2017) 176:57–66. doi: 10.1530/eje-16-0708
36. Lin HJ, Lin CC, Lin HM, Chen HJ, Lin CC, Chang CT, et al. Hypothyroidism is Associated With All-Cause Mortality in a National Cohort of Chronic Haemodialysis Patients. *Nephrol (Carlton)* (2018) 23:559–64. doi: 10.1111/nep.13049
37. Rhee CM, Kalantar-Zadeh K, Ravel V, Streja E, You AS, Brunelli SM, et al. Thyroid Status and Death Risk in US Veterans With Chronic Kidney Disease. *Mayo Clin Proc* (2018) 93:573–85. doi: 10.1016/j.mayocp.2018.01.024
38. Peng CC, Huang HK, Wu BB, Chang RH, Tu YK, Munir KM. Association of Thyroid Hormone Therapy With Mortality in Subclinical Hypothyroidism: A Systematic Review and Meta-Analysis. *J Clin Endocrinol Metab* (2021) 106:292–303. doi: 10.1210/clinem/dgaa777
39. den Hollander JG, Wulkan RW, Mantel MJ, Berghout A. Correlation Between Severity of Thyroid Dysfunction and Renal Function. *Clin Endocrinol (Oxf)* (2005) 62:423–7. doi: 10.1111/j.1365-2265.2005.02236.x
40. Available at: http://kostat.go.kr/portal/korea/kor_nw/1/6/2/index.board (Accessed 13 April, 2021).
41. Diez JJ, Iglesias P. Spontaneous Subclinical Hypothyroidism in Patients Older Than 55 Years: An Analysis of Natural Course and Risk Factors for the Development of Overt Thyroid Failure. *J Clin Endocrinol Metab* (2004) 89:4890–7. doi: 10.1210/jc.2003-032061
42. Maldonado LS, Murata GH, Hershman JM, Braunstein GD. Do Thyroid Function Tests Independently Predict Survival in the Critically Ill? *Thyroid* (1992) 2:119–23. doi: 10.1089/thy.1992.2.119
43. Molinaro S, Iervasi G, Lorenzoni V, Coceani M, Landi P, Srebot V, et al. Persistence of Mortality Risk in Patients With Acute Cardiac Diseases and Mild Thyroid Dysfunction. *Am J Med Sci* (2012) 343:65–70. doi: 10.1097/MAJ.0b013e31822846bd
44. Du Puy RS, Poortvliet RKE, Mooijaart SP, den Elzen WPJ, Jagger C, Pearce SHS, et al. Outcomes of Thyroid Dysfunction in People Aged Eighty Years and Older: An Individual Patient Data Meta-Analysis of Four Prospective Studies (Towards Understanding Longitudinal International Older People Studies Consortium). *Thyroid* (2020) 31(4):552–62. doi: 10.1089/thy.2020.0567
45. Thayakaran R, Adderley NJ, Sainsbury C, Torlinska B, Boelaert K, Šumilo D, et al. Thyroid Replacement Therapy, Thyroid Stimulating Hormone Concentrations, and Long Term Health Outcomes in Patients With Hypothyroidism: Longitudinal Study. *Bmj* (2019) 366:l4892. doi: 10.1136/bmj.l4892
46. Kim WG, Kim WB, Woo G, Kim H, Cho Y, Kim TY, et al. Thyroid Stimulating Hormone Reference Range and Prevalence of Thyroid Dysfunction in the Korean Population: Korea National Health and Nutrition Examination Survey 2013 to 2015. *Endocrinol Metab (Seoul)* (2017) 32:106–14. doi: 10.3803/EnM.2017.32.1.106

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Trends in Childhood Thyroid Cancer incidence in Korea and Its Potential Risk Factors

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Background: Although the incidence of thyroid cancer had been increasing until a few years ago, a decrease has been observed in the last years, probably due to the reduction of the screening tests in Korea. Childhood thyroid cancer has been increasing in the past with the same trend as in adults, but there have been few reports on recent trends. We analyzed the trends of thyroid cancer in Korean children and related factors.

Methods: From national statistics and cancer register database, the data of age-specific incidence rate in Korean childhood thyroid cancer from 1999 to 2017 was obtained, and levels of seaweed intake, the number of computed tomography (CT) and neck ultrasonography (US), obesity prevalence rate, and smoking and alcohol consumption rates in children were analyzed.

Results: The age-specific incidence of thyroid cancer in Korean children has increased in both genders between 1999 and 2017 (2.0 in 1999 vs. 7.2 in 2017, per population of 100,000), especially in the age group of 14-18 years (1.5 in 1999 vs. 5.5 in 2017, per population of 100,000). During the same period, levels of seaweed intake, number of CT scans and neck US, and prevalence of obesity in children increased significantly, while childhood smoking and alcohol consumption rates decreased.

Conclusion: Unlike the adult thyroid cancer in Korea, childhood thyroid cancer continues to increase, and the cause might be accompanied by actual increases due to the environmental factors such as excessive iodine intake, exposure to medical radiation, and increased obesity prevalence as well as the screening effect.

Keywords: childhood, thyroid, cancer, incidence, increase, Korea

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INTRODUCTION

The worldwide incidence of thyroid cancer has been increasing (1, 2). This is mainly due to earlier detection of small sized thyroid carcinomas using high-resolution ultrasonography (US) (3). However, the incidence of larger thyroid cancers is also increasing (1, 4). Therefore, many experts have emphasized that early detection or over-diagnosis cannot completely explain the observed increase in thyroid cancer (1, 4, 5).

The understanding of predisposing genetic factors is increasing (6, 7). Czene et al. has suggested that genetic factors play a major role in the pathogenesis of thyroid cancer and that the population living in East Asia, including Korea, is genetically susceptible to thyroid cancer (7). Environmental factors, such as iodine intake, increased exposure to radiation, and rising rates of obesity may be the potential candidates to explain this phenomenon (8–12).

In the past, the incidence of childhood thyroid cancer had been increasing, although it remained very low. Siegel et al. also reported that annual percentage changes in the incidence of childhood thyroid cancer between 2001 and 2009 were increasing with a range from 4.3% to 6.6% in the United States (13). Our research team previously reported an increasing trend of childhood thyroid cancer in Korea until 2012 and since most children are not screened for thyroid cancer, 72% of childhood thyroid cancers were detected by palpation rather than screening (14). However, some experts stated that the increased incidence of childhood thyroid cancer is due to overdiagnosis by US-based screening (15).

The incidence of thyroid cancer has been decreasing from around 2012 based on the Korea Central Cancer Registry (KCCR), as US screening tests have decreased in Korea (16). The question arises whether the incidence of childhood thyroid cancer has also decreased since 2012 as in adults. Therefore, in the present study, we evaluated the incidence of childhood thyroid cancer in Korea between 1999 and 2017 to investigate its changes after 2012. We also analyzed the trends in iodine intake, medical radiation, and obesity in children and adolescents, which were known to be associated with the development of thyroid cancer by using data from national statistics and cancer register database.

MATERIALS AND METHODS

Data from Statistics Korea (KOSTAT) and the KCCR were used to obtain incidences of thyroid cancer, brain tumors, and leukemia in total and childhood populations. The KCCR has been described in a previous study (14). Mid-year total and childhood populations were calculated as arithmetic averages of the resident registration populations on the first and last days of each year in supplementary data.

The total incidence of thyroid cancer by year was calculated as an age-adjusted incidence rate (population by age of resident registration in 2005). The annual incidence of childhood thyroid cancer was calculated as an age-specific incidence rate by dividing the number of newly diagnosed childhood thyroid cancer patients by the number of persons in the same age group (0-18 years). Incidences for brain tumors and leukemia were also calculated as age-specific incidence rates in supplementary data.

The Korea National Health and Nutrition Examination Survey (NHANES) has been conducted every three years since 1998. Since 2007, it has been operated as a regular annual survey conducted by the Korean Ministry of Health and Welfare (MOHW). This survey is administered to people above the age of 1 year in 11,520 households nationwide, and the consumption of each food group is assessed using a questionnaire in 192 districts and 4,416 households nationwide each year. Data on dietary iodine intake was obtained from the NHANES's standardized daily seaweed intake trend for each food group. Dried kelp is a food ingredient commonly used to make broth in Korea, and if the ingredients were discarded, the food was treated as not comsumed before 2013. Since 2013, as broth foods were added to the survey, the actual nutrient intake was more accurately reflected, The annual trend data for seaweed intake is the result of standardization with the 2005 estimated population to compensate for the impact of age structure differences.

The number of CT scans in children (0-18 years) was identified through CT cases registered in the HIRA database. The age-specific CT scan rate was calculated as the number of childhood CT scans per year divided by the mid-year childhood population from 2007 to 2018. Moreover, since neck US was claimed for insurance from mid-2013, the number of US cases in children (4-18 years) from 2014 to 2017 was identified from the HIRA database.

Data on rates of childhood (13-18 years) obesity prevalence, smoking, and alcohol consumption were based on a Youth Health Behavior Survey, which is a survey of 800 middle and high schools and approximately 75,000 students, conducted by the MOHW form 2005. Childhood obesity prevalence rate is indicated as the percentage of survey respondents who are above the 95th percentile according to the body mass index (BMI) growth chart by age for children and adolescents in 2017. The childhood smoking rate was calculated as the percentage of survey respondents who have smoked more than one day in the last 30 days, and the childhood alcohol consumption rate was calculated as the percentage of survey respondents who have drunk more than one drink in the last 30 days.

RESULTS

Trends in Incidence of Childhood Thyroid Cancer in Korea Between 1999 and 2017

From 1999 to 2017, the number of children with thyroid cancer per year ranges from 79 to 225. The age-adjusted incidence of thyroid cancer in Korea had gradually increased and peaked in 2012, but has been rapidly declining since 2013. The age-specific incidence of childhood (0-18 years) thyroid cancer in Korea also increased and peaked in 2013, followed by a decrease. However, it has been increasing again since 2015 (**Figure 1**). The age-specific incidence of childhood thyroid cancer saw a 3.6-fold increase from 2.0 per 100,000 children in 1999 to 7.2 per 100,000 children in 2017. As in adult, girls have higher incidence rates of thyroid cancer than boys, but while the rate of increase in incidence among girls was faster until 2012, the rate of increase in incidence in boys became faster from 2012 to 2017. Between 1999 and 2017, the 4.75-fold overall increase in the incidence rate in boys (0.8 vs. 3.8 per 100,000, respectively) was larger than the 3.1-fold increase seen in girls (3.5 vs. 10.8 per 100,000, respectively) (**Figure 2A**). According to the age-specific

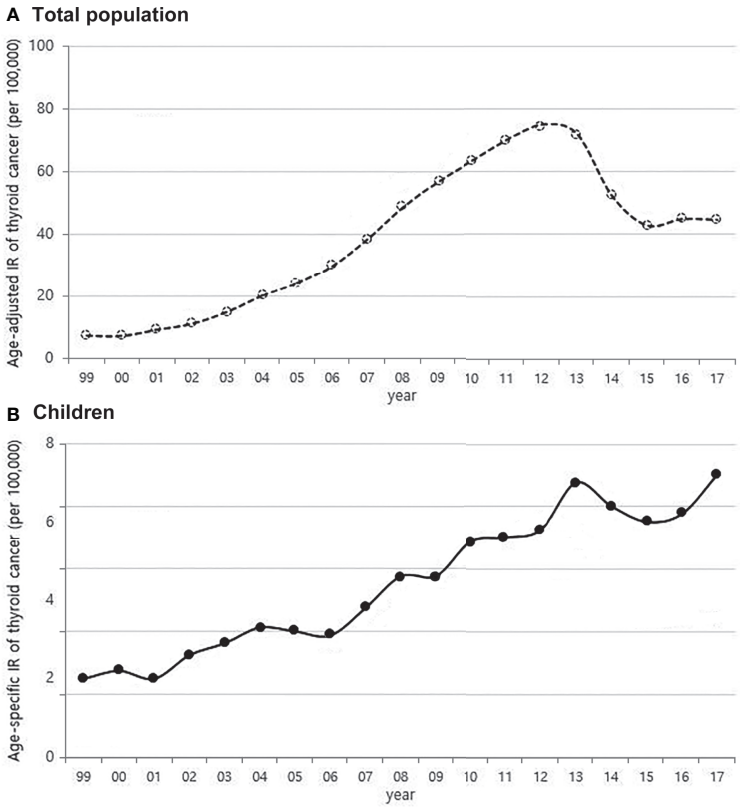

FIGURE 1 | Age-adjusted incidence of thyroid cancer in Korea **(A)** and age-specific incidence rate of childhood thyroid cancer (0-18 years) **(B)** (1999-2017, per population of 100,000). IR, incidence rate.

incidence rates, the age group of 14-18 years accounted for the majority, approximately 75%, of all childhood thyroid cancer cases, and this group's rate continued to increase from 1.5 per 100,000 in 1999 to 5.5 per 100,000 in 2017 (**Figure 2B**).

Environmental Factors That Might Result in an Increase in Childhood Thyroid Cancer

Although it is not possible to compare before 2013 (broth food was not included), but the age-standardized rates of seaweed intake, which accounts for the bulk of dietary iodine consumption in Korea, have risen since 2013 when broth foods were added to the survey, based on the Korea NHANES (**Figure 3**). The number of CT scans in children and adolescents (0-18 years) has been increasing every year from approximately 260,000 cases in 2007 to 490,000 cases in 2018 (**Figure 4**). Adolescent (13-18 years) obesity rates from 2006 to 2018 also showed a steady increase in both genders (from 5.9% in 2006 to 10.8% in 2018), which corresponds to the increase in incidence of childhood thyroid cancer (**Figures 5A, B**). On the other hand, adolescent (13-18 years) cigarette smoking and alcohol consumption rates tended to decrease from 2005 to 2018 (11.8% to 6.7% and 27.0% to 16.1%, respectively), corresponding to a negative correlation with the incidence of childhood thyroid cancer (**Figures 6A, B**).

Trends in Neck US Exams in Korean Children Between 2014 and 2018

The national health insurance guarantee of neck US has begun from mid-2013, and the age-specific number of US exams for children charged with HIRA from 2014 to 2018 has increased sharply in all age groups (**Figures 7A, B**). This is different from the overall trend in incidence of childhood thyroid cancer, especially those aged 14-18 years (**Figures 7B** and **2B**).

DISCUSSION

We investigated trends in the incidence of childhood thyroid cancer in Korea over the past 18 years and also analyzed the trends in previously considered potential risk factors for thyroid cancer, such as the dietary iodine intake, the number of CT scans performed, and obesity prevalence in children or adolescents, by using data representing the Korean population.

Several studies have shown that iodine intake levels that are lower or higher than recommended result in an increase in thyroid cancer. Mousavi et al. showed that there were high rates of thyroid cancer among first-generation immigrants to Sweden from areas with inadequate or excessive levels of iodine intake

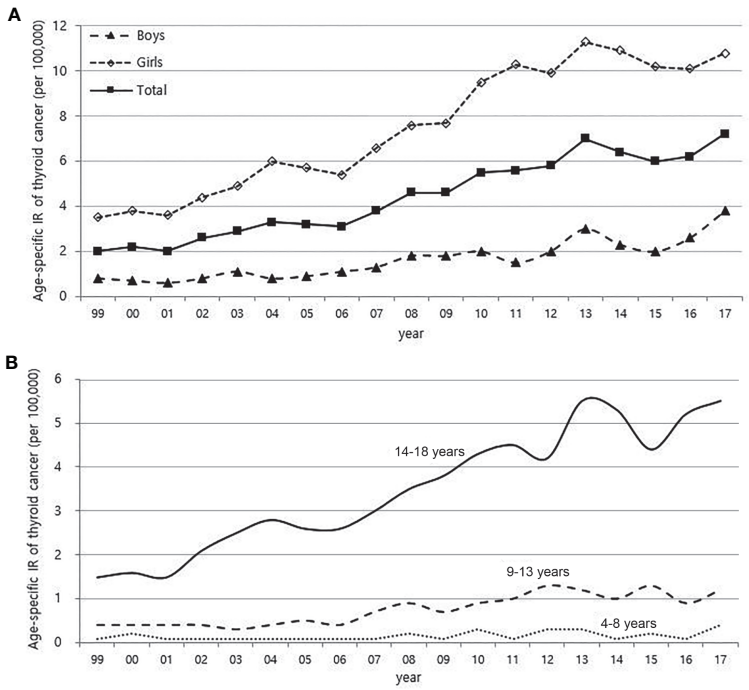

FIGURE 2 | Age-specific incidence rate of childhood (0-18 years) thyroid cancer according to gender **(A)** and age **(B)** (1999-2017, per population of 100,000). IR, incidence rate.

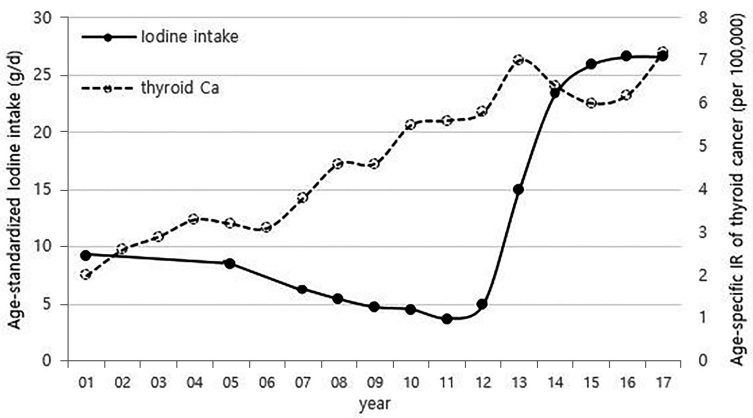

FIGURE 3 | Correlation between age-standardized iodine intake (2007-2017) and age-specific incidence rate of childhood thyroid cancer (0-18 years) by year in Korea (1999-2017, per population of 100,000). Broth foods were added to the survey since 2013. Ca, cancer; IR, incidence rate.

(17). Michikawa et al. demonstrated that the incidence of thyroid cancer was significantly higher in Japanese women, who consumed seaweed more than 3-4 times per week, than in those who consumed seaweed fewer than 2 days per week, with hazard ratio 1.71 (95% CI: 1.01-2.90, p = 0.04) (18). In fact, there is a high level of iodine consumption in Korean due to the large amounts of seaweed intake (19). Iodine consumption in Korean children is higher than in adult groups (**Figure S1**) (20), and children have a relatively smaller volume of thyroid gland than adults, thus, it is expected that high iodine intake can have a greater impact on the development of thyroid cancer in children.

Radiation exposure of the thyroid, particularly in childhood, is a clearly established risk factor for the development of thyroid

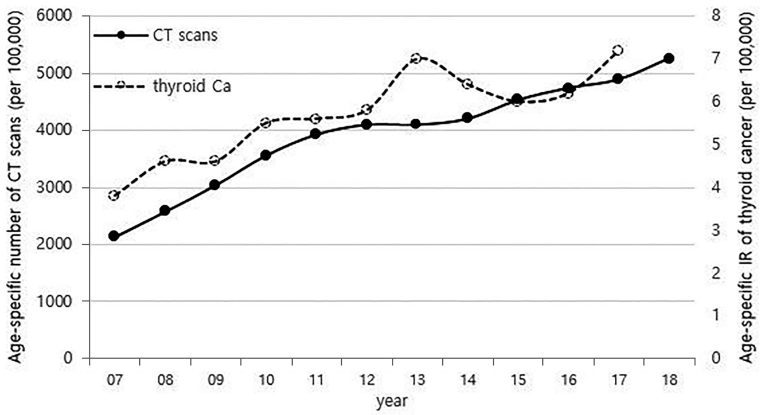

FIGURE 4 | Correlation between the age-specific number of CT scans in childhood (0-18 years) (2007-2018, per population of 100,000) and age-specific incidence rate of childhood thyroid cancer (0-18 years) by year in Korea (2007-2017, per population of 100,000). CT, computed tomography; Ca, cancer; IR, incidence rate.

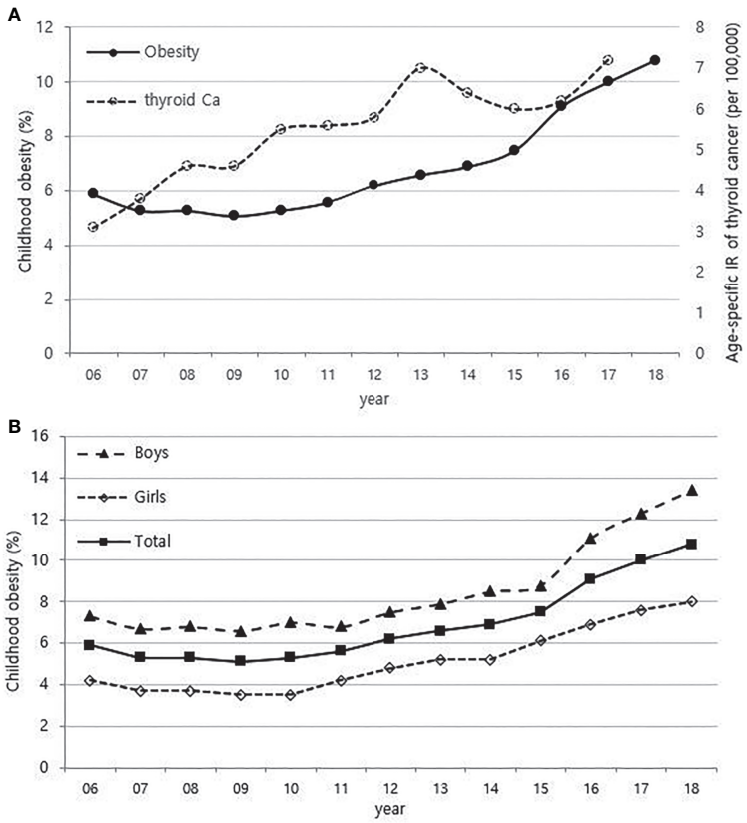

FIGURE 5 | Correlation between childhood obesity prevalence (13-18 years) (2006-2018) and age-specific incidence rate of childhood thyroid cancer (0-18 years) by year in Korea (2006-2017, per population of 100,000) **(A)**. Childhood obesity prevalence according to sex **(B)**. Ca, cancer; IR, incidence rate.

cancer, as demonstrated by the Chernobyl accident (21). There is a report that there was no significant increase in the incidence of pediatric thyroid cancer after the Fukushima Daiichi Nuclear Power Plant accident (22), but considering that the incidence of thyroid cancer increased rapidly after 4-5 years at the time of the Chernobyl accident, it is necessary to understand the latency phase. CT scans have been the largest contributor to radiation exposure in recent years. The childhood population in Korea

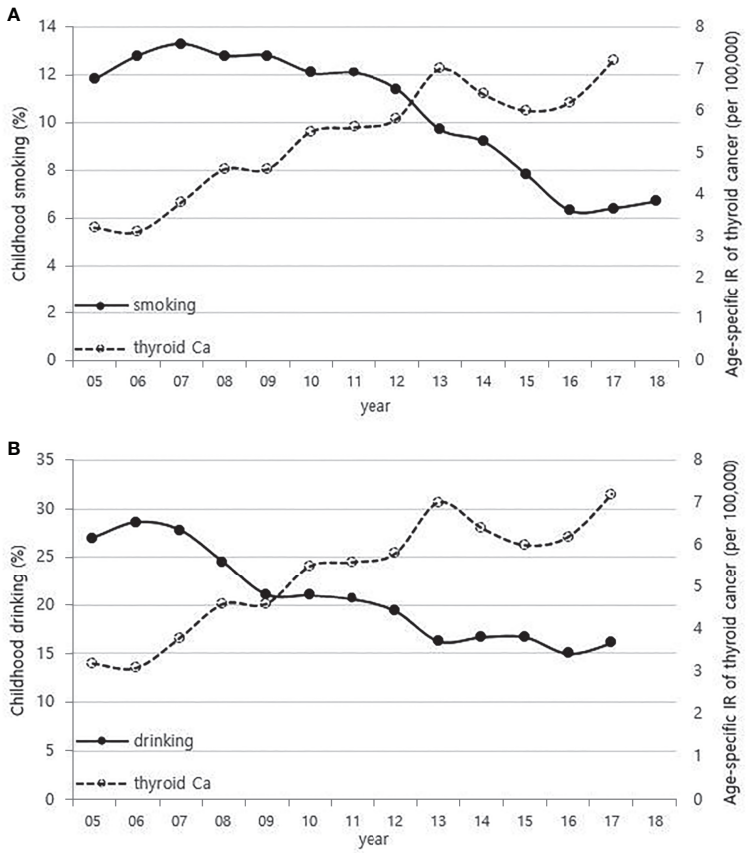

FIGURE 6 | Correlation between childhood smoking **(A)** and drinking **(B)** rates (13-18 years) (2005-2018) and age-specific incidence rate of childhood thyroid cancer (0-18 years) in Korea (2005-2017, per population of 100,000). Ca, cancer; IR, incidence rate.

steadily decreased (**Figure S2**), while the age-specific number of CT scans has steadily increased since 2007. The use of CT scans is rising more rapidly in the childhood population than in adults, and the thyroid glands of children are more sensitive to radiation than those of adults (23, 24). It is known that CT scans in children also increase the risk of developing brain tumors and leukemia as well as thyroid cancer, and the increase in risk is greatest for brain tumors (25, 26). However, while the incidence of childhood thyroid cancer is rapidly increasing, the incidence of brain tumors and leukemia in children has only been minimally increasing during the same period (**Figure S3**). Thus, the recent increase in childhood thyroid cancer in Korea may be associated with the increase in medical exposure, but it is not expected to be a major factor. In addition, as neck US exam of children has been also increasing recently (**Figure 7**), there must be some portion of the increase due to the incidental detection of small sized thyroid cancer. However, the increase in childhood thyroid cancer cannot be fully explained by the increase in incidental detection, and the true increase should be taken into account.

The relationship between obesity and thyroid cancer is still controversial, but there seems to be a positive association between BMI and the incidence of thyroid cancer, particularly in women (9, 27, 28). Thyroid cancer is almost three times more common in women than in men, and it has been suggested that the female sex hormones, especially estrogen, are related (29). Estrogen receptors are found in thyroid tissues. Therefore, the thyroid glands of women are more likely to be irritated by estrogen than those of men, causing inflammation and various thyroid diseases. There are also reports that estrogen promotes the proliferation of thyroid cancer cells (30, 31). In recent years, precocious puberty has been increasing with childhood obesity, and obesity also increases insulin resistance and inhibits sex hormone-binding proteins, which results in an increase in estrogen levels (32, 33). Since the prevalence of childhood obesity in Korea is rapidly increasing, exposure to estrogen at an early age might be contributing to the increase in childhood thyroid cancer. In particular, the prevalence of obesity in boys has been increasing more rapidly than in girls since 2015 in **Figure 2A** of this study, and this may be related to the recent steeper increase in the incidence of thyroid cancer in boys than in girls.

Cigarette smoking and alcohol consumption has an inverse association with thyroid cancer incidence. Many researchers

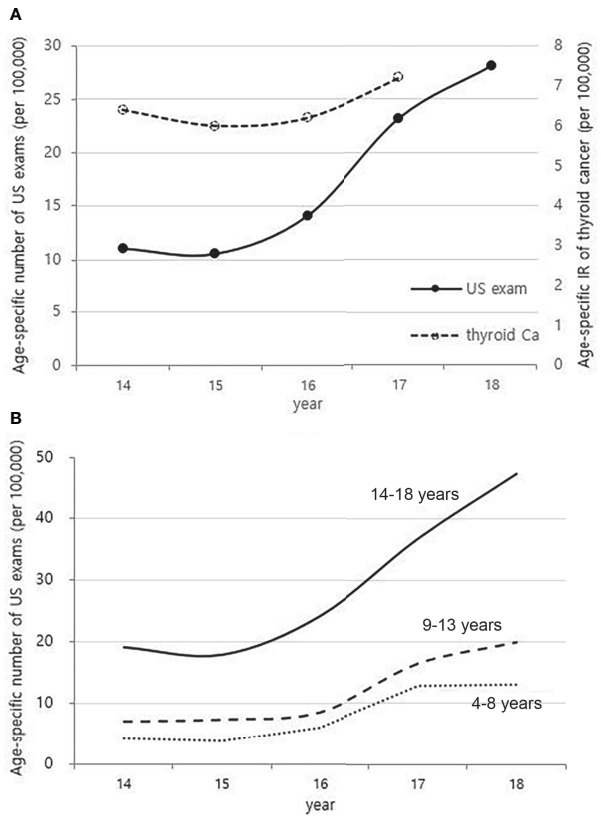

FIGURE 7 | Correlation between the age-specific number of neck US exams in childhood (4-18 years) (2014-2018, per population of 100,000) and age-specific incidence rate of childhood thyroid cancer (0-18 years) by year in Korea (2014-2017, per population of 100,000) **(A)**. Age-specific number of neck US exams in childhood (4-18 years) according to age groups (2014-2018, per population of 100,000) **(B)**. US, ultrasonography; Ca, cancer; IR, incidence rate.

have proposed that since higher thyroid-stimulating hormone (TSH) levels are associated with higher frequency and advanced stage of thyroid cancer (34), and low TSH levels are measured in smokers, suggesting that smokers have a lower risk of thyroid cancer (35, 36). Cho et al. found that men who are currently smoking had a lower risk of incident thyroid cancer even after adjusting for TSH and BMI in a cohort study of 96,855 Korean adults (37), and the reason is probably due to the anti-estrogenic effect of smoking (9, 35, 38). A meta-analysis showed that alcohol intake is responsible for reducing the risk of thyroid cancer (39), and Meinhold et al. demonstrated in a large prospective study that people who consume more than two alcoholic drinks a day have a reduced risk of thyroid cancer compared to those who do not drink alcohol (relative risk = 0.57, 95% CI 0.36–0.89, p-trend = 0.01) (40).

When considering the results from previous research and the positive correlations in overall trend between the incidence of thyroid cancer and dietary iodine consumption, the number of CT scans, and obesity prevalence that were observed among children in this study (**Figures 1**, **3**–**5**), we might be tempted to suggest that when there is a genetic susceptibility, excessive iodine, medical radiation, and obesity are likely to cause thyroid cancer by acting as driving factors in children.

There are several limitations in this study. First, since this research is an ecological study of the entire population, it is not known whether the individual children who were diagnosed with thyroid cancer actually had a high dietary iodine intake, underwent several CT scans, or gained weight. Therefore, the results cannot be interpreted at an individual level, but hypotheses for the next step of research have been presented. Second, considering the potentially long latency periods between exposure and disease onset, it was recommended to refer to the amount of dietary iodine intake, the number of childhood CT scan use, and the childhood obesity prevalence data before 2005. However, since such earlier data were not available, it is meaningful to keep track of the trend of childhood thyroid cancer after 2017. Third, this analysis is the lack of data on tumor detection methods and characteristics such as histology, stage, and size at diagnosis. In addition, since the increase in childhood thyroid cancer is mainly seen in the population of 14-18 years, further analysis of this age group would be more informative, but there was a limit to further analysis as only the nationwide data already collected and published were used. Nevertheless, because there is not enough research on the development of childhood thyroid cancer, the comparison of childhood thyroid cancer incidence trends with total thyroid cancer incidence and the analysis of trends in prevalence of potential risk factors during the same period are a potentially valuable contribution.

In conclusion, the incidence of childhood thyroid cancer is constantly increasing. Although the number of thyroid US exams in children is also increasing, it cannot be concluded that the increase is only due to the screening effect. In addition, we cannot rule out the possibility that a true increase due to the potential risk factors may be implied. Since the environmental factors that can reduce or prevent childhood thyroid cancer can be overlooked, further studies are needed on the causes of the increase in childhood thyroid cancer.

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 Institutional Review Board in Samsung Medical Center (SMC-IRB number: 2020-04-041). 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

JP conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. HP designed the data collection instruments, collected data. TK, SK, and HJ reviewed and revised the manuscript. JC conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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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.2021.681148/full#supplementary-material

REFERENCES

1. Enewold L, Zhu K, Ron E, Marrogi AJ, Stojadinovic A, Peoples GE, et al. Rising Thyroid Cancer Incidence in the United States by Demographic and Tumor Characteristics, 1980-2005. *Cancer Epidemiol Biomarkers Prev* (2009) 18:784–91. doi: 10.1158/1055-9965.EPI-08-0960
2. La Vecchia C, Malvezzi M, Bosetti C, Garavello W, Bertuccio P, Levi F, et al. Thyroid Cancer Mortality and Incidence: A Global Overview. *Int J Cancer* (2015) 136:2187–95. doi: 10.1002/ijc.29251
3. Udelsman R, Zhang Y. The Epidemic of Thyroid Cancer in the United States: The Role of Endocrinologists and Ultrasounds. *Thyroid* (2014) 24:472–9. doi: 10.1089/thy.2013.0257
4. Chen AY, Jemal A, Ward EM. Increasing Incidence of Differentiated Thyroid Cancer in the United States, 1988-2005. *Cancer* (2009) 115:3801–7. doi: 10.1002/cncr.24416
5. Chung JH. Unfounded Reports on Thyroid Cancer. *J Korean Med Sci* (2014) 29:1033–4. doi: 10.3346/jkms.2014.29.8.1033
6. Adjadj E, Schlumberger M, de Vathaire F. Germ-Line DNA Polymorphisms and Susceptibility to Differentiated Thyroid Cancer. *Lancet Oncol* (2009) 10:181–90. doi: 10.1016/s1470-2045(09)70020-8
7. Czene K, Lichtenstein P, Hemminki K. Environmental and Heritable Causes of Cancer Among 9.6 Million Individuals in the Swedish Family-Cancer Database. *Int J Cancer* (2002) 99:260–6. doi: 10.1002/ijc.10332
8. Vigneri R, Malandrino P, Vigneri P. The Changing Epidemiology of Thyroid Cancer: Why is Incidence Increasing? *Curr Opin Oncol* (2015) 27:1–7. doi: 10.1097/cco.0000000000000148
9. Meinhold CL, Ron E, Schonfeld SJ, Alexander BH, Freedman DM, Linet MS, et al. Nonradiation Risk Factors for Thyroid Cancer in the US Radiologic Technologists Study. *Am J Epidemiol* (2010) 171:242–52. doi: 10.1093/aje/kwp354
10. Guan H, Ji M, Bao R, Yu H, Wang Y, Hou P, et al. Association of High Iodine Intake With the T1799A BRAF Mutation in Papillary Thyroid Cancer. *J Clin Endocrinol Metab* (2009) 94:1612–7. doi: 10.1210/jc.2008-2390
11. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-Mass Index and Incidence of Cancer: A Systematic Review and Meta-Analysis of Prospective Observational Studies. *Lancet* (2008) 371:569–78. doi: 10.1016/s0140-6736(08)60269-x
12. Imaizumi M, Usa T, Tominaga T, Neriishi K, Akahoshi M, Nakashima E, et al. Radiation Dose-Response Relationships for Thyroid Nodules and Autoimmune Thyroid Diseases in Hiroshima and Nagasaki Atomic Bomb Survivors 55-58 Years After Radiation Exposure. *JAMA* (2006) 295:1011–22. doi: 10.1001/jama.295.9.1011
13. Siegel DA, King J, Tai E, Buchanan N, Ajani UA, Li J. Cancer Incidence Rates and Trends Among Children and Adolescents in the United States, 2001-2009. *Pediatrics* (2014) 134:e945–55. doi: 10.1542/peds.2013-3926
14. Cho YY, Jang HW, Joung JY, Park SM, Jeong DJ, Kim SW, et al. Trends in Thyroid Cancer Incidence in Korean Children (1999-2012) Based on Palpation and Nonpalpation Detection Methods. *Eur Thyroid J* (2015) 4:252–9. doi: 10.1159/000442047
15. Takano T. Overdiagnosis of Juvenile Thyroid Cancer. *Eur Thyroid J* (2020) 9:124–31. doi: 10.1159/000503323
16. Jung K-W, Won Y-J, Kong H-J, Lee ES. Cancer Statistics in Korea: Incidence, Mortality, Survival, and Prevalence in 2016. *Cancer Res Treat* (2019) 51:417–30. doi: 10.4143/crt.2019.138
17. Mousavi SM, Brandt A, Sundquist J, Hemminki K. Risks of Papillary and Follicular Thyroid Cancer Among Immigrants to Sweden. *Int J Cancer* (2011) 129:2248–55. doi: 10.1002/ijc.25867
18. Michikawa T, Inoue M, Shimazu T, Sawada N, Iwasaki M, Sasazuki S, et al. Seaweed Consumption and the Risk of Thyroid Cancer in Women: The Japan Public Health Center-Based Prospective Study. *Eur J Cancer Prev* (2012) 21:254–60. doi: 10.1097/CEJ.0b013e32834a8042
19. Han M-R, Ju DL, Park YJ, Paik H-Y, Song Y. An Iodine Database for Common Korean Foods and the Association Between Iodine Intake and Thyroid Disease in Korean Adults. *Int J Thyroidol* (2015) 8:170–82. doi: 10.11106/ijt.2015.8.2.170
20. Park SY, Kim HI, Oh HK, Kim TH, Jang HW, Chung JH, et al. Age- and Gender-Specific Reference Intervals of TSH and Free T4 in an Iodine-Replete Area: Data From Korean National Health and Nutrition Examination Survey IV (2013-2015). *PLoS One* (2018) 13:e0190738. doi: 10.1371/journal.pone.0190738
21. Pacini F, Vorontsova T, Demidchik EP, Molinaro E, Agate L, Romei C, et al. Post-Chernobyl Thyroid Carcinoma in Belarus Children and Adolescents: Comparison With Naturally Occurring Thyroid Carcinoma in Italy and France. *J Clin Endocrinol Metab* (1997) 82:3563–9. doi: 10.1210/jcem.82.11.4367
22. Iwaku K, Noh JY, Sasaki E, Suzuki N, Kameda T, Kobayashi S, et al. Changes in Pediatric Thyroid Sonograms in or Nearby the Kanto Region Before and After the Accident At the Fukushima Daiichi Nuclear Power Plant. *Endocr J* (2014) 61(9):875–81. doi: 10.1507/endocrj.ej14-0032
23. Sigurdson AJ, Ronckers CM, Mertens AC, Stovall M, Smith SA, Liu Y, et al. Primary Thyroid Cancer After a First Tumour in Childhood (the Childhood Cancer Survivor Study): A Nested Case-Control Study. *Lancet* (2005) 365:2014–23. doi: 10.1016/s0140-6736(05)66695-0
24. Brenner DJ, Hall EJ. Computed Tomography–An Increasing Source of Radiation Exposure. *N Engl J Med* (2007) 357:2277–84. doi: 10.1056/NEJMra072149
25. Mathews JD, Forsythe AV, Brady Z, Butler MW, Goergen SK, Byrnes GB, et al. Cancer Risk in 680,000 People Exposed to Computed Tomography Scans in Childhood or Adolescence: Data Linkage Study of 11 Million Australians. *BMJ* (2013) 346:f2360. doi: 10.1136/bmj.f2360
26. Shao YH, Tsai K, Kim S, Wu YJ, Demissie K. Exposure to Tomographic Scans and Cancer Risks. *JNCI Cancer Spectr* (2020) 4:pkz072. doi: 10.1093/jncics/pkz072
27. Xu L, Port M, Landi S, Gemignani F, Cipollini M, Elisei R, et al. Obesity and the Risk of Papillary Thyroid Cancer: A Pooled Analysis of Three Case-Control Studies. *Thyroid* (2014) 24:966–74. doi: 10.1089/thy.2013.0566
28. Son H, Lee H, Kang K, Lee I. The Risk of Thyroid Cancer and Obesity: A Nationwide Population-Based Study Using the Korea National Health Insurance Corporation Cohort Database. *Surg Oncol* (2018) 27:166–71. doi: 10.1016/j.suronc.2018.03.001
29. Rahbari R, Zhang L, Kebebew E. Thyroid Cancer Gender Disparity. *Future Oncol* (2010) 6:1771–9. doi: 10.2217/fon.10.127
30. Santin AP, Furlanetto TW. Role of Estrogen in Thyroid Function and Growth Regulation. *J Thyroid Res* (2011) 2011:875125. doi: 10.4061/2011/875125
31. Zeng Q, Chen GG, Vlantis AC, van Hasselt CA. Oestrogen Mediates the Growth of Human Thyroid Carcinoma Cells Via an Oestrogen receptor-ERK Pathway. *Cell Prolif* (2007) 40:921–35. doi: 10.1111/j.1365-2184.2007.00471.x
32. Ahmed ML, Ong KK, Dunger DB. Childhood Obesity and the Timing of Puberty. *Trends Endocrinol Metab* (2009) 20:237–42. doi: 10.1016/j.tem.2009.02.004

33. Holly JM, Smith CP, Dunger DB, Howell RJ, Chard T, Perry LA, et al. Relationship Between the Pubertal Fall in Sex Hormone Binding Globulin and Insulin-Like Growth Factor Binding Protein-I. A Synchronized Approach to Pubertal Development? *Clin Endocrinol (Oxf)* (1989) 31:277–84. doi: 10.1111/j.1365-2265.1989.tb01251.x
34. Boelaert K. The Association Between Serum TSH Concentration and Thyroid Cancer. *Endocr Relat Cancer* (2009) 16:1065–72. doi: 10.1677/erc-09-0150
35. Kreiger N, Parkes R. Cigarette Smoking and the Risk of Thyroid Cancer. *Eur J Cancer* (2000) 36:1969–73. doi: 10.1016/s0959-8049(00)00198-2
36. Kitahara CM, Linet MS, Beane Freeman LE, Check DP, Church TR, Park Y, et al. Cigarette Smoking, Alcohol Intake, and Thyroid Cancer Risk: A Pooled Analysis of Five Prospective Studies in the United States. *Cancer Causes Control* (2012) 23:1615–24. doi: 10.1007/s10552-012-0039-2
37. Cho A, Chang Y, Ahn J, Shin H, Ryu S. Cigarette Smoking and Thyroid Cancer Risk: A Cohort Study. *Br J Cancer* (2018) 119:638–45. doi: 10.1038/s41416-018-0224-5
38. Baron JA, La Vecchia C, Levi F. The Antiestrogenic Effect of Cigarette Smoking in Women. *Am J Obstet Gynecol* (1990) 162:502–14. doi: 10.1016/0002-9378(90)90420-c
39. Hong SH, Myung SK, Kim HS. Korean Meta-Analysis Study G. Alcohol Intake and Risk of Thyroid Cancer: A Meta-Analysis of Observational Studies. *Cancer Res Treat* (2017) 49:534–47. doi: 10.4143/crt.2016.161
40. Meinhold CL, Park Y, Stolzenberg-Solomon RZ, Hollenbeck AR, Schatzkin A, Berrington de Gonzalez A. Alcohol Intake and Risk of Thyroid Cancer in the NIH-AARP Diet and Health Study. *Br J Cancer* (2009) 101:1630–4. doi: 10.1038/sj.bjc.6605337

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Primary Cilia Mediate TSH-Regulated Thyroglobulin Endocytic Pathways

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Primary cilia are sensory organelles with a variety of receptors and channels on their membranes. Recently, primary cilia were proposed to be crucial sites for exocytosis and endocytosis of vesicles associated with endocytic control of various ciliary signaling pathways. Thyroglobulin (Tg) synthesis and Tg exocytosis/endocytosis are critical for the functions of thyroid follicular cells, where primary cilia are relatively well preserved. LRP2/megalin has been detected on the apical surface of absorptive epithelial cells, including thyrocytes. LRP2/megalin on thyrocytes serves as a Tg receptor and can mediate Tg endocytosis. In this study, we investigated the role of primary cilia in LRP2/megalin expression in thyroid gland stimulated with endogenous TSH using MMI-treated and *Tg-Cre;Ift88$^{flox/flox}$* mice. LRP2/megalin expression in thyroid follicles was higher in MMI-treated mice than in untreated control mice. MMI-treated mice exhibited a significant increase in ciliogenesis in thyroid follicular cells relative to untreated controls. Furthermore, MMI-induced ciliogenesis accompanied increases in LRP2/megalin expression in thyroid follicular cells, in which LRP2/megalin was localized to the primary cilium. By contrast, in *Tg-Cre;Ift88$^{flox/flox}$* mice, thyroid with defective primary cilia expressed markedly lower levels of LRP2/megalin. Serum Tg levels were elevated in MMI-treated mice and reduced in *Tg-Cre;Ift88$^{flox/flox}$* mice. Taken together, these results indicate that defective ciliogenesis in murine thyroid follicular cells is associated with impaired LRP2/megalin expression and reduced serum Tg levels. Our results strongly suggest that primary cilia harbors LRP2/megalin, and are involved in TSH-mediated endocytosis of Tg in murine thyroid follicles.

Keywords: LRP2/megalin, primary cilium, thyroglobulin endocytosis, ciliogenesis, thyroid follicular cell

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INTRODUCTION

Thyroglobulin (Tg), the most abundant thyroid-specific protein synthesized by follicular cells (thyrocytes), serves as the molecular template for the synthesis of thyroid hormones T4 and T3 at the thyrocyte–colloid interface. A major regulatory step in thyroid hormone release in mammalian thyroid follicular cells is Tg endocytosis. This process requires micropinocytosis, which includes nonspecific fluid-phase pinocytosis and receptor-mediated endocytosis. Tg internalized by receptors is handled by post-endocytic pathways that sort Tg molecules to undergo lysosomal degradation,

transcytosis, or recycling. Effective Tg endocytosis is primarily regulated by TSH and plays an important role in thyroid hormone release. In thyroid follicular cells, clathrin-coated pits, caveolae-dependent endocytosis, and low-density lipoprotein receptor protein 2 (LRP2, also known as megalin) are involved in receptor-mediated endocytic pathways of Tg (1, 2). LRP2/megalin has been detected on the apical surface of thyrocytes; it serves as a Tg receptor and can mediate Tg endocytosis (1, 2). Megalin knockout mice exhibit hypothyroidism, which is associated with reduced levels of serum Tg and free T4 (fT4) levels, and significantly elevated levels of serum TSH (3).

The primary cilia concentrate proteins, hormones, and ions so that they can exert their effects on the primary ciliary membrane. The ciliary pocket, a cytoplasmic invagination of the periciliary membrane, is a crucial site for exocytosis/endocytosis of vesicles for delivery and retrieval of ciliary membrane components; in addition, receptor-mediated endocytosis takes place at the ciliary pocket (4). Previously, we reported that the primary cilia of the mammalian thyroid follicles protrude from the apical surface of follicular cells toward the luminal colloid and present at the cell–colloid interface (5). In murine thyroid follicular cells, the primary cilium plays important roles in maintaining the globular follicular structure of the thyroid (6). Consequently, defective primary cilia in the murine thyroid results in irregular dilation of follicles and colloid Tg depletion (6). Interestingly, the morphological and functional alterations of the thyroid in *Tg-Cre;Ift88*$^{flox/flox}$ mice with defective primary cilia resembled those in megalin knockout mice (3).

The primary cilium is the key machinery involved in the transduction of the sonic Hedgehog (Shh) signaling pathway. The components of the Shh pathway, smoothened (Smo), patched 1 (Ptch1), GLI family zinc finger 1 (Gli1), GLI family zinc finger 2 (Gli2), and GLI family zinc finger 3 (Gli3), exhibit dynamic movements along the primary cilium (7, 8). In the central nervous system, the endocytic receptor LRP2/megalin mediates Shh signaling. LRP2/megalin forms a co-receptor complex with Ptch1 that promotes Shh binding and internalization of the Shh/Ptch1 complex and Shh pathway activation in the ciliary pocket of neuroepithelial cells (9). Therefore, we propose that induction of LRP2/megalin-mediated Tg endocytosis is followed by activation of the Shh signaling pathway in primary cilia of thyroid follicular cells.

In this study, we investigated whether the primary cilium of thyroid follicular cells plays a role in Tg endocytosis. We observed the primary cilia or ciliogenesis and LRP2/megalin expression in the murine thyroid gland with endogenous TSH stimulation and high rates of Tg endocytosis. In addition, we observed LRP2/megalin expression in thyroid follicular cells of *Tg-Cre;Ift88*$^{flox/flox}$ mice, which have no functional primary cilia due to loss of the *Ift88* gene.

MATERIALS AND METHODS

Mice

Mouse experiments were approved by the Institutional Animal Care and Use Committee of the Catholic Univ. of Korea Daejeon St. Mary's Hospital (approval ID, CMCDJ-AP-2019-002). Male C57BL/6J mice were purchased from DooYeol Biotech (Seoul, Korea). Mice were housed in temperature-controlled (22 ± 2°C) and light-controlled conditions (12 hours light/12 hours dark cycle, lights on at 7 am), and had free access to food and water. Twelve-week-old C57BL/6J mice were divided into two groups: Group 1 was an untreated control group; Group 2 received 0.05% methimazole (MMI, Sigma-Aldrich, 301507) in distilled drinking water for 4 weeks. MMI is used to establish hypothyroidism in experimental animals. Mouse weights were measured before the start of the experiment and after 4 weeks of MMI exposure.

Generation of Thyroid-Specific *Ift88*-Knockout Mice

Ift88$^{flox/flox}$ mice were obtained from Dr. Kim J (Korea Advanced Institute of Science and Technology, Republic of Korea), and *Thyroglobulin-Cre/+* (*Tg-Cre*) transgenic mice were obtained from Dr. Jukka Kero (University of Turku, Finland). The mice were maintained on the C57BL/6 genetic background. *Ift88*$^{flox/flox}$ mice were crossed with *Tg-Cre* transgenic mice to generate thyroid follicle-specific *Ift88*-knockout (*Tg-Cre;Ift88*$^{flox/flox}$) mice. Only 35-week-old male mice were used in this study (6 *Tg-Cre;Ift88*$^{flox/flox}$ and 6 *Tg-Cre;Ift88*$^{+/+}$ mice). The experiments using *Tg-Cre;Ift88* floxed mice received prior approval by the Institutional Animal Care and Use Committee of the Catholic Medical Center (approval ID, CRCC-BE-CMC-17013391).

Blood Collection and Thyroid Function Test

Retro-orbital blood collected from mice was allowed to clot by leaving the sample undisturbed for 30 minutes at room temperature. The clotted blood was centrifuged at 3000*g* for 10 minutes. Sera were separated and stored at -80°C prior to the hormonal assay. Serum fT4 and serum Tg levels were measured using radioimmunoassay (RIA) by Dr. Kun-Ho Kim (Chungnam National University Hospital, Republic of Korea). Serum TSH was measured using a specific mouse TSH RIA provided by Dr. Cheng SY (Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA).

Thyroid Extraction

We extracted mouse thyroid using a stereo microscope (Leica EZ4). The right lobe of the dissected thyroid gland was fixed in 10% neutral buffered formalin for 24 hours at room temperature, and then the tissue was embedded in a paraffin block. Tissue slices were subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry. The left lobe of the thyroid gland was stored at -80°C prior to RNA isolation.

RNA Isolation and RT-qPCR

Total RNA was extracted using TRIzol (Invitrogen). Complementary DNA (cDNA) was synthesized from the total RNA using M-MLV Reverse Transcriptase and oligo-dT primers (Invitrogen). Reverse transcription–quantitative polymerase chain reaction (RT-qPCR) was performed using QuantiTect

SYBR Green PCR Master Mix (QIAGEN). Each reaction was performed in triplicate. PCR primers are presented in **Supplementary Table S2**. Amplification conditions were as follows: 10 minutes at 95°C for enzyme activation, followed by 40 cycles of 95°C denaturation for 10 seconds, 60°C annealing for 30 seconds, and 72°C extension for 30 seconds. The cycle threshold (Ct) values for Gapdh RNA and RNA of target genes were measured and calculated using the Life Technologies 7500 software (Life Technologies, Foster City, CA USA). The bar graph data of target genes were normalized to Gapdh mRNA levels.

Detection of Primary Cilia With Immunofluorescence Staining

Paraffin-embedded 7 μm-thick tissue sections were incubated at 56°C for 5 hours. The sections were then deparaffinized in xylene and rehydrated through a graded series of ethanol baths. Antigens were retrieved in antigen retrieval buffer (0.01 M citric acid–sodium citrate, pH 6.0) by heating the sections in an autoclave at 121°C for 25 minutes. After washing, the sections were air-dried for 30 minutes and rewashed with 1× PBS. The sections were fixed with 4% paraformaldehyde in PBS for 15 minutes and then permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. Tissue sections were blocked with 5% bovine serum albumin in PBS for 30 minutes at room temperature. Thereafter, the sections were incubated with primary antibodies for 24 hours at 4°C. On the following day, the tissue-section slides were washed three times with 1× PBS and incubated at 4°C for 12 hours with secondary antibodies. Primary antibodies against acetylated α-tubulin (Cell signaling), ARL13B (ProteinTech Group), polyglutamylation modification (GT335, AdipoGen), γ-tubulin (Sigma-Aldrich), and thyroglobulin (Dako) were used. Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 or 568 (Invitrogen/Life Technologies) were used for indirect immunofluorescence. The stained slides were observed under a FluoView FV1000 microscope equipped with a charge-coupled device camera (Olympus Corp.). The frequency of primary cilia was determined as follows: 100 follicles of similar size were selected; follicular cells with acetylated α-tubulin–positive and γ-tubulin–positive cilia within each thyroid follicle were counted; and the average number of primary cilia per one follicle was calculated.

Immunohistochemistry Staining

Paraffin-embedded tissue sections (4 μm thick) were incubated at 56°C for 3 hours before immunohistochemistry. The sections were stained using a BenchMark GX automatic system (Ventana Medical Systems, Illkirch, France). All procedures, including antigen retrieval and blocking of endogenous peroxidase activity, were performed automatically by the Benchmark system. Primary antibodies against LRP2 (1:200, Biorbyt), caveolin-1 (1:100, BioVision), Thyroglobulin (1:200, Abcam), Shh (1:100, Bioss Inc.), PTCH1 (1:100, LifeSpan BioSciences), and GLI1 (1:100, Bioss Inc.) were used for immunohistochemistry. Tissue sections were incubated with primary antibody for 32 minutes at 42°C. Immunoperoxidase staining was performed on an LSAB NeuVision system (Ventana) and tissue sections were counterstained with hematoxylin. Tissue slides were analyzed on an OLYMPUS BX51 microscope.

Transmission Electron Microscopy

The mouse thyroid tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C for 4 hours. After washing in 0.1 M cacodylate, samples were post-fixed in 1% OsO_4 in cacodylate buffer (pH 7.2) containing 0.1% $CaCl_2$ for 1 hour at 4°C. Samples were analyzed by electron microscopy (Tecnai G2 Spirit Twin; FEI Company; Korea Basic Science Institute).

Immunoblot Analysis

Immunoblotting was carried out as previously described (10). The following primary antibodies were used: SHH (Bioss Inc.), PTCH1 (LifeSpan BioSciences), SMO (Abcam), GLI1 (Bioss Inc.), and GAPDH (Abcam).

Statistical Data Analysis

Serum levels of fT4, TSH, and Tg were assessed by comparing each group to control mice by one-way ANOVA Dunnett's *post hoc* test. Student's t-test was used to compare mRNA levels between experimental groups *versus* controls. Data are presented as means ± standard deviation (SD). P-value < 0.05 was considered statistically significant.

RESULTS

Histopathological and Functional Analysis of Thyroid in Methimazole (MMI)-Treated Mice

To investigate the characteristics of LRP2/megalin expression and Tg endocytosis in the murine thyroid with endogenous TSH stimulation, we observed thyroid follicles and colloid Tg in control and MMI-treated mice.

Oral administration of MMI elicited a significant decrease in serum levels of fT4 (control = 1.47 ± 0.31 ng/dL; MMI = 0.63 ± 0.20 ng/dL; P = 0.032) and an increase in serum TSH (control = 34.91 ± 20.53 ng/mL; MMI = 232.98 ± 187.91 ng/mL; P = 0.00004), resulting in primary hypothyroidism (**Figure 1A** and **Supplementary Table S1**). Serum Tg levels were 0.21 ± 0.13 ng/mL in the control group and 0.34 ± 0.029 ng/mL in the MMI-treated group, respectively. The serum Tg levels were higher in MMI-treated mice than in untreated controls (**Figure 1A**). The thyroid gland and body weight were larger in the MMI-treated group than in the control group (**Figures 1B-a, C-a** and **Supplementary Table S1**).

The thyroid of MMI-treated mice exhibited irregular-shaped follicles, enlarged follicular cells exhibiting cytoplasmic vacuoles with centrally located nuclei, and depletion of luminal colloid (**Figures 1B-b, c, d, C-b, c, d**). Luminal colloid of control thyroid follicles was homogeneously stained by Tg (**Figure 1D-a, b**), whereas in the MMI-treated group, luminal colloid showed reduced Tg staining density (**Figure 1E-a, b**). At times, little to no luminal colloid Tg was observed in the thyroid follicles of

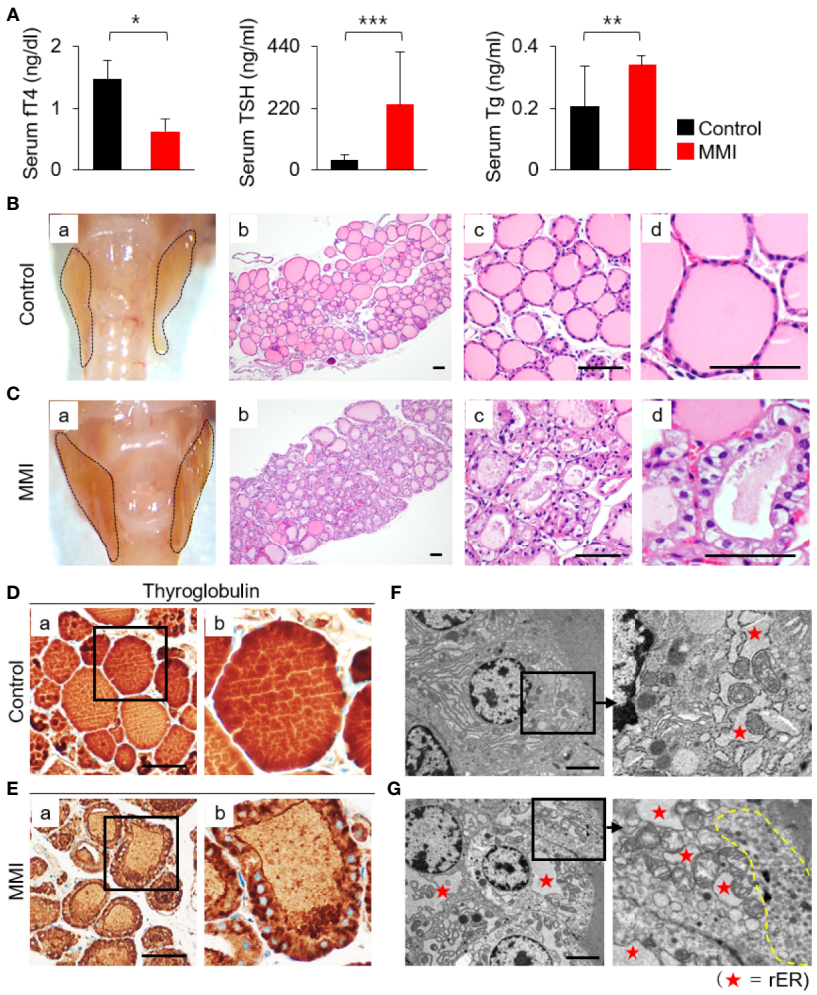

FIGURE 1 | Histopathological characterization and functional activity of thyroid in MMI-treated mice. **(A)** Serum fT4 levels were markedly lower in MMI-treated mice than in control mice ($P = 0.032$). In addition, serum TSH levels in MMI-treated mice were significantly higher ($P = 0.00004$), indicating that primary hypothyroidism in mice was properly induced by MMI treatment. Serum Tg levels were significantly higher in MMI-treated mice ($P = 0.005$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The size of the thyroid gland was larger in MMI-treated mice than controls **(B-a, C-a)**. **(B)** H&E-stained sections of the control thyroid gland showed round/ovoid follicles containing homogeneous colloids that were regularly distributed. Scale bar, 25 μm. **(C)** In the thyroid of MMI-treated mice, irregularly shaped follicles, consisting of enlarged follicular cells with cytoplasmic vacuoles, had reduced luminal colloids. H&E staining. Scale bar, 25 μm. **(D, E)** Follicular cell activity was analyzed by comparing the concentration of colloid Tg between the thyroid of MMI-treated mice and control groups. Scale bar, 25 μm. **(F, G)** Transmission electron micrographs of thyroid glands containing distended cisternae of the rough endoplasmic reticulum (rER, as indicated by star), as well as numerous endocytic vesicles and electron-dense lysosomes (indicated by dotted line) under the apical plasma membrane, from thyroid follicular cells of MMI-treated mice. Scale bar, 2 μm.

MMI-treated mice, yet the cytoplasm of follicular cells was densely stained with Tg (**Figure 1E-b**). These findings indicate that thyroid follicular cells of MMI-treated mice actively take up more colloid Tg due to elevated endogenous TSH stimulation in response to reduced concentrations of serum thyroid hormone, resulting in a smaller follicular lumen, reduced luminal colloid, and elevated cuboidal/columnar follicular cell formation.

Electron microscopic examination of thyroid glands in MMI-treated mice confirmed the increase in cytoplasmic vesicles. More endocytic vesicles and electron-dense lysosomes were found under the apical plasma membrane of thyroid follicular cells in MMI-treated mice than in the control group (**Figures 1F, G**). Further, thyroid follicular cells of MMI-treated mice exhibited hypertrophy relative to the control group (**Figures 1F, G**). In the thyroid follicular cells of MMI-treated mice, the rough endoplasmic reticulum (rER) exhibited distended cisternae, which were visible as cytoplasmic vacuoles under light microscopy (**Figure 1C-d**). Immunohistochemistry revealed cytoplasmic accumulation of Tg in mice treated with MMI (**Figure 1E-b**), but it was not prominent in control mice (**Figure 1D-b**).

Collectively, these morphological changes in MMI-treated mice resulted from increased TSH-stimulated functional activity of thyroid follicular cells.

Elevated Expression of Lrp2/Megalin in MMI-Treated Mouse Thyroid

Based on the morphological and functional characteristics, we investigated whether LRP2/megalin exhibited differential expression under the two experimental conditions. To assess the effects of MMI on receptor-mediated Tg endocytosis of thyroid follicular cells, we first measured the mRNA levels of *Lrp2/megalin*, *Clta* (clathrin light chain A), *Cltb* (clathrin light chain B), *Cltc* (clathrin heavy chain), and *Cav1* (caveolin-1). mRNA levels of *Lrp2/megalin*, *Clta*, and *Cltb* were higher in the thyroid of MMI-treated mice than the control group (**Figure 2A**). mRNA levels of *Cav1* and *Cltc* were elevated, but not significantly (**Figure 2A**).

Next, we observed the expression pattern of LRP2 in thyroid follicles in mice treated with MMI. LRP2 immunohistochemistry is restricted to the apical plasma membrane of thyroid follicular cells. However, LRP2 expression increased in both the apical plasma membrane and the cytoplasm in follicular cells of the MMI-treated group (**Figure 2B**). Interestingly, Tg immunofluorescence increased in the cytoplasm of follicular cells of MMI-treated mice (**Figure 2C**). These results are consistent with previous observations that Lrp2/megalin mediates Tg uptake under intense TSH stimulation, resulting in transcytosis of Tg from the colloid to the bloodstream (11).

Increased Ciliogenesis in Thyroid Follicles of MMI-Treated Mice

We next investigated whether there was an association between LRP2/megalin expression and ciliogenesis of primary cilia *in vivo*. To this end, we performed immunofluorescence analysis to assess changes in ciliogenesis associated with Tg endocytosis. Anti-acetylated α-tubulin and anti-γ-tubulin were used as proteins of the ciliary axoneme and the basal body, respectively (**Figure 3A**). The average frequencies of primary cilia in thyroid of control and MMI-treated mice were 37.80 ± 16.75% and 52.65 ± 9.57%, respectively (**Figure 3B**). Thus, the thyroid follicles in MMI-treated mice exhibited a significant increase in ciliogenesis relative to control thyroids ($P = 0.025$). At the same time, we examined changes in the mRNA expression of genes associated with ciliogenesis. The primary cilium is a dynamic organelle that repeatedly undergoes assembly and disassembly.

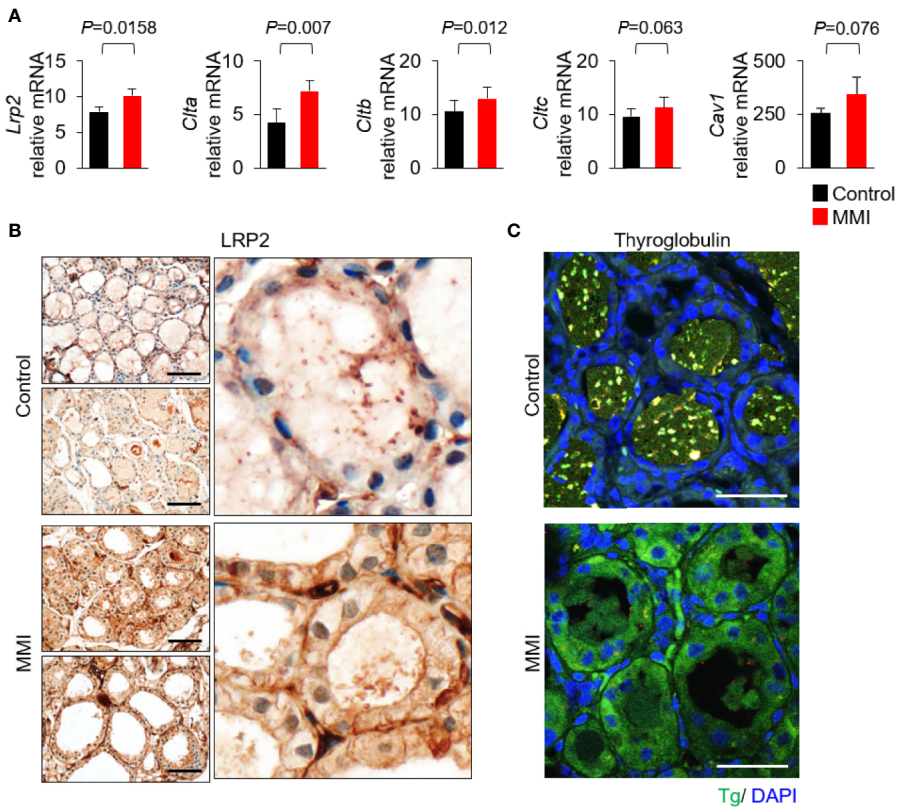

FIGURE 2 | Elevated expression of Lrp2/megalin in MMI-treated mouse thyroid. **(A)** mRNA levels of *Lrp2/megalin* were higher in the thyroid of MMI-treated mice than in the control group ($P = 0.0158$). Two genes associated with Tg endocytosis, *Clta* and *Cltb*, were significantly upregulated in the thyroid of MMI-treated mice relative to the control group (*Clta*, $P = 0.007$; *Cltb*, $P = 0.012$). mRNA levels of *Cav1* ($P = 0.076$) and *Cltc* ($P = 0.063$) were elevated, but the difference was not significant. **(B)** Immunohistochemical staining of LRP2/megalin at the apical plasma membrane of thyroid follicular cells in untreated control mice. In MMI-treated mice, LRP2/megalin was mainly cytoplasmic, but was also detected at the plasma membrane of thyroid follicular cells. Scale bar, 25 μm. **(C)** Immunofluorescence showing localization of Tg. Compared with the control, the level of Tg was markedly higher in the follicular cell cytoplasm of MMI-treated mice. Tg was absent from thyroid follicle lumen of MMI-treated mice but was detectable within the thyroid follicle lumen of the control group. Scale bar, 25 μm.

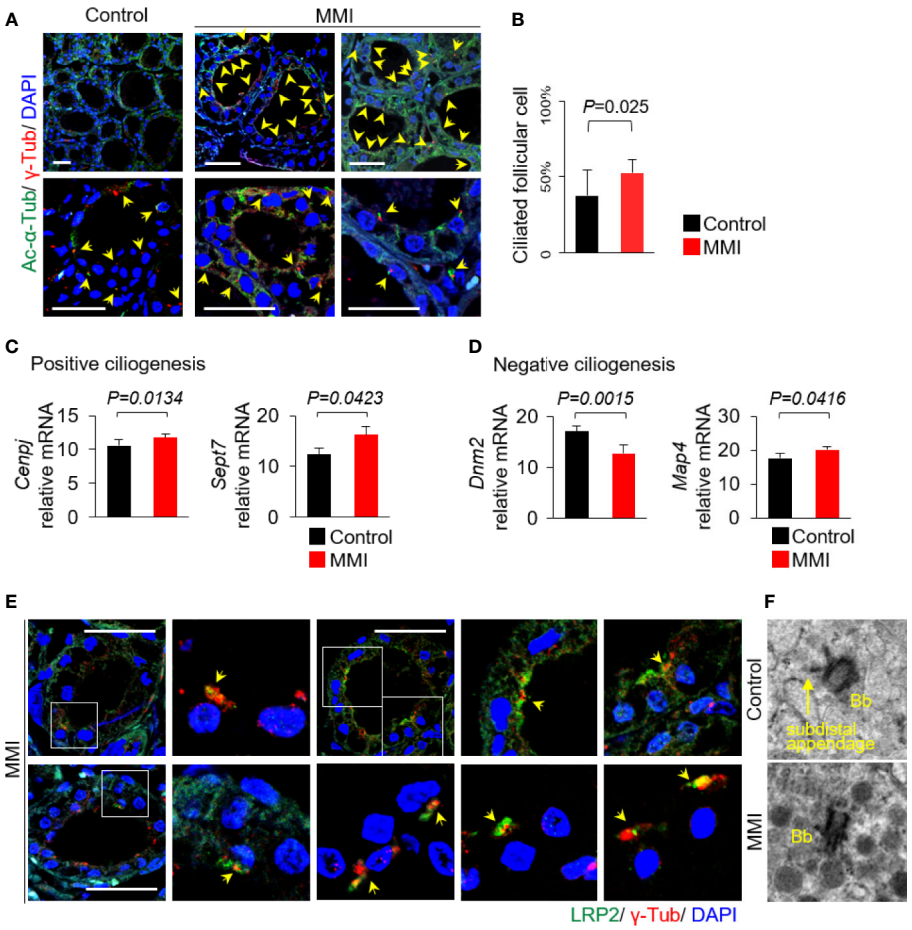

FIGURE 3 | Increased ciliogenesis in thyroid follicles of MMI-treated mice. **(A)** Primary cilia were confirmed by immunofluorescence staining with anti-acetylated α-tubulin (Ac-α-Tub, green) and anti-γ-tubulin (γ-Tub, red). Primary cilia are indicated by arrows. Scale bar, 20 μm. **(B)** Frequency of thyroid follicles containing primary cilia was 37.80 ± 16.75% in the control group and 52.65 ± 9.57% in MMI-treated mice. Ciliogenesis in the thyroid was higher in MMI-treated mice than in control mice (P = 0.025). Arrow indicates primary cilia. **(C)** Two genes involved in positive ciliogenesis, *Cenpj* (P = 0.0134) and *Sept7* (P = 0.0423), were expressed at significantly higher levels in the thyroid of MMI-treated mice than in the control group. **(D)** A gene involved in negative regulation of primary cilia assembly, *Dnm2*, was expressed at significantly lower levels in the thyroid of MMI-treated mice than in the control group (P = 0.0015). By contrast, Map4 was upregulated in the thyroid of MMI-treated mice (P = 0.0416). **(E)** In thyroid follicular cells of MMI-treated mice, LRP2/megalin (green) localized with γ-tubulin (red, basal body marker), indicating the presence of LRP2/megalin in the basal body of the primary cilium (arrow). Scale bar, 20 μm. **(F)** TEM revealed highly abundant endocytic vesicles and lysosomes primarily located near the basal body (Bb) in thyroid follicles of MMI-treated mice.

Cenpj (centromere protein J) and *Sept7* (septin 7), which are involved in positive regulation of primary cilia assembly, were expressed at significantly higher levels in the thyroid of MMI-treated mice than in the control group (**Figure 3C**). In addition, a gene encoding a negative regulator of primary cilia assembly, *Dnm2* (dynamin 2), was expressed at lower levels in the thyroid of MMI-treated mice than in the control group. However, *Map4* (microtubule-associated protein 4) was upregulated in the thyroid of MMI-treated mice (**Figure 3D**).

To clarify the relationship between ciliogenesis and LRP2/megalin expression in the thyroid follicles of MMI-treated mice, we evaluated LRP2 localization to primary cilia. Immunofluorescence microscopy revealed that LRP2 was localized with the basal body of primary cilia marked by γ-tubulin (**Figure 3E**). To further confirm the association between primary cilia and Tg endocytosis, we conducted transmission electron microscopy (TEM) in MMI-treated and untreated murine thyroid follicles. Interestingly, abundant endocytic vesicles and lysosomes were primarily located near the basal body in thyroid follicles of MMI-treated mice (**Figure 3F**).

Taken together, endogenous TSH stimulation resulted in increased ciliogenesis and expression of LRP2/megalin in thyroid follicular cells.

LRP2 Expression of Murine Thyroid Follicles Associated With Ciliogenesis

To investigate the *in vivo* functional roles of primary cilia, we recently produced *Tg-Cre;Ift88*$^{flox/flox}$ mice to characterize thyroid follicular cell-specific ciliary loss (6). IFT88 is a component of intraflagellar transport particle proteins, which

are required for ciliogenesis and ciliary transport (12). We observed the thyroid in *Tg-Cre;Ift88*$^{flox/flox}$ mice aged 7, 11, 14, 20, and 35 weeks. The 7, 11, 14, 20, and 35 weeks-old *Tg-Cre;Ift88*$^{flox/flox}$ mice exhibited focal histomorphological changes in thyroid follicles. However, 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ mice exhibited profound follicular changes, including irregular dilation. Therefore, we chose 35-week-old mice that were more suitable for this experiment. Frequency of primary cilia per thyroid follicle was 3.31 ± 3.00% in the *Tg-Cre;Ift88*$^{flox/flox}$ mice and 43.32 ± 7.72% in the *Tg-Cre;Ift88*$^{+/+}$ control mice. Primary cilia were rarely detected in thyroid follicles of *Tg-Cre;Ift88*$^{flox/flox}$ mice ($P < 0.0001$) (**Figure 4A**). Thirty-five-week-old *Tg-Cre;Ift88*$^{flox/flox}$ mice exhibited hypothyroidism with serum TSH elevation (**Figure 4B**). Serum Tg levels were significantly lower in *Tg-Cre;Ift88*$^{flox/flox}$ mice (0.128 ± 0.026 ng/mL) than in Tg-Cre; Ift88+/+ mice (0.230 ± 0.105 ng/mL, $P = 0.008$) (**Figure 4B**).

Histological examinations revealed that ciliary loss mediated by Ift88 deficiency in thyroid follicles of 35-week-old mice caused irregularly shaped follicles with luminal colloid Tg depletion in the entire thyroid (**Figure 4C**). Cytoplasmic Tg was barely detectable in thyroid follicular cells of *Tg-Cre;Ift88*$^{flox/flox}$ mice (**Figure 4C**). Here, we determined whether Tg depletion of thyroid follicles in *Tg-Cre;Ift88*$^{flox/flox}$ mice was due to an increased endocytosis by high TSH or decreased Tg synthesis. RT-qPCR of Tg mRNA was performed to confirm new Tg synthesis. As a result, there was no difference in thyroid Tg mRNA levels between *Tg-Cre;Ift88*$^{flox/flox}$ (14.623 ± 3.327) and *Tg-Cre;Ift88*$^{+/+}$ mice (14.587 ± 2.141, $P < 0.0001$) (**Figure 4D**). Therefore, the thyroid colloid Tg depletion in *Tg-Cre;Ift88*$^{flox/flox}$ mice was not caused by a decrease in Tg synthesis.

To clarify the relationship between defective primary cilia and Lrp2/megalin expression, we compared the expression of genes related to receptor-mediated Tg endocytosis in 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ thyroid relative to *Tg-Cre;Ift88*$^{+/+}$ control thyroids. First, murine thyroids with ciliary loss mediated by Ift88 deficiency exhibited significant downregulation of mRNA levels of *Clta*, *Cltb*, and *Cltc* relative to wild-type controls, although the difference in *Cltc* expression was not statistically significant (**Figure 4D**). mRNA levels of caveolin-1 (*Cav1*) and caveolin-2 (*Cav2*) were lower in the thyroid follicles of *Tg-Cre;Ift88*$^{flox/flox}$ than in those of *Tg-Cre;Ift88*$^{+/+}$ mice (**Figure 4D**). In particular, mRNA levels of Lrp2/megalin were markedly lower in 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ than *Tg-Cre;Ift88*$^{+/+}$ thyroids (**Figure 4E**). Immunohistochemistry revealed that the LRP2 was present on the apical plasma membrane of control thyroid follicles, whereas LRP2 was less expressed in the thyroid follicles of *Tg-Cre;Ift88*$^{flox/flox}$ mice (**Figure 4F**).

Therefore, loss of primary cilia in murine thyroid follicles was correlated with reduced expression of Lrp2/megalin, which was also associated with irregularly dilated follicles with colloidal Tg depletion and lower levels of serum Tg.

Interaction Between LRP2/Megalin, Tg Endocytosis, and SHH Signaling in the Primary Cilium of Thyroid Follicles

LRP2/megalin-mediated endocytosis controls Shh trafficking and signaling (13–15). The primary cilia, which play critical roles in signal transduction, are central organelles in the Shh signaling pathway (7, 8). The primary cilium can act as a positive or negative regulator of Shh signaling (16, 17). To elucidate the molecular mechanism of LRP2/megalin-mediated Tg endocytosis in primary cilia, we analyzed the expression levels of genes and proteins related to the SHH signaling pathway in the thyroid of MMI-treated and untreated control mice. Although *Shh* mRNA levels exhibited no significant change, the mRNA levels of *Smo*, *Ptch1*, *Gli1*, *Gli2*, and *Gli3* in the thyroid were higher in MMI-treated mice than in the control group (**Figure 5A**). Next, we monitored the expression of SHH signaling pathways in the thyroid of MMI-treated mice by immunohistochemistry and immunoblot assays for SHH, PTCH1, SMO, and GLI1. GLI1 immunohistochemistry was consistently higher in the cytoplasm and nucleus of thyroid follicular cells of MMI-treated mice relative to control, whereas SHH was barely detectable in the thyroid of MMI-treated mice and controls (**Figure 5B**). Immunoblotting revealed an increase of PTCH1 and GLI1 expressions in the thyroid of MMI-treated mice (**Figure 5C**), and it showed an increase in the expression of PTCH1 without a significant change in SHH (**Figure 5C**). These findings indicate that Shh signaling was upregulated in the thyroid of TSH-stimulated mice. Consistent with this, the PTCH1 is detected in primary cilium of controls, whereas it is not observed in primary cilium of MMI-treated mice (**Figure 5D**).

DISCUSSION

Here, we demonstrated that Lrp2/megalin is localized in the primary cilium of thyroid follicular cells. Endogenous TSH stimulation in MMI-treated mice increased Lrp2/megalin expression and Tg endocytosis. In addition, thyroid-specific cilium-deficient *Tg-Cre;Ift88*$^{flox/flox}$ mice exhibited a significant loss of Lrp2/megalin and a reduction in serum Tg despite the high TSH level. Hence, these results may link the functional role of thyroid primary cilium with Lrp2/megalin-mediated Tg endocytosis *in vivo*.

LRP2/megalin plays a role in Tg uptake in thyroid follicular cells. TSH-stimulated Tg uptake is an important determinant of thyroid hormone and Tg release into the bloodstream (18–20). We found that Lrp2/megalin in *Tg-Cre;Ift88*$^{flox/flox}$ mice exhibited a significant reduction in serum Tg levels despite having high levels of TSH. These findings indicate that cilium-deficient thyroid follicular cells impaired Tg uptake with TSH stimulation. In this study, we showed that *Tg-Cre;Ift88*$^{flox/flox}$ mice developed as irregularly dilated thyroid follicles with depleted colloid Tg. These follicular changes, accompanied by a reduced serum Tg and elevated serum TSH, suggest that absence of LRP2 leads to reduced thyroid hormone and Tg release.

Tg endocytosis can be mediated by a variety of cell membrane proteins, such as asialoglycoprotein receptor (21), N-acetylglucosamine receptor (22), several low-affinity receptors (23, 24) and through components of membrane rafts (25). Therefore, not all the endocytic effects can be directly linked to LRP2/megalin. Nevertheless, reductions of LRP2/megalin and serum Tg in thyroid follicles with ciliary loss make it possible to determine that LRP2/megalin is more suitable for elucidating the association between Tg endocytosis and primary cilia.

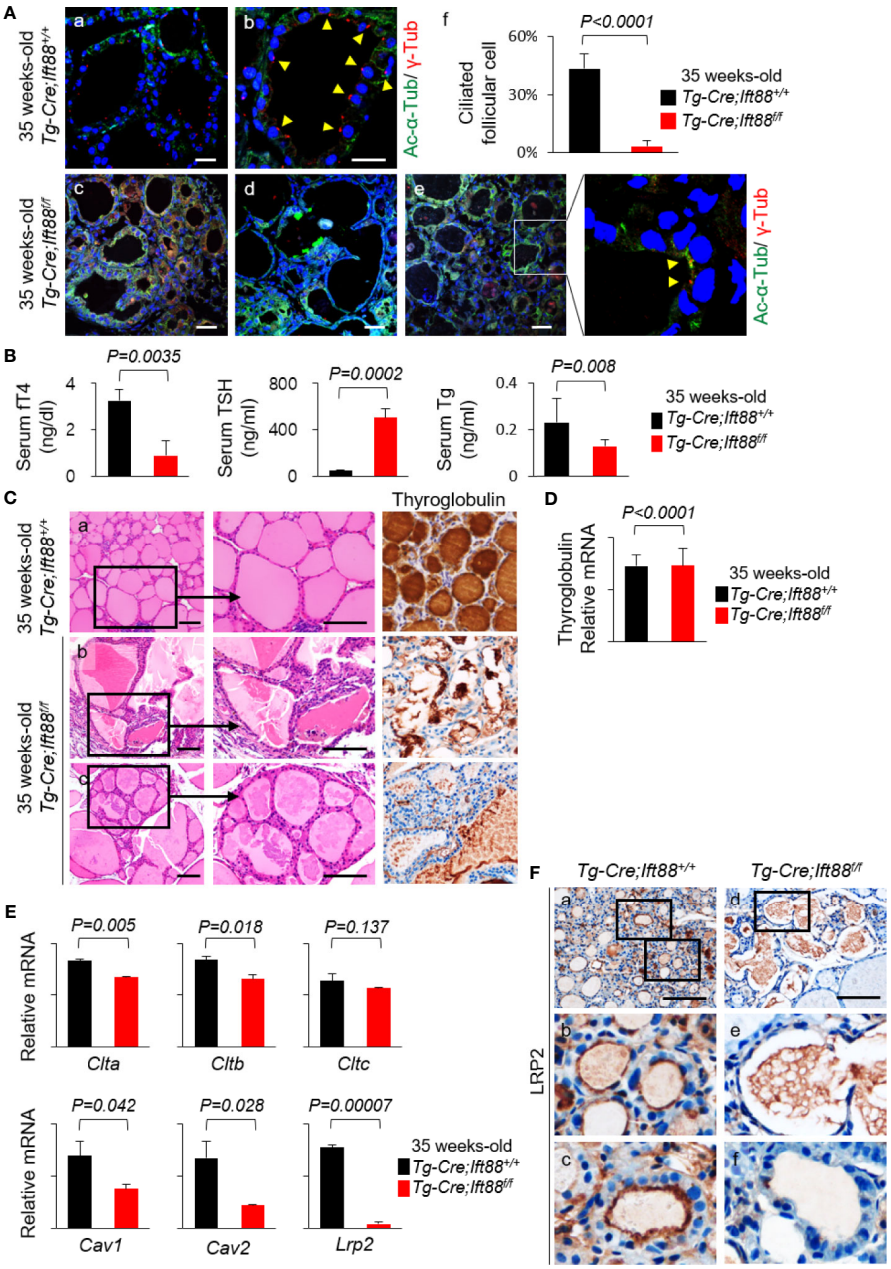

FIGURE 4 | LRP2 expression of murine thyroid follicles regulated by ciliogenesis. **(A)** Immunofluorescence analysis of primary cilia with acetylated α-tubulin (green) to detect the axoneme and γ-tubulin (red) to detect the basal body. Scale bar, 20 μm. Frequency of thyroid follicles containing primary cilia was 3.31 ± 3.00% in the *Tg-Cre;Ift88*$^{flox/flox}$ mice and 43.32 ± 7.72% in the *Tg-Cre;Ift88*$^{+/+}$ control mice. In the thyroid follicles of *Tg-Cre;Ift88*$^{flox/flox}$ mice, primary cilia were rarely detected ($P < 0.0001$). Arrowhead indicates primary cilia. **(B)** Serum fT4 levels were lower in 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ mice (0.89 ± 0.65 ng/dL) than in 35-week-old *Tg-Cre;Ift88*$^{+/+}$ control mice (3.25 ± 0.46 ng/dL, $P = 0.0035$). Serum TSH levels were significantly higher in 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ mice (505.31 ± 75.69 ng/mL) than in 35-week-old *Tg-Cre;Ift88*$^{+/+}$ mice (53.51 ± 3.25 ng/mL, $P = 0.0002$). Serum Tg levels were significantly lower in 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ mice (0.128 ± 0.026 ng/mL) than in 35-week-old *Tg-Cre;Ift88*$^{+/+}$ mice (0.230 ± 0.105 ng/mL, $P = 0.008$). **(C)** Thirty-five-week-old *Tg-Cre;Ift88*$^{flox/flox}$ thyroid had irregularly dilated follicles with colloidal Tg-negative lumens. Scale bar, 25 μm. **(D)** There was no difference in thyroid Tg mRNA levels between *Tg-Cre;Ift88*$^{flox/flox}$ and *Tg-Cre;Ift88*$^{+/+}$ mice (*Tg-Cre;Ift88*$^{flox/flox}$ = 14.623 ± 3.327; *Tg-Cre;Ift88*$^{+/+}$ = 14.587 ± 2.141; $P < 0.0001$). **(E)** mRNA levels of receptor-mediated Tg endocytosis-related genes *Clta*, *Cltb*, *Cltc*, and *Lrp2* were reduced in 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ thyroid with ciliary loss. mRNA levels of genes associated with caveolae-mediated endocytosis were lower in 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ thyroid than in *Tg-Cre;Ift88*$^{+/+}$ control thyroid (*Cav1*, P=0.042; *Cav2*, P=0.028). **(F)** Immunohistochemical staining of LRP2 was present on the apical plasma membrane of control thyroid follicles, whereas LRP2 was expressed at lower levels in thyroid follicles of 35-week-old *Tg-Cre;Ift88*$^{flox/flox}$ mice. Scale bar, 25 μm.

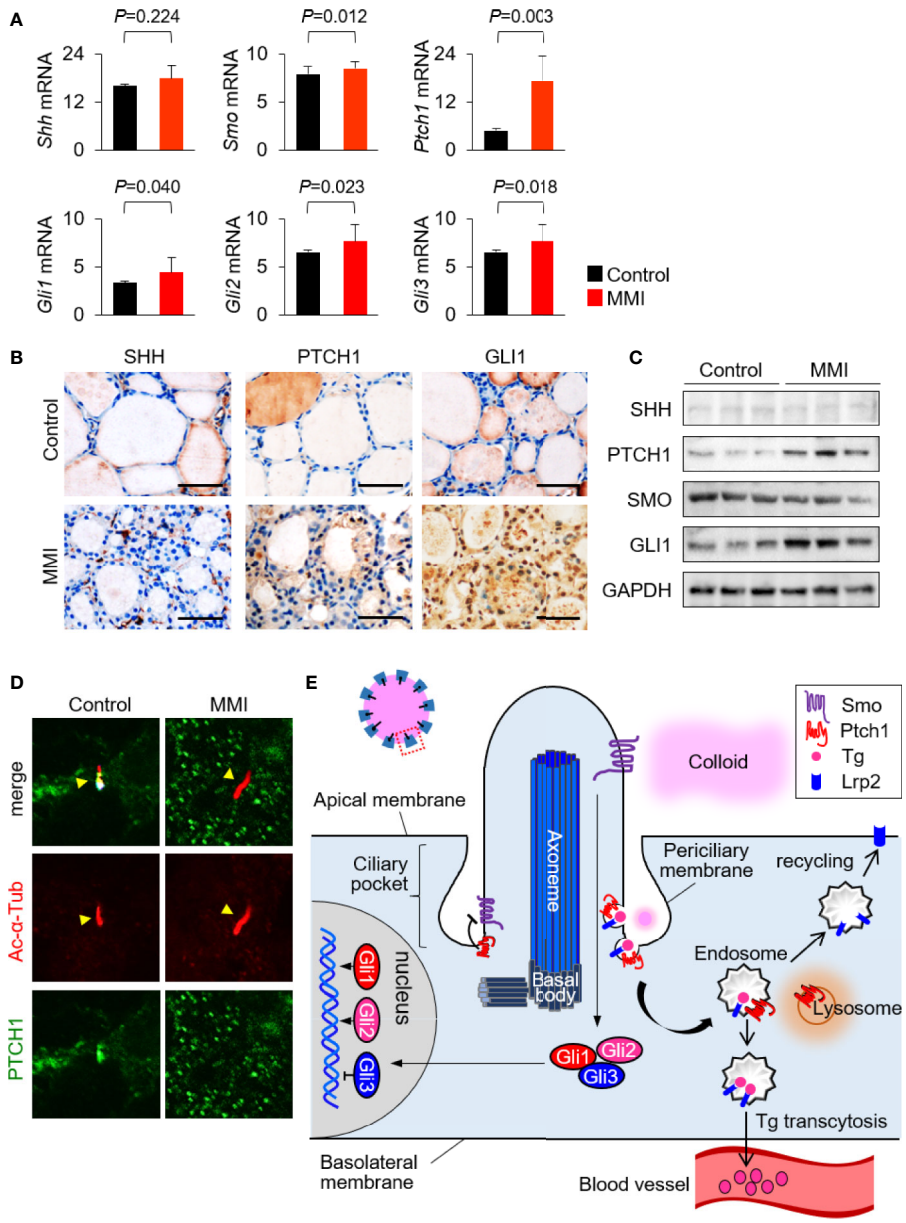

FIGURE 5 | Interaction between LRP2/megalin and SHH in primary cilium of murine thyroid follicles. **(A)** mRNA levels of *Smo*, *Ptch1*, *Gli1*, *Gli2*, and *Gli3* were higher in the thyroid of MMI-treated mice, which exhibited elevated ciliogenesis, than in the control group. **(B)** Immunohistochemistry of PTCH1 and GLI1 was consistently upregulated in the thyroid follicles of MMI-treated mice relative to control. SHH was barely detected in the thyroid of MMI-treated and control mice. Scale bar, 25 μm. **(C)** Immunoblot analysis revealed that PTCH1 and GLI1 levels were higher in MMI-treated mice than in controls. We observed no difference in the expressions of SHH and SMO. Immunoblot data are normalized against GAPDH levels in the same sample. **(D)** PTCH1 was localized in the primary cilium (arrow head) of thyroid follicular cells in the control group. In MMI-treated mice, loss of PTCH1 from the primary cilium (arrow head) was confirmed by immunofluorescence staining. **(E)** Proposed mechanisms for the relationship between receptor-mediated Tg endocytosis and Shh signaling at the primary cilium.

We observed a significant increase in Tg endocytosis and serum TSH levels in the thyroid follicular cells of MMI-treated mice. In addition, both the frequency of primary cilia and LRP2/megalin expression were elevated in the thyroid of MMI-treated mice. High TSH stimulation in follicular cells of MMI-treated mice may be responsible for the elevated frequency of primary cilia. TSH is required not only for the differentiated function of thyrocytes, but also for the stimulation of cell cycle progression and proliferation in various species (26). It remains to be determined how TSH stimulation is directly linked to increased ciliogenesis and LRP2/megalin expression of thyroid follicular cells.

In addition, our findings indicate that Shh/Gli1 signaling pathway contributes to Lrp2/megalin-mediated Tg endocytosis in primary cilia of thyroid follicular cells. The co-localization of

Lrp2 and Tg promoted activation of Ptch1 and translocation of Gli1 to the nucleus. We speculate that Lrp2/megalin-mediated Tg endocytosis activated by TSH stimulation may promote the internalization of Ptch1 near Lrp2/megalin, alleviating Ptch1-dependent inhibition of Smo activity and activating the Shh signaling pathway (**Figure 5E**).

In conclusion, we have demonstrated that ciliogenesis and LRP2/megalin expression are significantly elevated in the thyroid follicular epithelium of endogenous TSH-stimulated mice. Furthermore, these TSH-stimulated mice showed that LRP2 is localized to the primary cilium. *Tg-Cre;Ift88*$^{flox/flox}$ mice, which exhibited thyroid-specific ciliary loss, expressed dramatically lower levels of LRP2/megalin expression. Together, our results strongly suggest that LRP2/megalin-mediated endocytosis of Tg in murine thyroid follicles is regulated by ciliogenesis. This is the first *in vivo* study showing that the primary cilia of thyroid follicular cells are the site of LRP2/megalin-mediated Tg endocytosis.

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 animal study was reviewed and approved by CMCDJ-AP-2019-002.

AUTHOR CONTRIBUTIONS

JL, JYC, and MS conceived the work, designed and performed the experiments, and wrote the manuscript. HJS provided human thyroid tissue samples. Serum-free thyroxine and Tg levels were measured by K-HK. All authors contributed to the article and approved the submitted version.

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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.2021.700083/full#supplementary-material

REFERENCES

1. Marino M, Zheng G, Chiovato L, Pinchera A, Brown D, Andrews D, et al. Role of Megalin (Gp330) in Transcytosis of Thyroglobulin by Thyroid Cells. A Novel Function in the Control of Thyroid Hormone Release. *J Biol Chem* (2000) 275:7125–37. doi: 10.1074/jbc.275.10.7125
2. Marino M, Pinchera A, McCluskey RT, Chiovato L. Megalin in Thyroid Physiology and Pathology. *Thyroid* (2001) 11:47–56. doi: 10.1089/10507250150500667
3. Lisi S, Segnani C, Mattii L, Botta R, Marcocci C, Dolfi A, et al. Thyroid Dysfunction in Megalin Deficient Mice. *Mol Cell Endocrinol* (2005) 236:43–7. doi: 10.1016/j.mce.2005.03.009
4. Molla-Herman A, Ghossoub R, Blisnick T, Meunier A, Serres C, Silbermann F, et al. The Ciliary Pocket: An Endocytic Membrane Domain at the Base of Primary and Motile Cilia. *J Cell Sci* (2010) 123:1785–95. doi: 10.1242/jcs.059519
5. Lee J, Yi S, Kang YE, Chang JY, Kim JT, Sul HJ, et al. Defective Ciliogenesis in Thyroid Hurthle Cell Tumors Is Associated With Increased Autophagy. *Oncotarget* (2016) 7:79117–30. doi: 10.18632/oncotarget.12997
6. Lee J, Yi S, Chang JY, Kim JT, Sul HJ, Park KC, et al. Loss of Primary Cilia Results in the Development of Cancer in the Murine Thyroid Gland. *Mol Cells* (2019) 42:113–22. doi: 10.14348/molcells.2018.0430
7. Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF. Vertebrate Smoothened Functions at the Primary Cilium. *Nature* (2005) 437:1018–21. doi: 10.1038/nature04117
8. Rohatgi R, Milenkovic L, Scott MP. Patched1 Regulates Hedgehog Signaling at the Primary Cilium. *Science* (2007) 317:372–6. doi: 10.1126/science.1139740
9. Pedersen LB, Mogensen JB, Christensen ST. Endocytic Control of Cellular Signaling at the Primary Cilium. *Trends Biochem Sci* (2016) 41:784–97. doi: 10.1016/j.tibs.2016.06.002
10. Lee J, Yi S, Won M, Song YS, Yi HS, Park YJ, et al. Loss-Of-Function of IFT88 Determines Metabolic Phenotypes in Thyroid Cancer. *Oncogene* (2018) 37:4455–74. doi: 10.1038/s41388-018-0211-6
11. Marino M, Chiovato L, Mitsiades N, Latrofa F, Andrews D, Tseleni-Balafouta S, et al. Circulating Thyroglobulin Transcytosed by Thyroid Cells in Complexed With Secretory Components of Its Endocytic Receptor Megalin. *J Clin Endocrinol Metab* (2000) 85:3458–67. doi: 10.1210/jc.85.9.3458
12. Katoh Y, Terada M, Nishijima Y, Takei R, Nozaki S, Hamada H, et al. Overall Architecture of the Intraflagellar Transport (IFT)-B Complex Containing Cluap1/IFT38 as an Essential Component of the IFT-B Peripheral Subcomplex. *J Biol Chem* (2016) 291:10962–75. doi: 10.1074/jbc.M116.713883
13. McCarthy RA, Barth JL, Chintalapudi MR, Knaak C, Argraves WS. Megalin Functions as an Endocytic Sonic Hedgehog Receptor. *J Biol Chem* (2002) 277:25660–7. doi: 10.1074/jbc.M201933200
14. Morales CR, Zeng J, El Alfy M, Barth JL, Chintalapudi MR, McCarthy RA, et al. Epithelial Trafficking of Sonic Hedgehog by Megalin. *J Histochem Cytochem* (2006) 54:1115–27. doi: 10.1369/jhc.5A6899.2006
15. Ortega MC, Cases O, Merchan P, Kozyraki R, Clemente D, de Castro F. Megalin Mediates the Influence of Sonic Hedgehog on Oligodendrocyte Precursor Cell Migration and Proliferation During Development. *Glia* (2012) 60:851–66. doi: 10.1002/glia.22316
16. Wheway G, Nazlamova L, Hancock JT. Signaling Through the Primary Cilium. *Front Cell Dev Biol* (2018) 6:8. doi: 10.3389/fcell.2018.00008
17. Goetz SC, Ocbina PJ, Anderson KV. The Primary Cilium as a Hedgehog Signal Transduction Machine. *Methods Cell Biol* (2009) 94:199–222. doi: 10.1016/S0091-679X(08)94010-3
18. Romagnoli P, Herzog V. Transcytosis in Thyroid Follicle Cells: Regulation and Implications for Thyroglobulin Transport. *Exp Cell Res* (1991) 194:202–9. doi: 10.1016/0014-4827(91)90355-X
19. Herzog V. Transcytosis in Thyroid Follicle Cells. *J Cell Biol* (1983) 97:607–17. doi: 10.1083/jcb.97.3.607

20. Herzog V. Pathways of Endocytosis in Thyroid Follicle Cells. *Int Rev Cytol* (1984) 91:107–39. doi: 10.1016/S0074-7696(08)61315-7
21. Stockert RJ. The Asialoglycoprotein Receptor: Relationships Between Structure, Function, and Expression. *Physiol Rev* (1995) 75:591–609. doi: 10.1152/physrev.1995.75.3.591
22. Consiglio E, Shifrin S, Yavin Z, Ambesi-Impiombato FS, Rall JE, Salvatore G, et al. Thyroglobulin Interactions With Thyroid Membranes. Relationship Between Receptor Recognition of N-Acetylglucosamine Residues and the Iodine Content of Thyroglobulin Preparations. *J Biol Chem* (1981) 256:10592–9. doi: 10.1016/S0021-9258(19)68664-3
23. Giraud A, Siffroi S, Lanet J, Franc JL. Binding and Internalization of Thyroglobulin: Selectivity, pH Dependence, and Lack of Tissue Specificity. *Endocrinology* (1997) 138:2325–32. doi: 10.1210/endo.138.6.5195
24. Lemansky P, Herzog V. Endocytosis of Thyroglobulin Is Not Mediated by Mannose-6-Phosphate Receptors in Thyrocytes. Evidence for Low-Affinity-Binding Sites Operating in the Uptake of Thyroglobulin. *Eur J Biochem* (1992) 209:111–9. doi: 10.1111/j.1432-1033.1992.tb17267.x
25. Luo Y, Akama T, Okayama A, Yoshihara A, Sue M, Oda K, et al. A Novel Role for Flotillin-Containing Lipid Rafts in Negative-Feedback Regulation of Thyroid-Specific Gene Expression by Thyroglobulin. *Thyroid* (2016) 26:1630–9. doi: 10.1089/thy.2016.0187
26. Lee YJ, Park DJ, Shin CS, Park KS, Kim SY, Lee HK, et al. Microarray Analysis of Thyroid Stimulating Hormone, Insulin-Like Growth Factor-1, and Insulin-Induced Gene Expression in FRTL-5 Thyroid Cells. *J Korean Med Sci* (2007) 22:883–90. doi: 10.3346/jkms.2007.22.5.883

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Prognostic Value of Preoperative Serum Calcitonin Levels for Predicting the Recurrence of Medullary Thyroid Carcinoma

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Background: Serum calcitonin level is a useful biomarker for predicting primary tumor size, the extent of lymph node, and distant metastasis in patients with medullary thyroid carcinoma (MTC). However, the association between preoperative serum calcitonin levels and long-term oncologic outcomes has not yet been established. The aims of this study were to determine the preoperative serum calcitonin cut-off value for predicting disease recurrence and to evaluate its prognostic value.

Methods: Patients with MTC (n = 169) who were treated at a tertiary referral hospital in Korea between 1995 and 2019 were enrolled. To determine the preoperative serum calcitonin cut-off value for predicting structural recurrence, the maximum of the standardized log-rank statistics of all possible cut-off values was used. Multivariable Cox regression analysis was used to determine prognostic factors for disease-free survival.

Results: The overall disease-free survival rate was 75.7%. The preoperative serum calcitonin cut-off value that predicted structural recurrence was 309 pg/mL. Preoperative serum calcitonin levels of > 309 pg/mL were the strongest independent predictor of disease recurrence (hazard ratio (HR) 5.33, 95% confidence interval (85% CI) 1.67–16.96; P = 0.005). Lateral lymph node metastasis (HR 3.70, 95% CI 1.61–8.51; P = 0.002) and positive resection margins (HR 3.57, 95% CI 1.44–8.88; P = 0.006) were also significant predictors of disease recurrence.

Conclusions: The preoperative serum calcitonin cut-off value is useful in clinical practice. It is also the best predictive factor for disease-free survival. Preoperative serum calcitonin levels may help determine the optimal postoperative follow-up strategy for patients with MTC.

Keywords: calcitonin, biomarker, medullary carcinoma, recurrence, prognosis

INTRODUCTION

Medullary thyroid carcinomas are a subtype of neuroendocrine tumors that are derived from the parafollicular cells of the thyroid gland, and secrete several hormones and peptides including calcitonin and carcinoembryonic antigen (1). Medullary thyroid carcinomas account for 3%–5% of thyroid carcinomas, with a mean age-standardized incidence of 0.19/100,000 per year (2, 3).

Tumor markers play an important role in screening for early malignancy, diagnosis, prognosis, and surveillance following curative surgery (4). Calcitonin is a tumor marker for medullary thyroid carcinoma. Measurement of serum calcitonin levels in patients with newly diagnosed, histologically confirmed medullary thyroid carcinoma is recommended by the American Thyroid Association (5). Previous studies (6–8) have shown that preoperative basal serum calcitonin levels correlate with primary tumor size, the extent of disease, and postoperative calcitonin normalization. The calcitonin doubling time has also been reported to be an independent predictor of the prognosis of medullary thyroid carcinoma. However, it is difficult to apply in practice because at least four measurements over a 2-year period are required to calculate the calcitonin doubling time (9–11). Postoperative serum calcitonin levels can also predict the recurrence of medullary thyroid carcinoma (12, 13). However, the prognostic value of preoperative basal serum calcitonin cut-off level for predicting the recurrence of medullary thyroid carcinoma has yet to be evaluated.

The aims of this study were to determine the preoperative serum calcitonin cut-off value for predicting structural recurrence and to evaluate its usefulness as a prognostic biomarker for recurrence in patients with medullary thyroid carcinoma.

MATERIALS AND METHODS

Study Population and Ethics

The medical records of 246 patients with medullary thyroid carcinoma who were treated at the Samsung Medical Center, Seoul, Korea between 1995 and 2019 were retrospectively reviewed. Patients were excluded if preoperative serum calcitonin levels were unavailable (n = 71), they had inoperable advanced disease (n = 3), or follow-up data were missing (n = 3). After exclusion, 169 patients were included in the final analysis. The study design was approved by the Institutional Review Board of Samsung Medical Center (approval number: 2020-07-007). The requirement for informed consent was waived owing to the retrospective nature of the study.

Study Outcomes and Definitions

The primary outcome of this study was to determine the preoperative serum calcitonin cut-off value for predicting structural recurrence. Structural recurrence was defined as a newly identified structural disease in the thyroid bed or neck lymph nodes or distant metastasis. A diagnosis of structural disease in the thyroid bed and/or neck lymph nodes by imaging studies was confirmed cytologically or pathologically. Distant metastases were detected by chest and/or abdominopelvic computed tomography, magnetic resonance imaging, whole-body bone scintigraphy, and 19-fluorodeoxyglucose positron emission tomography (PET) and/or were pathologically confirmed. The secondary outcomes were factors associated with disease-free survival, which was defined as the time from initial surgery to the date of first structural recurrence or last follow-up.

Measurements of Serum Calcitonin Levels

The preoperative serum calcitonin levels were all measured by immunoradiometric assay: MEDGENIX CT-U.S.-IRMA kit (BioSource Europe S.A., Belgium) from 1995 to 2005, DSL-7700 ACTIVE IRMA kit (Diagnostic Systems Laboratories, Inc., Webster, TX) from 2005 to 2007. Since then it was replaced by current immunoradiometric assay (CT-US-IRMA, DIAsource ImmunoAssays SA, Louvain-la-Neuve, Belgium). All samples were measured in duplicate. The intra- and interassay coefficients of variation were 2.4%–3.4% and 3.6%–5.4%, respectively. The detection limit was 0.9 pg/mL.

Statistical Analyses

Continuous variables were presented as means ± standard deviation or medians (interquartile range) and analyzed using the Student's t-test or Kruskal-Wallis test, as appropriate. Categorical variables were presented as absolute numbers and percentages and analyzed using the chi-square test or Fisher's exact test. In the derivation of the preoperative serum calcitonin cut-off value for predicting structural recurrence, running log-rank statistics were applied after removing outliers (the upper and lower 10% of patients). The preoperative serum calcitonin value that coincided with the highest log-rank statistic was chosen as the optimal cut-off value (14). A multivariable Cox proportional hazard model was constructed using backward elimination with a univariable inclusion criterion of $P < 0.1$ to assess the independent effects of covariates on disease-free survival. Statistical analyses were conducted using SPSS for Windows version 25.0 (IBM Corp., Chicago, IL, USA) and R version 4.0.1 (The R Foundation for Statistical Computing,

Vienna, Austria; http://www.R-project.org/). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics

The clinicopathological features of all patients with medullary thyroid carcinoma ($n = 169$) are summarized in **Table 1**. The mean ± standard deviation age was 49.4 ± 14.5 years; 112 patients (65.1%) were female. The median (interquartile range) follow-up was 84 (39.5–127.5) months. One hundred and sixty-seven (98.8%) and 162 patients (95.9%) underwent total thyroidectomy and central neck dissection, respectively. The primary tumor size distribution was as follows: less than or equal to 2.0 cm, 118 patients (69.8%); greater than 2.0 cm but less than or equal to 4.0 cm, 38 patients (22.5%); and greater than 4.0 cm, 13 patients (7.7%). Gross extrathyroidal extension was present in 18 patients (10.7%). Seven patients (4.1%) had positive resection margins. Central and lateral neck lymph node metastases were detected in 71 (42.0%) and 62 patients (36.7%), respectively. Ninety-four patients (55.6%) had preoperative serum calcitonin levels of > 309 pg/mL.

TABLE 1 | Baseline characteristics.

Characteristics	Patients ($n = 169$)
Age, years (mean ± SD)	49.4 ± 14.5
Sex, *n* (%)	
female	112 (65.1)
male	60 (34.9)
Tumor type, *n* (%)	
sporadic	139 (82.2)
hereditary (MEN2A)	30 (17.8)
Extent of surgery, *n* (%)	
total thyroidectomy	167 (98.8)
subtotal/near total thyroidectomy	2 (1.2)
Initial CND, *n* (%)	
yes	162 (95.9)
no	7 (4.1)
Tumor size, cm, *n* (%)	
≤2.0	118 (69.8)
>2.0 and ≤4.0	38 (22.5)
>4.0	13 (7.7)
Extrathyroidal extension, *n* (%)	
none/micro	151 (89.3)
gross	18 (10.7)
Resection margin, *n* (%)	
negative	162 (95.9)
positive	7 (4.1)
Central LNM, *n* (%)	
no	98 (58.0)
yes	71 (42.0)
Lateral LNM, *n* (%)	
no	107 (63.3)
yes	62 (36.7)
Preoperative serum calcitonin (pg/mL), *n* (%)	
≤309	75 (44.4)
>309	94 (55.6)
Median follow-up, month (median, IQR)	84 (39.5-127.5)

SD, standard deviation; MEN2A, multiple endocrine neoplasia type 2A; CND, central lymph node dissection; LNM, lymph node metastasis; IQR, interquartile range.

Clinicopathological Characteristics According to Preoperative Serum Calcitonin Levels

Clinicopathological characteristics were evaluated according to preoperative serum calcitonin levels (**Table 2**). Among 169 patients, 75 (44.4%) had preoperative serum calcitonin levels of < 309 pg/mL; the remaining 94 patients (55.6%) had preoperative serum calcitonin levels of > 309 pg/mL. Preoperative serum calcitonin levels of > 309 pg/mL were significantly associated with male sex ($P = 0.045$), a larger primary tumor size ($P < 0.001$), gross extrathyroidal extension ($P = 0.012$), and central and lateral neck lymph node metastases ($P < 0.001$).

Preoperative Serum Calcitonin Cut-Off Value for Predicting Structural Recurrence

Maximally selected log-rank statistics were applied to establish a preoperative serum calcitonin cut-off value of prognostic significance. The highest log-rank statistic coincided with a preoperative serum calcitonin level of 309 pg/mL (**Figure 1**). Kaplan–Meier curves were constructed to examine disease-free survival according to the defined preoperative serum calcitonin level (cut-off value: 309 pg/mL; **Figure 2**). The overall disease-free survival rate was 75.7%. The 10-year disease-free survival rate of patients with preoperative serum calcitonin levels of > 309 pg/mL was significantly lower than that of patients with preoperative serum calcitonin levels of < 309 pg/mL (52.9% *vs.* 92.9%, respectively; log-rank test, $P < 0.001$) (**Table 3**). Cancer-specific survival was also examined according to the defined preoperative serum calcitonin level. It also showed that preoperative serum calcitonin levels of > 309 pg/mL were associated with significantly poorer outcomes (log-rank test, $P = 0.028$; **Additional File 1: Supplementary Figure 1**).

Factors Associated With Structural Recurrence

Clinical characteristics, including preoperative serum calcitonin level (≤ 309 or > 309 pg/mL), age at diagnosis, sex, extent of surgery, tumor type, primary tumor size, central and lateral neck lymph node metastasis, extrathyroidal extension, and resection margin, were analyzed as independent variables in multivariable Cox regression analysis (**Table 4**). Univariable analysis showed that preoperative serum calcitonin levels of > 309 pg/mL, a larger primary tumor size, the presence of regional lymph node metastases, gross extrathyroidal extension, and positive resection margins were associated with an increased risk of disease recurrence ($P < 0.001$). In multivariable analysis, preoperative serum calcitonin levels remained significantly associated with disease-free survival (hazard ratio: 5.33, 95% confidence interval: 1.67–16.96; $P = 0.005$). Lateral lymph node metastasis (hazard ratio: 3.70, 95% confidence interval: 1.61–8.51; $P = 0.002$) and positive resection margins (hazard ratio: 3.57, 95% confidence interval: 1.44–8.88; $P = 0.006$) were also independent predictors of disease-free survival.

TABLE 2 | Clinicopathological characteristics according to preoperative serum calcitonin levels.

Characteristics	Calcitonin level (pg/mL)		*P*-value
	≤ 309	> 309	
Age, years (mean ± SD)	50.2 (12.1)	48.7 (16.2)	0.528
Sex, *n* (%)			
female	55 (73.3)	55 (58.5)	0.045
male	20 (26.7)	39 (41.5)	
Tumor type, *n* (%)			
sporadic	65 (86.7)	74 (78.7)	0.179
hereditary (MEN2A)	10 (13.3)	20 (21.3)	
Extent of surgery, *n* (%)			
total thyroidectomy	73 (97.3)	94 (100.0)	0.195
	Subtotal/near total thyroidectomy	2 (2.7)	0 (0.0)
Initial CND, *n* (%)			
Yes	71 (94.7)	91 (96.8)	0.701
No	4 (5.3)	3 (3.2)	
Tumor size, cm, *n* (%)			
≤2.0	70 (93.3)	48 (51.1)	<0.001
>2.0 and ≤4.0	4 (5.3)	34 (36.2)	
>4	1 (1.3)	12 (12.8)	
Extrathyroidal extension, *n* (%)			
none/micro	72 (96.0)	79 (84.0)	0.012
gross	3 (4.0)	15 (16.0)	
Resection margin, *n* (%)			
negative	74 (98.7)	88 (93.6)	0.134
Positive	1 (1.3)	6 (6.4)	
Central LNM, *n* (%)			
no	61 (81.3)	37 (39.4)	<0.001
yes	14 (18.7)	57 (60.6)	
Lateral LNM, *n* (%)			
no	68 (90.7)	39 (41.5)	<0.001
yes	7 (9.3)	55 (58.5)	
Follow-up, months, median (IQR)	96 (58–123)	80 (30.5-150.5)	0.953

SD, standard deviation; MEN2A, multiple endocrine neoplasia type 2A; CND, central lymph node dissection; LNM, lymph node metastasis; IQR, interquartile range.

DISCUSSION

Herein, we examined the relationship between preoperative serum calcitonin levels and the prognosis of patients with medullary thyroid carcinoma. We defined a specific preoperative serum calcitonin cut-off value of 309 pg/mL for predicting structural recurrence. We also showed that preoperative serum calcitonin levels are an accurate predictor of clinical outcomes in patients with medullary thyroid carcinoma.

Cancer biomarkers have shown potential applications in cancer detection and management (4, 15, 16). As a biomarker for medullary thyroid carcinoma (17), the routine measurement of serum calcitonin levels for detecting medullary thyroid carcinoma in patients with thyroid nodules is controversial. However, the guidelines suggest that serum calcitonin levels should be measured whenever a preoperative diagnosis of medullary thyroid carcinoma is suspected (5, 18–20). The measurement of preoperative serum calcitonin levels is simple to perform. It has been widely used for predicting the extent of regional lymph node metastases, and helps to determine the initial extent of surgery (5, 6, 21, 22). Furthermore, previous reports (7, 8, 23–25) have shown that preoperative serum calcitonin levels are associated with primary tumor size, distant metastasis, and postoperative biochemical cure.

Predicting biochemical cure is important, because postoperative biochemical cure is associated with a favorable outcome (26). Some studies have reported that preoperative serum calcitonin levels are useful for predicting disease prognosis. Cohen et al. (23) suggested the preoperative serum calcitonin levels of < 50 pg/mL could predict postoperative calcitonin normalization. Machens et al. (7) suggested that preoperative serum calcitonin levels of > 500 pg/mL could best predict the failure to achieve biochemical cure. However, they only estimated preoperative serum calcitonin levels to predict biochemical cure. Surgical resection for disease recurrence is only considered when structural recurrence has been confirmed by imaging studies (5). Thus, the preoperative serum calcitonin cut-off value for predicting structural recurrence is more important for surgical decision-making than the cut-off value for predicting biochemical cure. Yen et al. (27) investigated the relationship between preoperative serum calcitonin levels and structural recurrence-free survival. However, only a relatively small number of patients with medullary thyroid carcinoma were enrolled, and recurrence-free survival was only evaluated according to the median preoperative serum calcitonin level of enrolled

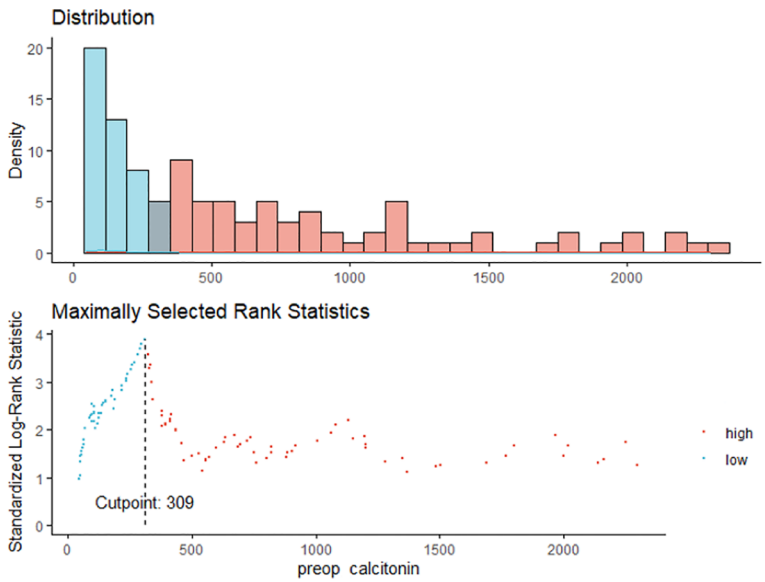

FIGURE 1 | The maximum of the standardized log-rank statistics for preoperative serum calcitonin cut-off value.

patients. In this study, we defined an optimal cut-off value for preoperative serum calcitonin levels and confirmed that it had prognostic value for structural recurrence.

Previous studies (26, 28–33) have shown that age, sex, the extent of the primary tumor, extrathyroidal extension, and postoperative gross residual disease are significantly associated with oncologic outcomes. Similar factors were identified in this study. In multivariable analysis, lateral lymph node metastasis and positive resection margins were significant prognostic factors. Preoperative serum calcitonin levels of > 309 pg/mL were a poor prognostic factor for disease-free survival, and this association was maintained after adjustment for conventional risk factors for the recurrence of medullary thyroid carcinoma.

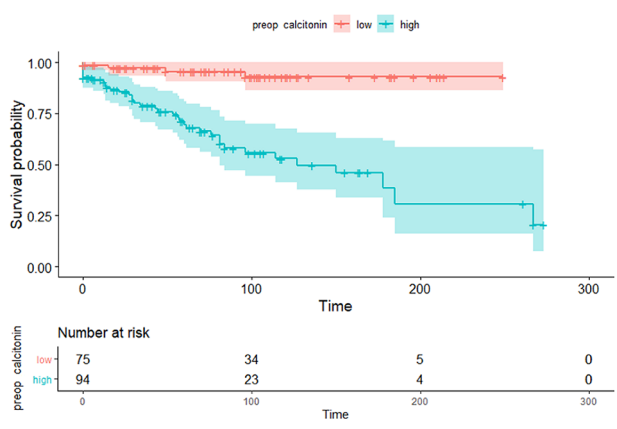

FIGURE 2 | Kaplan–Meier curves of disease-free survival according to the preoperative serum calcitonin cut-off value (309 pg/mL) ($P < 0.001$).

The hazard ratio for a cut-off value of 309 pg/mL as a predictor for disease recurrence was 5.33, which was higher than those for conventional risk factors. Therefore, preoperative serum calcitonin levels may be a strong predictor of disease recurrence.

We also postulated that preoperative serum calcitonin levels would play an important role in predicting cancer-specific survival. A preoperative serum calcitonin cut-off value of 309 pg/mL was closely correlated with cancer-specific survival. There were no cancer-specific deaths in patients with preoperative serum calcitonin levels of < 309 pg/mL. The 5-, 10-, and 20-year cancer-specific survival rates in patients with preoperative serum calcitonin levels of > 309 pg/mL were 95.6%, 90.2%, and 70.3%, respectively. However, further multivariable analysis to identify factors affecting cancer-specific survival was not performed because only a small number of patients died from medullary thyroid carcinoma.

Postoperative biochemical remission of serum calcitonin and post-operative calcitonin doubling time were known as an important factors for oncologic outcome (13, 34). Furthermore, dynamic risk stratification has been demonstrated to be clinically valuable also in MTC (35). However, when physicians only used postoperative serum calcitonin level as predictive factor, hindsight bias which is the common tendency for people to perceive past events as having been more predictable than they actually were would be occurred (36). On the other hand, measuring preoperative calcitonin level is a simple and easy way that can be used before everything happens. Since postoperative serum calcitonin has been widely used to predict clinical outcome in real-world practice, we calculate the sensitivity and specificity for structural recurrence of each of preoperative and postoperative serum calcitonin. The sensitivity for a preoperative calcitonin level of 309 pg/mL as a predictor for structural recurrence was 90.2%, and specificity was 55.5%.

TABLE 3 | Disease-free survival and cancer-specific survival according to the preoperative serum calcitonin cut-off value of 309 pg/mL.

Calcitonin	No of patients	No of recurrences (%)	Disease-free survival (%)			
			5-year	10-year	15-year	20-year
≤309 pg/mL	75	4 (5.3)	95.5	92.9	–	–
>309 pg/mL	94	37 (39.4)	69.7	52.9	38.3	30.7
all	169	41 (24.3)	81.5	71.2	62.3	57.1
Calcitonin	**No of patients**	**No of deaths (%)**	**Cancer-specific survival (%)***			
			5-year	10-year	15-year	20-year
≤309 pg/mL	72	0 (0.0)	–	–	–	–
>309 pg/mL	93	7 (7.5)	95.6	90.2	90.2	70.3
all	165	7 (4.2)	97.6	94.6	94.6	77.6

**Cancer-specific survival was calculated after the exclusion of four patients deaths from other causes.*

The sensitivity for a postoperative calcitonin level of 10 pg/mL (7) as a predictor for structural recurrence was 80.5%, and specificity was 84.3%. The sensitivity was higher in preoperative serum calcitonin level. Using the optimal cut-off value for preoperative serum calcitonin levels for preoperative risk stratification can guide the initial resection strategy, and helpful for the determining postoperative screening test frequency for local and distant metastases. Similarly, if a patient with differentiated thyroid carcinoma was classified into high risk group according to 2009 American Thyroid Association Initial Risk Stratification System, it would not be completely reassuring even if the patient was subsequently classified into excellent response when re-evaluated using dynamic risk stratification (37).

The strength of this study lies in its relatively large number of patients with medullary thyroid carcinoma who were recruited from a single tertiary hospital. The optimal cut-off value for preoperative serum calcitonin levels was determined using maximally selected log-rank statistics, which is an appropriate

TABLE 4 | Multivariable analysis of disease-free survival.

Characteristics	Unadjusted		Adjusted	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age, years	0.98 (0.96-1.00)	0.104		
Sex				
male	1 (reference)			
female	0.61 (0.33-1.13)	0.118		
Extent of surgery*				
Subtotal/near total thyroidectomy	1 (reference)			
total thyroidectomy	non-estimable	–		
Initial CND				
no	1 (reference)			
yes	3.61 (0.48-27.02)	0.211		
Tumor type				
sporadic	1 (reference)			
hereditary	0.55 (0.21-1.40)	0.207		
Primary tumor size, cm		(<0.001)		(0.022)
≤2.0	1 (reference)		1 (reference)	
>2.0 and ≤4.0	1.85 (0.90-3.82)	0.095	0.53 (0.23-1.18)	0.119
>4.0	5.90 (2.71-12.84)	<0.001	1.78 (0.78-4.09)	0.177
Central LN metastasis				
no	1 (reference)		1 (reference)	
yes	5.30 (2.54-11.03)	<0.001	1.42 (0.52-3.86)	0.497
Lateral LN metastasis				
no	1 (reference)		1 (reference)	
yes	7.35 (3.47-15.59)	<0.001	3.70 (1.61-8.51)	0.002
Extrathyroidal extension				
none/micro	1 (reference)		1 (reference)	
Gross	4.72 (2.33-9.55)	<0.001	1.50 (0.64-3.54)	0.353
Resection margin				
negative	1 (reference)		1 (reference)	
positive	8.78 (3.82-20.18)	<0.001	3.57 (1.44-8.88)	0.006
Calcitonin cut-off value, pg/mL				
≤309	1 (reference)		1 (reference)	
>309	9.53 (3.39-26.84)	<0.001	5.33 (1.67-16.96)	0.005

**Non-estimable because all recurred patients underwent total thyroidectomy. CND, central lymph node dissection; LN, lymph node; HR, hazard ratio; 95% CI, 95% confidential interval.*

statistical methodology for assessment of biomarker in survival endpoints (14, 16, 38). This study has several limitations. First is its retrospective design. Second, although this study included a relatively large number of patients with medullary thyroid carcinoma, patients were recruited from a single tertiary referral hospital. Thus, the study population may be prone to selection bias. Because of the rarity of medullary thyroid carcinoma, a multicenter prospective study is needed to validate to our findings. Third, we tried to evaluate the preoperative carcinoembryonic antigen (CEA) level concurrently, however we could not get enough information about preoperative CEA level in this cohort.

In conclusion, this study defined a preoperative serum calcitonin level of 309 pg/mL as a useful threshold for predicting disease recurrence. It also has clinical implications for long-term cancer-specific survival.

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 study design was approved by the Institutional Review Board of Samsung Medical Center (approval number: 2020-07-007). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HP, SYP, and THK conceptualized and designed the study. HP, SYP, and S-YW analyzed the data and made the figures. HP drafted the manuscript. JP, JHCho, and MKC, data curation. SWK, JHChu, and JYC acquired and interpreted the data, and revised the manuscript. THK coordinated, and critically reviewed the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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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.2021.749973/full#supplementary-material

REFERENCES

1. Utiger RD. Medullary Thyroid Carcinoma, Genes, and the Prevention of Cancer. *N Engl J Med* (1994) 331:870–1. doi: 10.1056/nejm199409293311309
2. Fagin JA, Wells SAJr. Biologic and Clinical Perspectives on Thyroid Cancer. *N Engl J Med* (2016) 375:2307. doi: 10.1056/NEJMc1613118
3. Mathiesen JS, Kroustrup JP, Vestergaard P, Stochholm K, Poulsen PL, Rasmussen AK, et al. Incidence and Prevalence of Sporadic and Hereditary MTC in Denmark 1960-2014: A Nationwide Study. *Endocr Connect* (2018) 7:829–39. doi: 10.1530/EC-18-0157
4. Duffy MJ. Tumor Markers in Clinical Practice: A Review Focusing on Common Solid Cancers. *Med Princ Pract* (2013) 22:4–11. doi: 10.1159/000338393
5. Wells SAJr., Asa SL, Dralle H, Elisei R, Evans DB, Gagel RF, et al. Revised American Thyroid Association Guidelines for the Management of Medullary Thyroid Carcinoma. *Thyroid* (2015) 25:567–610. doi: 10.1089/thy.2014.0335
6. Machens A, Dralle H. Biomarker-Based Risk Stratification for Previously Untreated Medullary Thyroid Cancer. *J Clin Endocrinol Metab* (2010) 95:2655–63. doi: 10.1210/jc.2009-2368
7. Machens A, Schneyer U, Holzhausen HJ, Dralle H. Prospects of Remission in Medullary Thyroid Carcinoma According to Basal Calcitonin Level. *J Clin Endocrinol Metab* (2005) 90:2029–34. doi: 10.1210/jc.2004-1836
8. Yip DT, Hassan M, Pazaitou-Panayiotou K, Ruan DT, Gawande AA, Gaz RD, et al. Preoperative Basal Calcitonin and Tumor Stage Correlate With Postoperative Calcitonin Normalization in Patients Undergoing Initial Surgical Management of Medullary Thyroid Carcinoma. *Surgery* (2011) 150:1168–77. doi: 10.1016/j.surg.2011.09.043
9. Laure Giraudet A, Al Ghulzan A, Auperin A, Leboulleux S, Chehboun A, Troalen F, et al. Progression of Medullary Thyroid Carcinoma: Assessment With Calcitonin and Carcinoembryonic Antigen Doubling Times. *Eur J Endocrinol* (2008) 158:239–46. doi: 10.1530/EJE-07-0667
10. American Thyroid Association Guidelines Task Force, Kloos RT, Eng C, Evans DB, Francis GL, Gagel RF, et al. Medullary Thyroid Cancer: Management Guidelines of the American Thyroid Association. *Thyroid* (2009) 19:565–612. doi: 10.1089/thy.2008.0403
11. Meijer JA, le Cessie S, van den Hout WB, Kievit J, Schoones JW, Romijn JA, et al. Calcitonin and Carcinoembryonic Antigen Doubling Times as Prognostic Factors in Medullary Thyroid Carcinoma: A Structured Meta-Analysis. *Clin Endocrinol (Oxf)* (2010) 72:534–42. doi: 10.1111/j.1365-2265.2009.03666.x
12. Cho YY, Jang HW, Jang JY, Kim TH, Choe JH, Kim JH, et al. Clinical Outcomes of Patients With Hypercalcitoninemia After Initial Treatment for Medullary Thyroid Cancer and Postoperative Serum Calcitonin Cutoffs for Predicting Structural Recurrence. *Head Neck* (2016) 38:1501–8. doi: 10.1002/hed.24469
13. Jung KY, Kim SM, Yoo WS, Kim BW, Lee YS, Kim KW, et al. Postoperative Biochemical Remission of Serum Calcitonin is the Best Predictive Factor for Recurrence-Free Survival of Medullary Thyroid Cancer: A Large-Scale Retrospective Analysis Over 30 Years. *Clin Endocrinol (Oxf)* (2016) 84:587–97. doi: 10.1111/cen.12852
14. Lausen B, Lerche R, Schumacher M. Maximally Selected Rank Statistics for Dose-Response Problems. *Biom J* (2002) 44:131–47. doi: 10.1002/1521-4036(200203)44:2<131::AID-BIMJ131>3.0.CO;2-Z
15. Henry NL, Hayes DF. Cancer Biomarkers. *Mol Oncol* (2012) 6:140–6. doi: 10.1016/j.molonc.2012.01.010
16. Yotsukura S, Mamitsuka H. Evaluation of Serum-Based Cancer Biomarkers: A Brief Review From a Clinical and Computational Viewpoint. *Crit Rev Oncol Hematol* (2015) 93:103–15. doi: 10.1016/j.critrevonc.2014.10.002
17. Bae YJ, Schaab M, Kratzsch J. Calcitonin as Biomarker for the Medullary Thyroid Carcinoma. *Recent Results Cancer Res* (2015) 204:117–37. doi: 10.1007/978-3-319-22542-5_5
18. Perros P, Boelaert K, Colley S, Evans C, Evans RM, Gerrard Ba G, et al. Guidelines for the Management of Thyroid Cancer. *Clin Endocrinol (Oxf)* (2014) 81(Suppl 1):1–122. doi: 10.1111/cen.12515

19. Haddad RI, Nasr C, Bischoff L, Busaidy NL, Byrd D, Callender G, et al. NCCN Guidelines Insights: Thyroid Carcinoma, Version 2.2018. *J Natl Compr Canc Netw* (2018) 16:1429–40. doi: 10.6004/jnccn.2018.0089
20. Pacini F, Schlumberger M, Dralle H, Elisei R, Smit JW, Wiersinga W, et al. European Consensus for the Management of Patients With Differentiated Thyroid Carcinoma of the Follicular Epithelium. *Eur J Endocrinol* (2006) 154:787–803. doi: 10.1530/eje.1.02158
21. Park H, Park J, Choi MS, Kim J, Kim H, Shin JH, et al. Preoperative Serum Calcitonin and Its Correlation With Extent of Lymph Node Metastasis in Medullary Thyroid Carcinoma. *Cancers (Basel)* (2020) 12(10):2894. doi: 10.3390/cancers12102894
22. Gimm O. Extent of Surgery in Clinically Evident But Operable MTC - When is Central and/or Lateral Lympadenectomy Indicated? *Thyroid Res* (2013) 6 Suppl 1:S3. doi: 10.1186/1756-6614-6-S1-S3
23. Cohen R, Campos JM, Salaun C, Heshmati HM, Kraimps JL, Proye C, et al. Preoperative Calcitonin Levels are Predictive of Tumor Size and Postoperative Calcitonin Normalization in Medullary Thyroid Carcinoma. Groupe D'Etudes Des Tumeurs a Calcitonine (GETC). *J Clin Endocrinol Metab* (2000) 85:919–22. doi: 10.1210/jcem.85.2.6556
24. Machens A, Lorenz K, Dralle H. Prediction of Biochemical Cure in Patients With Medullary Thyroid Cancer. *Br J Surg* (2020) 107:695–704. doi: 10.1002/bjs.11444
25. Miyauchi A, Matsuzuka F, Kuma K, Takai S, Nakamoto K, Nakamura K, et al. Evaluation of Surgical Results and Prediction of Prognosis in Patients With Medullary Thyroid Carcinoma by Analysis of Serum Calcitonin Levels. *World J Surg* (1988) 12:610–5. doi: 10.1007/BF01655862
26. Pellegriti G, Leboulleux S, Baudin E, Bellon N, Scollo C, Travagli JP, et al. Long-Term Outcome of Medullary Thyroid Carcinoma in Patients With Normal Postoperative Medical Imaging. *Br J Cancer* (2003) 88:1537–42. doi: 10.1038/sj.bjc.6600930
27. Yen TW, Shapiro SE, Gagel RF, Sherman SI, Lee JE, Evans DB. Medullary Thyroid Carcinoma: Results of a Standardized Surgical Approach in a Contemporary Series of 80 Consecutive Patients. *Surgery* (2003) 134:890–9. doi: 10.1016/s0039-6060(03)00408-2
28. Kuo EJ, Sho S, Li N, Zanocco KA, Yeh MW, Livhits MJ. Risk Factors Associated With Reoperation and Disease-Specific Mortality in Patients With Medullary Thyroid Carcinoma. *JAMA Surg* (2018) 153:52–9. doi: 10.1001/jamasurg.2017.3555
29. Brierley J, Tsang R, Simpson WJ, Gospodarowicz M, Sutcliffe S, Panzarella T. Medullary Thyroid Cancer: Analyses of Survival and Prognostic Factors and the Role of Radiation Therapy in Local Control. *Thyroid* (1996) 6:305–10. doi: 10.1089/thy.1996.6.305
30. Dottorini ME, Assi A, Sironi M, Sangalli G, Spreafico G, Colombo L. Multivariate Analysis of Patients With Medullary Thyroid Carcinoma. Prognostic Significance and Impact on Treatment of Clinical and Pathologic Variables. *Cancer* (1996) 77:1556–65. doi: 10.1002/(sici)1097-0142(19960415)77:8<1556::Aid-cncr20>3.0.Co;2-y
31. Modigliani E, Cohen R, Campos JM, Conte-Devolx B, Maes B, Boneu A, et al. Prognostic Factors for Survival and for Biochemical Cure in Medullary Thyroid Carcinoma: Results in 899 Patients. The GETC Study Group. Groupe D'etude Des Tumeurs a Calcitonine. *Clin Endocrinol (Oxf)* (1998) 48:265–73. doi: 10.1046/j.1365-2265.1998.00392.x
32. Pelizzo MR, Boschin IM, Bernante P, Toniato A, Piotto A, Pagetta C, et al. Natural History, Diagnosis, Treatment and Outcome of Medullary Thyroid Cancer: 37 Years Experience on 157 Patients. *Eur J Surg Oncol* (2007) 33:493–7. doi: 10.1016/j.ejso.2006.10.021
33. Raue F, Kotzerke J, Reinwein D, Schroder S, Roher HD, Deckart H, et al. Prognostic Factors in Medullary Thyroid Carcinoma: Evaluation of 741 Patients From the German Medullary Thyroid Carcinoma Register. *Clin Investig* (1993) 71:7–12. doi: 10.1007/BF00210956
34. Miyauchi A, Onishi T, Morimoto S, Takai S, Matsuzuka F, Kuma K, et al. Relation of Doubling Time of Plasma Calcitonin Levels to Prognosis and Recurrence of Medullary Thyroid Carcinoma. *Ann Surg* (1984) 199:461–6. doi: 10.1097/00000658-198404000-00014
35. Lindsey SC, Ganly I, Palmer F, Tuttle RM. Response to Initial Therapy Predicts Clinical Outcomes in Medullary Thyroid Cancer. *Thyroid* (2015) 25:242–9. doi: 10.1089/thy.2014.0277
36. Kahneman D. Thinking, Fast and Slow: Macmillan. New York: Farrar, Straus and Giroux (2011) p. 199–208.
37. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, et al. 2015 American Thyroid Association Management Guidelines for Adult Patients With Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* (2016) 26:1–133. doi: 10.1089/thy.2015.0020
38. Ray P, Le Manach Y, Riou B, Houle TT. Statistical Evaluation of a Biomarker. *Anesthesiology* (2010) 112:1023–40. doi: 10.1097/ALN.0b013e3181d47604

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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Non-Conventional Clinical Uses of TSH Receptor Antibodies: The Case of Chronic Autoimmune Thyroiditis

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Anti TSH receptor antibodies (TSHrAb) are a family of antibodies with different activity, some of them stimulating thyroid function (TSAb), others with blocking properties (TBAb), it is a common finding that antibodies with different function might coexist in the same patient and can modulate the function of the thyroid. However, most of the labs routinely detect all antibodies binding to the TSH receptor (TRAb, i.e. TSH-receptor antibodies detected by binding assay without definition of functional property). Classical use of TSHr-Ab assay is in Graves' disease where they are tested for diagnostic and prognostic issues; however, they can be used in specific settings of chronic autoimmune thyroiditis (CAT) as well. Aim of the present paper is to highlight these conditions where detection of TSHr-Ab can be of clinical relevance. Prevalence of TSHrAb is different in in the 2 main form of CAT, i.e. classical Hashimoto's thyroiditis and in atrophic thyroiditis, where TBAb play a major role. Simultaneous presence of both TSAb and TBAb in the serum of the same patient might have clinical implication and cause the shift from hyperthyroidism to hypothyroidism and vice versa. Evaluation of TRAb is recommended in case of patients with Thyroid Associated Orbitopathy not associated with hyperthyroidism. At present, however, the most relevant recommendation for the use of TRAb assay is in patients with CAT secondary to a known agent; in particular, after treatment with alemtuzumab for multiple sclerosis. In conclusion, the routine use of anti-TSH receptor antibodies (either TRAb or TSAb/TBAb) assay cannot be suggested at the present for diagnosis/follow up of patients affected by CAT; there are, however, several conditions where their detection can be clinically relevant.

Keywords: chronic autoimmune thyroiditis, TSH-receptor blocking antibodies, TSH-receptor stimulating antibodies, Hashimoto's thyroiditis (HT), atrophic thyroiditis

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INTRODUCTION

Hashimoto's thyroiditis is a chronic autoimmune disorder that has been described for the first time by Hakaru Hashimoto in 1912 (1). His description: "*a massive growth of lymphatic elements, primarily lymphoid follicles*" …. "*this condition was a 'destructive affectation of the thyroid … here and there infiltrated with clumps of cells … which are found to be composed of leucocytes*" depicts the spectrum of the chronic lymphocytic thyroiditis which is the most common type of chronic

autoimmune thyroiditis (CAT). Nowadays the term Hashimoto's thyroiditis (HT) is commonly and erroneously used to identify all forms of CAT and is considered the most common cause of hypothyroidism as well as the most common autoimmune and endocrine disease (2). However at least one different form of disease is classified within the CAT, i.e. the atrophic thyroiditis (AT). In 1873 Sir William Withney Gull in his seminal study "On a cretinoid state supervening in adult life in women" illustrated the following picture: "*Her face altering from oval to round … the tongue broad and thick, voice guttural, and the pronunciation as if the tongue were too large for the mouth (cretinoid)… In the cretinoid condition in adults which I have seen, the thyroid was not enlarged … at a first hasty glance there might be supposed to be a general slight oedema of it.*" (3) which is considered the first description of the atrophic form of CAT.

Distinction of the two main forms of CAT has not relevance only for a proper categorization of the disease, but also because the role of the anti TSH receptor antibodies (TSHrAb) is various and has a different weight in the two forms. Recognizing the 2 forms might be difficult, the most distinctive clinical feature being thyroid atrophy which appears at the clinical onset in AT while it appears after a long-standing disease in HT. Of course, it might be difficult to notice this temporal distinction in clinical practice.

The first evidence for a serum thyroid-stimulating factor has been described in 1956 by Adams and Purves (4) and in 1958 by Mc Kenzie (5). Much work has been done from then, particularly in the last 2-3 decades; it is a major improvement that measurement of TSHrAb is suggested in the management of Graves' disease according to the 2016 American Thyroid Association (6) while only in the 2011 version it was recommended TSHrAb measurement only as "an alternative means to diagnose GD", to be used when a thyroid scan and uptake is unavailable or contraindicated (7). Moreover, in the "2019 European Thyroid Association guidelines on the Management of Thyroid Dysfunction following Immune Reconstitution Therapy" (8) TSHrAb assay is considered a cornerstone both of diagnosis and follow up. After the cloning and sequencing of the TSH receptor (TSHr) (9–11) we now know that TSHrAb are a family of antibodies with different activity, some of them stimulating thyroid function (TSAb), others with blocking properties (TBAb) and finally some neutral, i.e. with no effect on TSH binding and no effect on cAMP levels [TBII (12–14)], which are also called "cleavage" Abs because they recognize linear epitopes within the hinge region of the TSH receptor and might be involved in several signalling processes also including thyroid cell apoptosis (15, 16). For the purposes of this review, this last family of Abs have a minimal role. It is also of little clinical interest to know if a proportion of TBAb are indeed "weak" TSAb and their blocking activity is only due to preventing the binding of more potent TSH receptor agonist, like TSH or "potent" TSAb, as suggested by some Authors (17); from a clinical point of view they exert a blocking effect.

However, a major problem in interpretation of the role of TSHrAb arises from the observation that most of the labs routinely detect all antibodies binding to the TSH receptor without distinction of the functional properties.

Indeed, two types of assays have been historically used in the clinical setting: assays only measuring TSHrAb binding to the TSHr without functional discrimination (usually termed TRAb) and bioassays measuring functional activity of TSHrAb (12–14) (**Figure 1**). While the former, during decades, have been based on the inhibition by TSHR antibodies of radiolabeled/fluorescent TSH binding to thyroid membranes, human TSHr or known monoclonal human TSHR autoantibody, the latter measures the activation of transduction pathways after the binding of a ligand to the TSHr (17, 18). A further clarification is needed for the

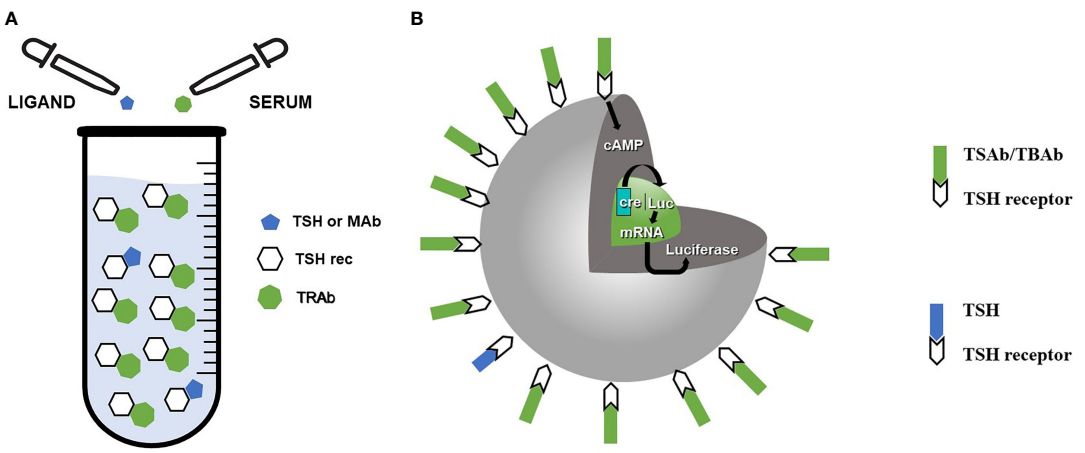

FIGURE 1 | Different methods of TSHrAb assay. **(A)** Assays employing competition by TRAb for binding of a ligand (TSH or monoclonal antibody) to the TSHR. **(B)** Bioassays involving cultured thyroid cells or nonthyroidal cells expressing the TSH receptor. Binding of TSAb or TBAb modulate cAMP production. In the depicted assay cell lines lacking TSHR are double transfected with a luciferase reporter gene under the transcriptional control of cAMP-responsive elements (CRE-Luc) and the TSH receptor. TSAb/TBAb levels in patient sera are determined by measuring the production of luciferase.

functional assay: it can reflect a net sum of TSAb and TBAb activities (and therefore give an estimate of the stimulus present on thyroid cell at the time of the blood test) or differentiate (by using different cells or protocols) between TSAb or TBAb activities (12, 19). Differently from Thyroglobulin antibodies (TgAb) and Thryroid peroxidase antibodies (TPOAb), which have no major role in the pathogenesis of autoimmune thyroid diseases, TSHrAbs are directly causative of Graves' disease and are also involved in the pathogenesis of CAT. It is therefore obvious that an assay which gives a quantitative measure of the stimulus/inhibition performed on thyroid cells has more clinical advantages than an assay just measuring the Abs binding to the TSH receptor. Indeed, bioassay have clinical advantages in Graves' disease because they have prognostic value (20) and are helpful and predictive in Graves' patients during pregnancy/postpartum, as well as for extrathyroidal manifestations (21). It is beyond the aims of the present review to discuss advantages/disadvantages of the two different types of assays; a recent review (18) is exhaustively summarizing this issue. It is fair to admit, however, that binding assays are easier to handle also because commercially automated assays are available, and this is probably the main reason for their predominance (12–14, 18).

While the presence of TSAb in Graves patient is clearly causative and the amount of TSAb is correlated to the clinical entity of the disease, the role of TSHrAb (both TSAb and TBAB) in CAT is less defined. Marked lymphocytic infiltration (mainly T lymphocytes) of the thyroid is the hallmark of Hashimoto's thyroiditis and apoptotic destruction and cell death of thyroid cells are probably the main mechanisms of damage leading to hypothyroidism. A minor contribution can also be attributed to TPOAb and TgAb which can be able to fix the complement and induce antibody-mediated complement-dependent cytotoxicity (ACDC) [revised in (18)]. A role for TSHrAb, and especially for TBAb which can block the TSH receptor and therefore inhibit both thyroid hormone synthesis and production and cell growth has been proposed especially for the atrophic form of thyroiditis. More speculative, as discussed later, is the role of TSAb.

CLINICAL USES OF TSHr Ab IN CAT

Aiming to better understand the role of TSHr Ab in chronic autoimmune thyroiditis, however, the impossibility to know in many studies whether TSAb or TBAb are involved, because binding assays have been used, it's a major limit. It is a common finding that antibodies with different function might coexist in the same patient and can modulate the function of the thyroid (15–18, 22, 23). The final evidence for the contemporaneous presence of both kind of Abs has been obtained by the cloning of a stimulating (K1-18) and a blocking (K1-70) monoclonal Ab in the serum of the same patient (24).

According to an extensive review about the role of TSH receptor in clinical settings published in the year 2000 (14), mean prevalence of TRAb varies was 9% in HT and 12% in AT. However, when TBAb are separately evaluated mean prevalence was 12% and 33% in HT and AT respectively (**Table 1**). In a study by Cho et al. (25) TRAb were detected in 6.3% and in 48% and TBAb in 10.5% and 59% in goitrous and atrophic form respectively. More recent data have shown 9% and 25% TBAb positivity in adults patients with HT and AT, respectively (26), and a positive rate for TBAb of 38% in children with AT (27). Finally, a study from Kahaly (28), evaluating patients with autoimmune thyroiditis without distinguishing between the 2 two main subgroups, showed TRAb positivity in 9.4% of sera and TBAb positivity in 9.3%; interestingly TSAb and TBAb positivity has been registered in 6/67 sera, thus meaning that contemporaneous presence of both TSAb and TBAb can be found not only in Graves' disease but also in CAT.

Hashitoxicosis is usually referred to a transient phase of hyperthyroidism in patients with chronic autoimmune thyroiditis; it is usually attributed to destruction of thyroid cells and the release of stored hormones within the bloodstream. However, in 1971 Fatourechi et al. (29) described patients with hyperthyroidism indistinguishable from Graves' disease (including elevated radioiodine uptake and TRAb positivity) but with histological features of Hashimoto's thyroiditis; the hyperthyroidism was lasting few months and the patients later became hypothyroid. It has been therefore supposed that hyperthyroidism was attributable to the presence of TSAb. From then it has been generally accepted that a small percentage of hyperthyroid presentation of chronic autoimmune thyroiditis is due to the presence of TSAb. Contemporaneous presence of both TSAb and TBAb has been advocated as a mechanism by which euthyroidism can be maintained in a small subset of patients (28). Presence of TSAb during Hashimoto's thyroiditis has also relevance in newborns and in pediatric age. Indeed, thyrotoxicosis in a newborn from a mother with chronic autoimmune thyroiditis treated with L-T4 has been described

TABLE 1 | Nomenclature of TSH-receptor antibodies and Prevalence of TRAb and TBAb in Hashimoto's thyroiditis and atrophic thyroiditis according to previous studies.

Nomenclature			Definition			
TSHr-Ab			All TSH receptor antibodies without definition of assay method or functional properties			
TRAb			TSH-receptor antibodies detected by binding assay without definition of functional property			
TSAb			TSH-receptor stimulating antibodies which act as agonists by stimulating thyroid growth and thyroid hormone synthesis			
TBAb			TSH receptor blocking antibodies which act as antagonists, by blocking the action of the TSH			
	Orgiazzi J et al. (14)		**Cho BJ et al.** (25)		**Takasu N et al.** (26)	
	HT	AT	HT	AT	HT	AT
TRAb % (range)	9 (0-44)	12 (0-54)	6.3	48	–	–
TBAb % (range)	12 (0-44)	33 (0-62)	10.5	59	9	25

HT, Hashimoto's thyroiditis; AT, atrophic thyroiditis.

(30); the presence of TSAb has been documented and it was the cause of hyperthyroidism lasting only until TSAb normalized. Transient central hypothyroidism, possibly due to TSH suppression, then developed and the newborn required L-T4 treatment.

TBAb have a clearer role in CAT; as previously briefly disclosed, TBAb are supposed to have a central role in the etiology of the atrophic form of chronic autoimmune thyroiditis. They inhibit the metabolic pathways activated by the interaction of TSH with its receptor and then prevent both growth and hormone synthesis. It is therefore easily comprehensible that their presence is associated to a more severe form of hypothyroidism, especially in children (28, 31). In a population of patients with subclinical hypothyroidism, overt hypothyroidism and atrophic thyroiditis TBAb positivity has been detected in 12.5, 23.3 and 34% of sera, respectively (32). The same group of Authors, by using a different assay, also reported TBAb positivity in 46, 3, 9.4 and 36% of sera from patients with atrophic thyroiditis, euthyroid, subclinical or overt hypothyroid Hashimoto's thyroiditis, respectively (33). In pediatric population presence of TBAb may be even more relevant, if 9% of children with CAT had TBAb posititvity which was corelated to higher values of TSH (31, 34). It is surprisingly interesting, however, to note that TBAb are also linked to a transient form of CAT, with spontaneous remission of hypothyroidism. Indeed, in a 11-years follow up in sera from Hashimoto's thyroiditis the disappearance (35) of TBAb was linked to recovery of euthyroidism in about 40% of patients; it was only detected in patients with the goitrous form of CAT, while all the patients showing features of the atrophic form of thyroiditis remain hypothyroid. It is therefore accepted that in a small amount of patients with CAT and positivity for TRAb a spontaneous recovery from hypothyroidism can occur (36) and that TBAb in this small subset of patients are causative of the transient hypothyroidism (37). Of course, the small number of patients occurring this eventuality does not grant systematic assay of TBAb in all patients with Hashimoto's thyroiditis. Transient positivity of TBAb has also relevance in congenital hypothyroidism. In 1980 Matsuura et al. described the first report of neonatal transient hypothyroidism correlated to the presence of maternal TBAb (38). Prevalence of congenital hypothyroidism due to the presence of TBAb is supposed to be approximately 1:180000 newborns (36) and reviewed in (39).

An intriguing, although infrequent phenomenon is the shift from hypothyroidism to hyperthyroidism and vice versa. It is supposed to be, at least partially, correlated to a shift of TBAb to TSAb or vice versa and has been matter of an extensive review (17). The evolution from hyperthyroidism to hypothyroidism is somehow a more frequent phenomenon which can be attributed to different causes; contemporaneous presence of Graves' disease and Hashimoto's thyroiditis is not difficult to understand, whether we keep in mind that these are the two far ends of the same disease called thyroid autoimmunity. It is therefore conceivable to imagine that, while TSAb stimulate thyrocytes to overwork, slower mechanisms of thyroid destruction can take place and with time prevail, especially when amount of TSAb decreases either spontaneously or due to anti-thyroid drugs (ATD). Shift from TSAb to TBAb can therefore be only a minority phenomenon in the evolution from hyperthyroidism to hypothyroidism. The opposite (i.e. the evolution from hypothyroidism to hyperthyroidism) is a very sporadic phenomenon which requires the development of TSAb acting on an already damaged thyroid or the shift from TBAb to TSAb. Both mechanisms have been described; in 2012 (26) Takasu and Matsushita reported the development of TSAb positivity in 2/34 (5.9%) of patients with TBAb positivity over a 10 years follow up. On the opposite few patients developed hyperthyroidism following the appearance of TSAb (40, 41) [and reviewed in (17)] without a previous TBAb presence; this phenomenon was accompanied by an increase of TgAb and TPOAb titer. There is no difference in age or sex ratio, most of the patients have been described in Japan, possibly due to the more common use of TRAb assay. The common feature of all patients developing TSAb is the long time, up to 20 years (41), treatment with L-T4. A possible link to TSAb development is difficult to imagine; a decrease of TBAb amount similarly to what happen for TgAb and TPOAb in long-time L-T4 treated patients (42), can be supposed but it remains difficult to understand why TSAb should behave oppositely to TBAb. Easier to understand is the phenomenon by which a newborn from a mother with chronic autoimmune thyroiditis should initially be hypothyroid and then develop hyperthyroidism. In 1983 Zakarjia et al. (43) described delayed onset of hyperthyroidism in a newborn from a mother with both TBAb and TSAb. The phenomenon has been attributed to the earlier disappearance of TBAb from the serum with the longer lasting presence of TSAb causing hyperthyroidism.

Thyroid Associated Orbitopathy (TAO) generally occurs in patients with hyperthyroidism due to Graves' disease, and it sometimes occurs in euthyroid and hypothyroid patients. Mild orbitopathy, mainly consisting in mild upper eyelid retraction, has been reported to be common in chronic autoimmune thyroiditis (44), while severe ophthalmopathy was considered very infrequent. However, a recent study (45) evaluating 700 patients with CAT revealed that 44 (6%) of them had TAO, and 15/44 (34% of this subset and 2.1% of the whole CAT population) had an active/severe disease. Interestingly 30/44 (68.2%) of patients with CAT and TAO had TSAb positive values, while only 36/656 (5,5%) of patients with CAT and no TAO were TSAb positive; even more significant, all the 15 patients with active/severe disease had positive TSAb values. Although cases of severe thyroid-associated orbitopathy have been documented in Hashimoto's thyroiditis (46), the recently published Guidelines for the medical management of TAO (47) suggest the opportunity to assay TRAb in all patients with TAO and either Graves' disease or Hashimoto's thyroiditis for diagnostic and prognostic purposes.

In a very unusual scenario CAT is associated with encephalopathy and this association, later defined "steroid-responsive encephalopathy associated with autoimmune thyroiditis (SREAT)" (48) has been described for the first time in 1966 (49). A serological distinctive feature of the disease is the contemporary presence of one or more thyroid antibodies

(TgAb, TPOAb and TRAb) and antibodies against brain-expressed autoantigens; amongst the latter anti-alpha-enolase antibodies are the most frequent (50). Interestingly a recent study (50) has shown homologies between TSH-R and alpha-enolase; keeping in mind that main thyroid antigens (Tg, TPO and TSHr) are also expressed in the central nervous system and that alpha-enolase is expressed also in the thyroid, a pathogenic contribution of TRAb might be speculated.

Secondary CAT is a quite new clinical entity where onset of CAT is secondary to a known agent; interferon alpha or beta, interleukin-2, thalidomide, amiodarone, radiation therapy, lithium, immune checkpoint inhibitors, and thyrosine kinases inhibitors are some examples (2, 8). Central to the topic of this review is the onset of thyroid autoimmunity caused by immune reconstitution therapy, i.e., the restoration of immune cells after a depletion phase. This is particularly the case for three different therapies, i.e. following alemtuzumab, after highly active antiretroviral therapy (HAART) and finally after bone marrow transplantation (BMT) or hematopoietic stem cell transplantation (HSCT) (8). Of particular interest is the case of alemtuzumab treatment in patients with multiple sclerosis (MS); indeed, the first peculiarity to be noted is that high prevalence of thyroid dysfunction has only been observed in patients affected by MS (51, 52) but not when alemtuzumab has been used for rheumatoid arthritis, B-cell chronic lymphocytic leukaemia or transplantation (53–55). Therefore, a common genetic background can be postulated. Mechanism of action of alemtuzumab is based on its binding to CD52 antigen which is expressed on almost all T and B lymphocytes; after binding a rapid cell lysis is induced and lymphocytes almost disappear from the bloodstream. Then a quicker recovery of B cells (3-12 months) is observed while CD8+ and CD4+ T lymphocytes recover later (within 20 and 36 months respectively) [reviewed in (56)]; this gap might explain the onset of B-cell related thyroid autoimmunity. Interestingly, no onset of autoimmune disorders has been observed after B-cell only depleting immunomodulatory agent, such as rituximab (57). Aside from mechanistic explanation, a huge number (34 to 41%) of patients treated with alemtuzumab for MS (8, 56, 58) develop thyroid autoimmunity, mainly hyperthyroidism (from 4,7 to 33%), but also hypothyroidism (5 to 13,8%) and destructive thyroiditis (4%) as well. These data are even more impressive if compared with those observed in MS patients treated with interferon-beta (6.5%). Two sets of data have particular relevance to the topic of these review: 1) 70% of patients developing thyroid dysfunction had positive TRAb values and this value rose up to 76.7% in patients with hypothyroidism, thus suggesting the presence of TBAb, which has been confirmed by following studies showing TBAb as the cause of hypothyroidism in 50% of MS patients (59). 2) there is a high possibility to observe a shift from hyperthyroidism to hypothyroidism and vice versa (19 to 52%) and this change very often (up to 70%) occurs when TRAb are present. For these reasons the European Thyroid Association in its guidelines (8) does not recommend TRAb assay before alemtuzumab treatment or during routine follow up, but strongly recommend its use when thyroid dysfunction is disclosed (recommendations 11 and 14). It is also suggested that TRAb assay might be performed in hypothyroid patients with a previous detection of Abs-positivity, because of the possible transitory presence of TBAb thus resulting in recovery from hypothyroidism.

CONCLUDING REMARKS

The routine use of anti-TSH receptor antibodies (either TRAb or TSAb/TBAb) assay cannot be suggested at the present for diagnosis/follow up of patients affected by CAT. Indeed, prevalence of TRAb is too low in CAT to be a useful tool for diagnosis; moreover, even when detected they do not modify the strategy of treatment. There are, however, several conditions where their detection can be relevant: 1) due to the possibility of disappearance of TBAb which can be causative of transient hypothyroidism in goitrous form of CAT (35), HT patients with long standing stability of low dose L-T4 treatment might deserve evaluation of TBAb because of the high rate of recovery (40%) in patients whose hypothyroidism was originally due to the presence of TBAb. It has to be noted, however, that commercial test of TBAb is hard to find [revised in (18)]; 2) when a shift from hypothyroidism to hyperthyroidism is observed in a patient with long time treatment with L-T4; 3) in patients with alternating hyper/hypothyroidism phases; 4) in a newborn from a mother with CAT and a contradictory clinical setting; 5) in patients with CAT and suspected TAO. Assay of TSHrAB (either TRAb or, better, TSAb/TBAb) is, on the contrary, mandatory when treatment with immune reconstitution therapy (especially in MS patients) is performed and thyroid dysfunction is detected.

Finally, it must be noted that studies on the clinical role of TSHrAb in CAT are scarce and available data are very rarely based on studies on numerous population; therefore, assay of TSAb and particularly of TBAB should deserve more clinical studies to better understand their role.

AUTHORS CONTRIBUTIONS

GN: substantial contributions to the conception and design of the work; reviewing the literature; drafting the work. IB: substantial contributions to the conception of the work; revising the work critically for important intellectual content. GD; substantial contributions to the design of the work; revising the work critically for important intellectual content. CG: substantial contributions to the design of the work; revising the work critically for important intellectual content. All authors contributed substantially to the article and approved the submitted version.

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REFERENCES

1. Hashimoto H. Zur Kenntniss Der Lymphomatosen Veranderung Der Schilddruse (Struma Lymphomatosa). *Arch Klin Chir* (1912) 97:219–48.
2. Caturegli P, De Remigis A, Rose NR. Hashimoto Thyroiditis: Clinical and Diagnostic Criteria. *Autoimmun Rev* (2014) 13(4-5):391–7. doi: 10.1016/j.autrev.2014.01.007
3. Gull WW. On a Cretinoid State Supervening in Adult Life in Women. *Trans Clin Soc London* (1874) 7:180–5.
4. Adams DD. *Abnormal Responses in the Assay of Thyrotropin*. Dunedin: Proc Univ Otago Med School (1956) pp. 34.
5. McKenzie JM. Humoral Factors in the Pathogenesis of Graves' Disease. *Physiol Rev* (1968) 48(1):252–310. doi: 10.1152/physrev.1968.48.1.252
6. Ross DS, Burch HB, Cooper DS, Greenlee MC, Laurberg P, Maia AL, et al. 2016 American Thyroid Association Guidelines for Diagnosis and Management of Hyperthyroidism and Other Causes of Thyrotoxicosis. *Thyroid* (2016) 26(10):1343–421. doi: 10.1089/thy.2016.0229
7. Bahn Chair RS, Burch HB, Cooper DS, Garber JR, Greenlee MC, Klein I, et al. Hyperthyroidism and Other Causes of Thyrotoxicosis: Management Guidelines of the American Thyroid Association and American Association of Clinical Endocrinologists. *Thyroid* (2011) 21(6):593–646. doi: 10.1089/thy.2010.0417
8. Muller I, Moran C, Lecumberri B, Decallonne B, Robertson N, Jones J, et al. 2019 European Thyroid Association Guidelines on the Management of Thyroid Dysfunction Following Immune Reconstitution Therapy. *Eur Thyroid J* (2019) 8(4):173–85. doi: 10.1159/000500881
9. Parmentier M, Libert F, Maenhaut C, Lefort A, Gérard C, Perret J, et al. Molecular Cloning of the Thyrotropin Receptor. *Science* (1989) 246 (4937):1620–2. doi: 10.1126/science.2556796
10. Nagayama Y, Kaufman KD, Seto P, Rapoport B. Molecular Cloning, Sequence and Functional Expression of the cDNA for the Human Thyrotropin Receptor. *Biochem Biophys Res Commun* (1989) 165(3):1184–90. doi: 10.1016/0006-291x(89)92727-7
11. Akamizu T, Ikuyama S, Saji M, Kosugi S, Kozak C, McBride OW, et al. Cloning, Chromosomal Assignment, and Regulation of the Rat Thyrotropin Receptor: Expression of the Gene is Regulated by Thyrotropin, Agents That Increase cAMP Levels, and Thyroid Autoantibodies. *Proc Natl Acad Sci USA* (1990) 87(15):5677–81. doi: 10.1073/pnas.87.15.5677
12. Giuliani C, Saji M, Bucci I, Napolitano G. Bioassays for TSH Receptor Autoantibodies, From FRTL-5 Cells to TSH Receptor-LH/CG Receptor Chimeras: The Contribution of Leonard D. Kohn. *Front Endocrinol (Lausanne)* (2016) 7:103. doi: 10.3389/fendo.2016.00103
13. Kohn LD, Giuliani C, Montani V, Napolitano G, Ohmori M , Ohta M, et al. Antireceptor Immunity. In: *Thyroid Immunity*. RG Landes Texas: Biomedical Publishers Austin/Georgetown (1995). p. 115–70.
14. Orgiazzi J. Anti-TSH Receptor Antibodies in Clinical Practice. *Endocrinol Metab Clin North Am* (2000) 29(2):339–55. doi: 10.1016/s0889-8529(05)70135-3
15. Michalek K, Morshed SA, Latif R, Davies TF. TSH Receptor Autoantibodies. *Autoimmun Rev* (2009) 9(2):113–6. doi: 10.1016/j.autrev.2009.03.012
16. Morshed SA, Davies TF. Graves' Disease Mechanisms: The Role of Stimulating, Blocking, and Cleavage Region TSH Receptor Antibodies. *Horm Metab Res* (2015) 47(10):727–34. doi: 10.1055/s-0035-1559633
17. McLachlan SM, Rapoport B. Thyrotropin-Blocking Autoantibodies and Thyroid-Stimulating Autoantibodies: Potential Mechanisms Involved in the Pendulum Swinging From Hypothyroidism to Hyperthyroidism or Vice Versa. *Thyroid* (2013) 23(1):14–24. doi: 10.1089/thy.2012.0374
18. Diana T, Olivo PD, Kahaly GJ. Thyrotropin Receptor Blocking Antibodies. *Horm Metab Res* (2018) 50(12):853–62. doi: 10.1055/a-0723-9023
19. Li Y, Kim J, Diana T, Klasen R, Olivo PD, Kahaly GJ. A Novel Bioassay for Anti-Thyrotrophin Receptor Autoantibodies Detects Both Thyroid-Blocking and Stimulating Activity. *Clin Exp Immunol* (2013) 173(3):390–7. doi: 10.1111/cei.12129
20. Giuliani C, Cerrone D, Harii N, Thornton M, Kohn LD, Dagia NM, et al. A TSHR-LH/CGR Chimera That Measures Functional Thyroid-Stimulating Autoantibodies (TSAb) can Predict Remission or Recurrence in Graves' Patients Undergoing Antithyroid Drug (ATD) Treatment. *J Clin Endocrinol Metab* (2012) 97(7):E1080–7. doi: 10.1210/jc.2011-2897
21. Kahaly GJ, Bartalena L, Hegedüs L, Leenhardt L, Poppe K, Pearce SH. 2018 European Thyroid Association Guideline for the Management of Graves' Hyperthyroidism. *Eur Thyroid J* (2018) 7(4):167–86. doi: 10.1159/000490384
22. Rapoport B, Chazenbalk GD, Jaume JC, McLachlan SM. The Thyrotropin (TSH) Receptor: Interaction With TSH and Autoantibodies. *Endocr Rev* (1998) 19(6):673–716. doi: 10.1210/edrv.19.6.0352
23. Ando T, Latif R, Davies TF. Thyrotropin Receptor Antibodies: New Insights Into Their Actions and Clinical Relevance. *Best Pract Res Clin Endocrinol Metab* (2005) 19(1):33–52. doi: 10.1016/j.beem.2004.11.005
24. Evans M, Sanders J, Tagami T, Sanders P, Young S, Roberts E, et al. Monoclonal Autoantibodies to the TSH Receptor, One With Stimulating Activity and One With Blocking Activity, Obtained From the Same Blood Sample. *Clin Endocrinol (Oxf)* (2010) 73(3):404–12. doi: 10.1111/j.1365-2265.2010.03831.x
25. Cho BY, Kim WB, Chung JH, Yi KH, Shong YK, Lee HK, et al. High Prevalence and Little Change in TSH Receptor Blocking Antibody Titres With Thyroxine and Antithyroid Drug Therapy in Patients With Non-Goitrous Autoimmune Thyroiditis. *Clin Endocrinol (Oxf)* (1995) 43(4):465–71. doi: 10.1111/j.1365-2265.1995.tb02619.x
26. Takasu N, Matsushita M. Changes of TSH-Stimulation Blocking Antibody (TSBAb) and Thyroid Stimulating Antibody (TSAb) Over 10 Years in 34 TSBAb-Positive Patients With Hypothyroidism and in 98 TSAb-Positive Graves' Patients With Hyperthyroidism: Reevaluation of TSBAb and TSAb in TSH-Receptor-Antibody (TRAb)-Positive Patients. *J Thyroid Res* (2012) 2012:182176. doi: 10.1155/2012/182176
27. Nagasaki K, Nakamura A, Yamauchi T, Kamasaki H, Hara Y, Kanno J, et al. Investigation of TSH Receptor Blocking Antibodies in Childhood-Onset Atrophic Autoimmune Thyroiditis. *Clin Pediatr Endocrinol* (2021) 30 (2):79–84. doi: 10.1297/cpe.30.79
28. Diana T, Krause J, Olivo PD, König J, Kanitz M, Decallonne B, et al. Prevalence and Clinical Relevance of Thyroid Stimulating Hormone Receptor-Blocking Antibodies in Autoimmune Thyroid Disease. *Clin Exp Immunol* (2017) 189(3):304–9. doi: 10.1111/cei.12980
29. Fatourechi V, McConahey WM, Woolner LB. Hyperthyroidism Associated With Histologic Hashimoto's Thyroiditis. *Mayo Clin Proc* (1971) 46(10):682–9.
30. Kiefer FW, Klebermass-Schrehof K, Steiner M, Worda C, Kasprian G, Diana T, et al. Fetal/Neonatal Thyrotoxicosis in a Newborn From a Hypothyroid Woman With Hashimoto Thyroiditis. *J Clin Endocrinol Metab* (2017) 102 (1):6–9. doi: 10.1210/jc.2016-2999
31. Feingold SB, Smith J, Houtz J, Popovsky E, Brown RS. Prevalence and Functional Significance of Thyrotropin Receptor Blocking Antibodies in Children and Adolescents With Chronic Lymphocytic Thyroiditis. *J Clin Endocrinol Metab* (2009) 94(12):4742–8. doi: 10.1210/jc.2009-1243
32. Chiovato L, Vitti P, Bendinelli G, Santini F, Fiore E, Capaccioli A, et al. Detection of Antibodies Blocking Thyrotropin Effect Using Chinese Hamster Ovary Cells Transfected With the Cloned Human TSH Receptor. *J Endocrinol Invest* (1994) 17(10):809–16. doi: 10.1007/bf03347782
33. Chiovato L, Vitti P, Santini F, Lopez G, Mammoli C, Bassi P, et al. Incidence of Antibodies Blocking Thyrotropin Effect *In Vitro* in Patients With Euthyroid or Hypothyroid Autoimmune Thyroiditis. *J Clin Endocrinol Metab* (1990) 71 (1):40–5. doi: 10.1210/jcem-71-1-40
34. Kawahara K, Tsukimoto I, Yokoya S. Atrophic Autoimmune Thyroiditis With Positive Thyroid Stimulation Blocking Antibody in a Prepubertal Boy. *Clin Pediatr Endocrinol* (2000) 9(2):105–11. doi: 10.1297/cpe.9.105
35. Takasu N, Yamada T, Takasu M, Komiya I, Nagasawa Y, Asawa T, et al. Disappearance of Thyrotropin-Blocking Antibodies and Spontaneous Recovery From Hypothyroidism in Autoimmune Thyroiditis. *N Engl J Med* (1992) 326(8):513–8. doi: 10.1056/nejm199202203260803
36. Takasu N, Yoshimura Noh J. Hashimoto's Thyroiditis: TGAb, TPOAb, TRAb and Recovery From Hypothyroidism. *Expert Rev Clin Immunol* (2008) 4 (2):221–37. doi: 10.1586/1744666x.4.2.221
37. Zöphel K, Roggenbuck D, Schott M. Clinical Review About TRAb Assay's History. *Autoimmun Rev* (2010) 9(10):695–700. doi: 10.1016/j.autrev.2010.05.021
38. Matsuura N, Yamada Y, Nohara Y, Konishi J, Kasagi K, Endo K, et al. Familial Neonatal Transient Hypothyroidism Due to Maternal TSH-Binding Inhibitor Immunoglobulins. *N Engl J Med* (1980) 303(13):738–41. doi: 10.1056/nejm198009253031306

39. Bucci I, Giuliani C, Napolitano G. Thyroid-Stimulating Hormone Receptor Antibodies in Pregnancy: Clinical Relevance. *Front Endocrinol (Lausanne)* (2017) 8:137. doi: 10.3389/fendo.2017.00137
40. Takasu N, Yamada T, Sato A, Nakagawa M, Komiya I, Nagasawa Y, et al. Graves' Disease Following Hypothyroidism Due to Hashimoto's Disease: Studies of Eight Cases. *Clin Endocrinol (Oxf)* (1990) 33(6):687–98. doi: 10.1111/j.1365-2265.1990.tb03906.x
41. Kamath C, Young S, Kabelis K, Sanders J, Adlan MA, Furmaniak J, et al. Thyrotrophin Receptor Antibody Characteristics in a Woman With Long-Standing Hashimoto's Who Developed Graves' Disease and Pretibial Myxoedema. *Clin Endocrinol (Oxf)* (2012) 77(3):465–70. doi: 10.1111/j.1365-2265.2012.04397.x
42. Schumm-Draeger PM, Padberg S, Heller K. Prophylactic Levothyroxine Therapy in Patients With Hashimoto's Thyroiditis. *Exp Clin Endocrinol Diabetes* (1999) 107 Suppl 3:S84–7. doi: 10.1055/s-0029-1212157
43. Zakarija M, McKenzie JM, Munro DS. Immunoglobulin G Inhibitor of Thyroid-Stimulating Antibody is a Cause of Delay in the Onset of Neonatal Graves' Disease. *J Clin Invest* (1983) 72(4):1352–6. doi: 10.1172/jci111091
44. Tjiang H, Lahooti H, McCorquodale T, Parmar KR, Wall JR. Eye and Eyelid Abnormalities are Common in Patients With Hashimoto's Thyroiditis. *Thyroid* (2010) 20(3):287–90. doi: 10.1089/thy.2009.0199
45. Kahaly GJ, Diana T, Glang J, Kanitz M, Pitz S, König J. Thyroid Stimulating Antibodies Are Highly Prevalent in Hashimoto's Thyroiditis and Associated Orbitopathy. *J Clin Endocrinol Metab* (2016) 101(5):1998–2004. doi: 10.1210/jc.2016-1220
46. Yoshihara A, Yoshimura Noh J, Nakachi A, Ohye H, Sato S, Sekiya K, et al. Severe Thyroid-Associated Orbitopathy in Hashimoto's Thyroiditis. Report of 2 Cases. *Endocr J* (2011) 58(5):343–8. doi: 10.1507/endocrj.k11e-019
47. Bartalena L, Kahaly GJ, Baldeschi L, Dayan CM, Eckstein A, Marcocci C, et al. The 2021 European Group on Graves' Orbitopathy (EUGOGO) Clinical Practice Guidelines for the Medical Management of Graves' Orbitopathy. *Eur J Endocrinol* (2021) 185(4):G43–67. doi: 10.1530/eje-21-0479
48. Sawka AM, Fatourechi V, Boeve BF, Mokri B. Rarity of Encephalopathy Associated With Autoimmune Thyroiditis: A Case Series From Mayo Clinic From 1950 to 1996. *Thyroid* (2002) 12(5):393–8. doi: 10.1089/105072502760043477
49. Brain L, Jellinek EH, Ball K. Hashimoto's Disease and Encephalopathy. *Lancet* (1966) 2(7462):512–4. doi: 10.1016/s0140-6736(66)92876-5
50. Benvenga S, Guarneri F. Homology Between TSH-R/Tg/TPO and Hashimoto's Encephalopathy Autoantigens. *Front Biosci (Landmark Ed)* (2020) 25:229–41. doi: 10.2741/4804
51. Cohen JA, Coles AJ, Arnold DL, Confavreux C, Fox EJ, Hartung HP, et al. Alemtuzumab Versus Interferon Beta 1a as First-Line Treatment for Patients With Relapsing-Remitting Multiple Sclerosis: A Randomised Controlled Phase 3 Trial. *Lancet* (2012) 380(9856):1819–28. doi: 10.1016/s0140-6736(12)61769-3
52. Coles AJ, Twyman CL, Arnold DL, Cohen JA, Confavreux C, Fox EJ, et al. Alemtuzumab for Patients With Relapsing Multiple Sclerosis After Disease-Modifying Therapy: A Randomised Controlled Phase 3 Trial. *Lancet* (2012) 380(9856):1829–39. doi: 10.1016/s0140-6736(12)61768-1
53. Isaacs JD, Greer S, Sharma S, Symmons D, Smith M, Johnston J, et al. Morbidity and Mortality in Rheumatoid Arthritis Patients With Prolonged and Profound Therapy-Induced Lymphopenia. *Arthritis Rheum* (2001) 44(9):1998–2008. doi: 10.1002/1529-0131(200109)44:9<1998::Aid-art348>3.0.Co;2-t
54. Lundin J, Porwit-MacDonald A, Rossmann ED, Karlsson C, Edman P, Rezvany MR, et al. Cellular Immune Reconstitution After Subcutaneous Alemtuzumab (Anti-CD52 Monoclonal Antibody, CAMPATH-1H) Treatment as First-Line Therapy for B-Cell Chronic Lymphocytic Leukaemia. *Leukemia* (2004) 18(3):484–90. doi: 10.1038/sj.leu.2403258
55. Haynes R, Harden P, Judge P, Blackwell L, Emberson J, Landray MJ, et al. Alemtuzumab-Based Induction Treatment Versus Basiliximab-Based Induction Treatment in Kidney Transplantation (the 3C Study): A Randomised Trial. *Lancet* (2014) 384(9955):1684–90. doi: 10.1016/s0140-6736(14)61095-3
56. Rotondi M, Molteni M, Leporati P, Capelli V, Marinò M, Chiovato L. Autoimmune Thyroid Diseases in Patients Treated With Alemtuzumab for Multiple Sclerosis: An Example of Selective Anti-TSH-Receptor Immune Response. *Front Endocrinol (Lausanne)* (2017) 8:254. doi: 10.3389/fendo.2017.00254
57. Memon AB, Javed A, Caon C, Srivastawa S, Bao F, Bernitsas E, et al. Long-Term Safety of Rituximab Induced Peripheral B-Cell Depletion in Autoimmune Neurological Diseases. *PloS One* (2018) 13(1):e0190425. doi: 10.1371/journal.pone.0190425
58. Daniels GH, Vladic A, Brinar V, Zavalishin I, Valente W, Oyuela P, et al. Alemtuzumab-Related Thyroid Dysfunction in a Phase 2 Trial of Patients With Relapsing-Remitting Multiple Sclerosis. *J Clin Endocrinol Metab* (2014) 99(1):80–9. doi: 10.1210/jc.2013-2201
59. Muller I, Willis M, Healy S, Nasser T, Loveless S, Butterworth S, et al. Longitudinal Characterization of Autoantibodies to the Thyrotropin Receptor (TRAb) During Alemtuzumab Therapy: Evidence That TRAb May Precede Thyroid Dysfunction by Many Years. *Thyroid* (2018) 28(12):1682–93. doi: 10.1089/thy.2018.0232

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Effect of Thyroid-Stimulating Hormone Suppression on Muscle Function After Total Thyroidectomy in Patients With Thyroid Cancer

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Context: Thyroid-stimulating hormone (TSH) suppression is recommended to reduce tumor recurrence following surgery for differentiated thyroid cancer (DTC). However, prolonged subclinical hyperthyroidism caused by levothyroxine treatment has deleterious effects on various organs.

Objective: To evaluate the relationships of TSH concentration with muscle mass, muscle strength, and physical performance related to sarcopenia in patients with DTC undergoing TSH suppression following surgery.

Methods: We studied 134 patients of >60 years who were undergoing TSH suppression therapy following surgery for DTC. We evaluated muscle mass and muscle function-related parameters and diagnosed sarcopenia using the threshold for Asian people.

Results: The participants were 68.3 ± 7.2 years old and 36/134 (26.9%) were diagnosed with sarcopenia. They were allocated to high-TSH and low-TSH groups using a threshold concentration of 0.40 μU/mL, and grip strength was significantly lower in the low-TSH group. The data were further analyzed according to age and sex, and in the low-TSH group, male participants and those of <70 years were found to have significantly lower grip strength.

Conclusions: Low-TSH concentrations is associated with low grip strength, and this is most pronounced in individuals of <70 years of age. Therefore, muscle function should be considered an adverse effect of TSH suppression in patients with DTC who undergo TSH suppression therapy, especially in men of <70 years.

Keywords: thyroid-stimulating hormone, sarcopenia, thyroidectomy, thyroid cancer, muscle function and physical activity

INTRODUCTION

The standard treatment for differentiated thyroid cancer (DTC) consists of thyroid surgery, with or without postoperative radioiodine therapy and thyroid-stimulating hormone (TSH) suppression therapy, according to the estimated risk of recurrence (1–3). For those treated using thyroidectomy and for some who undergo thyroid lobectomy alone, TSH suppression therapy, usually conducted alongside levothyroxine administration, is necessary to restore euthyroidism and is used to inhibit cancer recurrence, which suggests that DTCs are TSH-dependent tumors (4–6). Because aggressive TSH suppression therapy is of little or no benefit to most patients with DTC, levothyroxine is not routinely administered following unilateral lobectomy. Furthermore, clinical guidelines recommend that the oncologic benefits of TSH suppression are weighed against the cardiovascular and musculoskeletal risks (7).

Sarcopenia is defined as an age-related, involuntary, progressive loss of muscle mass and strength, and it affects approximately 50% of adults aged ≥80 years (8). Sarcopenia is frequently accompanied by long-standing physiologic consequences of metabolic disorders and malignancies in patients, and together these have various adverse sequelae, including physical disability, poor quality of life, and death (9, 10). Patients with advanced thyroid cancer who are treated using molecularly targeted therapies often experience a significant loss of skeletal muscle mass, irrespective of the rate of disease progression (11), but no previous studies have evaluated the relationships of thyroid hormone or TSH concentration with sarcopenia in patients with indolent DTC.

Hyperthyroidism and hypothyroidism affect multiple body systems, including skeletal muscle, because this is a principal target tissue of thyroid hormones. Although thyroid hormone excess in overt hyperthyroidism induces osteoporosis and myopathy, and impairs physical performance, subclinical hyperthyroidism, defined as a TSH concentration below the normal range, alongside normal serum thyroxine concentration, is also associated with bone loss and a higher risk of fracture (12, 13). Although most studies have shown the effects of overt or subclinical hyperthyroidism on the heart or bone tissue, there is no research on the effects of subclinical hyperthyroidism on physical and functional muscle profiles and sarcopenia incidence in patients with DTC. Furthermore, it remains to be elucidated how excessive T4 supplementation-induced subclinical hyperthyroidism affects muscle mass and function after total thyroidectomy in differentiated thyroid cancer patients. In addition, recent studies have shown that the TSH receptor is expressed in skeletal muscle cells (14, 15). TSH also directly modulates muscle metabolism, independently of thyroid hormone, as has been demonstrated with respect to bone remodeling (16). Therefore, we hypothesized that, even though the role of TSH receptor signaling in skeletal muscle is poorly understood, TSH suppression may be associated with higher risks of the loss and/or dysfunction of muscle in patients with DTC who are administering levothyroxine. In the present study, we aimed to evaluate the relationship of TSH concentration with muscle mass, muscle strength, and physical performance in older patients with DTC.

MATERIALS AND METHODS

Study Participants

We performed a cross-sectional study of a sample of Korean patients of >60 years of age who were undergoing TSH suppression therapy following surgery for DTC between April 2019 and April 2020. These participants were attending the Outpatient department of the hospital for the management of DTC and TSH suppression therapy. Anthropometric measurements were made after an overnight fast. Height (in centimeters) and body mass (in kilograms) were measured, and body mass index (BMI) was calculated as body mass divided by the square of height (kg/m^2). Individuals with liver cirrhosis, renal failure, stroke sequelae, myocardial infarction, or angina were excluded because these may affect muscle metabolism. After the exclusion of ineligible individuals, 134 eligible participants were enrolled.

Ethical Considerations

The Institutional Review Board (2019-06-063) of Chungnam National University Hospital approved the research protocol. Written informed consent was obtained from all the participants. The interviews and examinations were performed in accordance with the principles of the Declaration of Helsinki. The authors certify their compliance with the ethical guidelines for authorship and publishing in the journal.

Assessment of Sarcopenia

Information regarding the demographic characteristics and medical and surgical histories of the participants was collected through detailed interviews and reviews of medical records by experienced nurses. Body composition, including muscle mass (whole-body lean mass minus bone mineral content), was evaluated using a bioelectrical impedance analyzer (InBody S10; InBody, Seoul, Korea) at frequencies of 1, 5, 50, 250, 500, and 1,000 kHz (17). Appendicular skeletal muscle mass (ASM) was calculated as the sum of the muscle mass of all four limbs. Skeletal muscle mass index (SMI) was calculated as ASM/height^2 (kg/m^2) (18). Hand-grip strength on the dominant side was measured using a Jamar hydraulic hand dynamometer (Patterson Medical, Warrenville, IL, USA) (19). Participants were instructed to sit comfortably, bend their elbow to 90°, and grip the dynamometer as firmly as possible. The maximum value was recorded after all the tests were conducted twice at 1 min intervals or more. We also measured gait speed over a 4 m distance and the time taken to complete five chair-stands (20). The participants also underwent a short physical performance battery (SPPB), which consisted of repeated chair-stands, and assessments of balance when standing and gait speed (21). In the standing balance test, which comprised side-by-side, semi-tandem, and tandem stances, the participants were instructed to stand for up to 10 sec. Higher SPPB scores (range 0 to 12 points) are indicative of superior function of the lower extremities.

Sarcopenia was diagnosed using the 2019 Consensus Guidelines of the Asian Working Group for Sarcopenia (22).

Briefly, older patients with low muscle mass (SMI <7.0 kg/m^2 for men and <5.7 kg/m^2 for women) and low muscle strength (hand-grip strength <28 kg for men and <18 kg for women), and/or poor physical performance (gait speed <1.0 m/s, five-time chair-stand test ≥12 s, or SPPB score ≤9 points) were diagnosed as having sarcopenia.

Thyroid Function and Biochemical Measurements

Blood samples were collected from an antecubital vein and centrifuged at 400 g for 5 min at 4°C, and then the supernatants were carefully collected. Samples showing hemolysis or clotting were discarded. The serum samples were stored at −80°C until analyzed. Serum TSH and free T4 (FT4) concentrations were measured using electrochemiluminescence immunoassays (Roche Diagnostics, Mannheim, Germany) 24 h after sampling. Serum TSH was measured using an E-TSH kit (Roche Diagnostics; reference range: 0.35–5.50 mIU/L) and serum FT4 was measured using an E-Free T4 kit (Roche Diagnostics; reference range: 0.89–1.76 ng/mL).

Statistical Analysis

Clinical data are presented as means ± standard deviations (SDs) or as numbers and percentages unless otherwise specified. The chi-square and Fischer's exact tests were used to analyze categorical data. The normality of continuous variables was assessed using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. If the normality and homogeneity of variance assumptions were satisfied, then independent *t*-tests were used to compare the means of two groups and ANOVA was used to compare the means of three groups. If the normality assumption but not the homogeneity of variance assumption was satisfied, unpaired *t*-tests and Kruskal-Wallis tests were substituted. If neither assumption was satisfied, Mann-Whitney *U*-tests and Kruskal-Wallis tests were used for continuous clinical and biochemical data, as appropriate. A two-tailed $p < 0.05$ was considered to represent statistical significance.

Statistical analyses were performed using R software version 4.0.4 (R Project for Statistical Computing, Vienna, Austria).

RESULTS

Baseline Characteristics of the Study Participants

Supplementary Table 1 lists the baseline characteristics of the 134 study participants, of whom 109 (81.3%) were women, and the mean ± SD age was 68.33 ± 7.19 years. Their mean serum free T4 concentration was 1.42 ± 0.25 ng/mL and their mean TSH concentration was 0.84 ± 0.98 μIU/mL during thyroid function testing. Their mean BMI was 24.32 ± 3.24 kg/m^2, their mean skeletal muscle mass was 21.54 ± 4.53 kg, and their mean SMI was 8.68 ± 1.15 kg/m^2. According to the 2019 Consensus Guidelines of the Asian Working Group for Sarcopenia, 36 of the 134 (26.9%) participants were diagnosed with sarcopenia. Their mean grip strength, an index of muscle strength, was 21.54 ± 5.40 kg. With respect to indices of physical performance, their mean gait speed was 4.34 ± 1.38 m/s, their mean five-time chair-stand test result was 8.57 ± 3.52 s, and their mean SPPB score was 11.04 ± 1.75 points.

Effects of Age on Muscle Function in Older Patients Undergoing Total Thyroidectomy

We allocated the participants to two groups on the basis of age (**Table 1**): an over-70s group (n=55) and an under-70s group (n=79). There were 66 (83.5%) and 43 (78.2%) women in each group, respectively ($P = 0.433$). The mean FT4 concentrations were 1.38 ± 0.26 ng/mL in the over-70s group and 1.45 ± 0.23 ng/mL in the under-70s group ($P = 0.059$). The mean TSH concentrations were 0.74 ± 0.96 μIU/mL in the over-70s group and 0.91 ± 0.99 μIU/mL in the under-70s group ($P = 0.530$). Twenty-three participants (41.8%) were diagnosed with sarcopenia in the over-70s group and 13 (16.5%) in the under-70s group ($P = 0.001$). However, there were no differences in skeletal muscle mass or SMI between the two groups ($P = 0.095$ and 0.212, respectively). Moreover, there were no differences in grip strength or the five-time chair-stand test result between the two groups ($P = 0.325$ and 0.115, respectively), but the gait speed in the over-70s group (4.78 ± 1.33 s) was higher than that in the under-70s group (4.03 ± 1.34 s) ($P = 0.002$). There was also a lower SPPB score in the over-70s group (10.40 ± 2.20 points) than in the under-70s group (11.49 ± 1.18 points) ($P = 0.001$).

Relationship Between Serum TSH Concentration and Muscle Function in Older Patients Undergoing Total Thyroidectomy

Next, we allocated the participants to a high-TSH group (0.40–4.0 μU/mL; n = 69) and a low-TSH group (<0.40 μU/mL; n = 65) (**Table 2**). There was no significant differences in the sex ratio or the prevalence of sarcopenia between the high-TSH (73.9%) and low-TSH groups (72.3%) ($P = 0.988$). In addition, there were no differences between the groups with respect to the results of the five-time chair-stand test, gait speed, or SPPB score ($P = 0.295$, 0.297, and 0.612, respectively). However, hand-grip strength was significantly lower in the low-TSH group (20.30 ± 3.96 kg) than in the high-TSH group (22.72 ± 6.27 kg) ($P = 0.007$). Logistic regression analysis was performed to confirm the relationship between hand-grip strength and TSH, and the Akaike information criterion (AIC) was used to assess a model's maximum likelihood estimation. The AIC of the logistic model was 172.9. There was an odds ratio of 1.1563 for grip strength for the high-TSH group, meaning that the probability of being in the high-TSH group increased 1.1563 times for each increase of 1 in grip strength (**Table 3**). However, there was no relationships of free T4 or T3 concentrations with muscle function or physical performance in older patients who had undergone total thyroidectomy (**Supplementary Tables 2**, **3**).

Subgroup Analyses of the Data on the Bases of Age and Sex

As shown in **Figure 1**, we calculated correlations to characterize the relationships among all the clinical variables. Grip strength

TABLE 1 | Clinical characteristics of the study sample, categorized according to age (N = 134).

Parameter	60-70 years (N = 79)	>70 years (N = 55)	*P*-value
Sex			0.577
Male	13 (16.5)	12 (21.8)	
Female	66 (83.5)	43 (78.2)	
Sarcopenia			**0.002**
Absent	66 (83.5)	32 (58.2)	
Present	13 (16.5)	23 (41.8)	
Body mass index (kg/m^2)	24.22 ± 3.39	24.47 ± 3.02	0.502
Skeletal muscle mass (kg)	21.92 ± 4.66	20.98 ± 4.31	0.095
Skeletal muscle index (kg/m^2)	8.75 ± 1.22	8.59 ± 1.04	0.212
Free T4 (ng/mL)	1.45 ± 0.23	1.38 ± 0.26	0.059
T3 (ng/mL)	1.39 ± 0.19	1.36 ± 0.19	0.664
TSH (μIU/mL)	0.91 ± 0.99	0.74 ± 0.96	0.530
Grip strength (kg)	21.99 ± 5.48	20.91 ± 5.25	0.325
Chair-stand test result (s)	8.17 ± 3.47	9.21 ± 3.52	0.115
B-score	3.99 ± 0.11	3.91 ± 0.35	0.113
Chair score	3.76 ± 0.69	3.62 ± 0.74	0.269
Gait speed (s)	4.03 ± 1.34	4.78 ± 1.33	**0.002**
Gait speed score	3.84 ± 0.52	3.46 ± 0.79	**0.003**
SPPB score	11.49 ± 1.18	10.40 ± 2.20	**0.001**

Data are mean ± SD or number (%). The chi-square or t-tests were used to compare the groups, as appropriate. Significant differences between the age groups are highlighted in bold. TSH, thyroid stimulating hormone; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; SPPB, short physical performance battery.

tended to be lower in participants of <70 years and in men with a low-TSH concentration. The correlation coefficient for the relationship between TSH concentration and grip strength was 0.19, implying a weak positive correlation between the two variables (**Figure 1**). We next determined whether grip strength is related to age or sex in participants with a low-TSH concentration. First, we allocated the participants to two groups on the basis of being <70 or >70 years old, and then further allocated them to high- and low-TSH groups. In the over-70s group, there was no difference in hand-grip strength between the high- and low-TSH groups, but in the under-70s group, grip strength was significantly lower in participants with low-TSH concentration than in those with high-TSH (**Figure 2**). However, there were no relationships between five-time chair-stand test performance and serum TSH concentration in either the over-70s or under-70s groups. Next, the participants were categorized according to sex and the muscle function of each sex was compared between low- and high-TSH groups. There was no difference in grip strength according to TSH concentration in women, but it was significantly lower in men with a low-TSH concentration. As for the results of the analysis according to age, there was no relationship between five-time chair-stand performance or TSH concentration in either men or women (**Figure 3**).

TABLE 2 | Clinical characteristics of the study sample, categorized according to serum TSH concentration (N = 134).

Parameter	<0.40 μU/ml (N = 65)	≥0.40 μU/ml (N = 69)	*P*-value
Sex			0.244
Male	9 (13.8)	16 (23.2)	
Female	56 (86.2)	53 (76.8)	
Sarcopenia			0.988
Absent	47 (72.3)	51 (73.9)	
Present	18 (27.7)	18 (26.1)	
Age (years)	68.42 ± 7.45	68.25 ± 6.99	0.641
Body mass index (kg/m^2)	24.24 ± 3.07	24.40 ± 3.41	0.780
Skeletal muscle mass (kg)	21.09 ± 3.81	21.96 ± 5.10	0.428
Skeletal muscle index (kg/m^2)	8.59 ± 0.92	8.77 ± 1.33	0.423
Free T4 (ng/mL)	1.52 ± 0.27	1.33 ± 0.19	**0.000**
T3 (ng/mL)	1.42 ± 0.21	1.34 ± 0.16	0.126
Grip strength	20.30 ± 3.96	22.72 ± 6.27	**0.007**
Chair-stand test result (s)	8.91 ± 3.44	8.24 ± 3.58	0.295
B-score	3.95 ± 0.28	3.96 ± 0.21	0.949
Chair score	3.67 ± 0.73	3.75 ± 0.70	0.538
Gait speed (s)	4.21 ± 1.07	4.46 ± 1.62	0.297
Gait speed score	3.68 ± 0.64	3.69 ± 0.70	0.903
SPPB score	11.02 ± 1.74	11.07 ± 1.78	0.612

Data are mean ± SD or number (%). The chi-square or t-tests were used to compare the groups, as appropriate. Significant differences between the TSH groups are highlighted in bold. TSH, thyroid stimulating hormone; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; SPPB, short physical performance battery.

TABLE 3 | Results of the logistic regression analysis of potential predictors of serum TSH concentration.

No interaction (AIC = 172.9)			
Parameter	**OR**	**2.50%**	**97.50%**
(Intercept)	1.4385	0.0066	328.5889
Sarcopenia	1.4095	0.4244	4.7348
Grip strength (kg)	1.1563	1.0444	1.2945
Age (years)	0.4897	0.2105	1.1024
Free T4 (ng/mL)	0.1646	0.0657	0.3818
Skeletal muscle (kg)	1.1047	0.8679	1.4087
SPPB score	0.9153	0.6653	1.2496
Skeletal muscle index (kg/m^2)	0.6526	0.2744	1.5235

SPPB, short physical performance battery.

DISCUSSION

In the present study, we have compared the prevalence of sarcopenia, muscle strength, and physical performance in patients with DTC who had high or low serum TSH concentrations. We found that those with low-TSH concentrations who were 60–70 years old had lower grip strength than those who were > 70 years old.

The degree of TSH suppression in patients with DTC should be optimized to reduce tumor recurrence while minimizing the risk of toxicity associated with subclinical hyperthyroidism. TSH suppression significantly increases the postoperative risks of atrial fibrillation and osteoporosis in patients with DTC (23), and subclinical hyperthyroidism is associated with higher cardiovascular morbidity and mortality in older patients with DTC (24, 25). Furthermore, postmenopausal women with DTC and subclinical hyperthyroidism are at higher risk of osteoporosis, whereas the risks for men and premenopausal women are not affected by this (26). Therefore, we believe that measurement of bone mineral density should be recommended for postmenopausal women with DTC when TSH is being suppressed. The American Thyroid Association recommends that serum TSH should be suppressed to low concentrations (0.1–0.5 mU/L) for 5–10 years in high-risk groups only (3). However, although the major academic societies have made recommendations regarding TSH suppression-related adverse effects on heart and bone, on the basis of published evidence, the optimal maintenance TSH concentration for the preservation of muscle strength and physical performance in patients with DTC has not been determined. Therefore, our finding that TSH suppression may have adverse effects on skeletal muscle function in elderly patients with DTC is of great relevance. Moreover, we suggest that subclinical hyperthyroidism may be implicated as a modifier of muscle function in individuals with total thyroidectomy of <70 years of age.

Sarcopenia, which is associated with both low absolute muscle mass and poor muscle function, is a problem in elderly patients and increases in prevalence with age. Moreover, the prevalence of DTC also increases with age. Therefore, the effects of postoperative thyroid function on muscle function and physical performance in our aging societies are important. We have studied older adults with DTC (age 68.33 ± 7.19 years) and found a prevalence of sarcopenia of 26.9%, according to the diagnostic criteria of the Asian Working Group (22). This prevalence of sarcopenia in older patients of DTC is comparable with that of community-dwelling individuals of >65 years when SMI (ASM/height^2) is used as an index of sarcopenia, with thresholds of 7.09 kg/m^2 for men and 5.27 kg/m^2 for women (27). To evaluate the relationship of age with TSH suppression-related muscle function, we further allocated the participants to 60–70 years old and > 70 years old groups. As expected, the prevalence of sarcopenia in the latter group (41.8%)

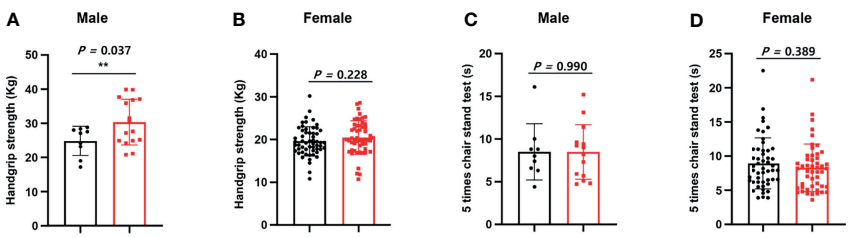

FIGURE 1 | Relationships of grip strength and chair-stand test result with serum TSH concentration and sex. **(A)** We allocated the participants to two groups on the basis of sex. Grip strength in men was significantly lower in the low-TSH group than in the high-TSH group. **(B)** However, grip strength in women was similar in the high-TSH and low-TSH groups. **(C)** The results of the chair-stand test were analyzed in the same way. The results of the test were similar in men in the high-TSH and low-TSH groups. **(D)** The results of the test were also similar in women in the high-TSH and low-TSH groups. ** in the figure indicates p-value < 0.01.

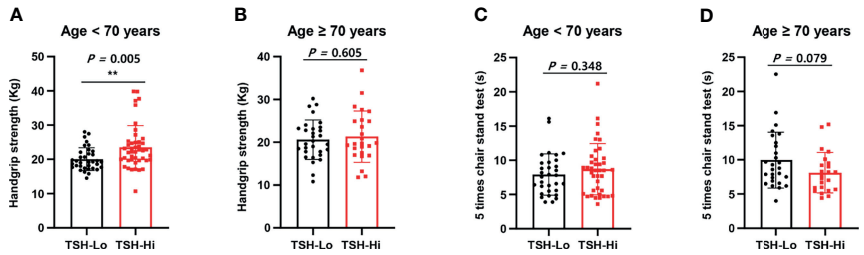

FIGURE 2 | Relationships of grip strength and chair-stand test results with serum TSH concentration and age. **(A)** Participants were allocated to two groups on the basis of age: <70 years and >70 years. Grip strength in the under-70s was significantly lower in the low-TSH group (TSH-Lo, <0.40 μU/mL). **(B)** Grip strength in the over-70s was similar in the high- (TSH-Hi, 0.40–4.0 μU/mL) and low-TSH groups. **(C)** The chair-stand test results were similarly analyzed. Among the under-70s, there was no difference between the low- and high-TSH groups. **(D)** Among the over-70s, there was also no difference between the low- and high-TSH groups. ** in the figure indicates p-value < 0.01.

was higher than that in the former group (16.5%). Intriguingly, we found that TSH suppression was associated with low hand-grip strength in the 60–70-year-old participants but not in the older participants. This suggests that the frailty of the older patients may conceal or prevent TSH suppression-related muscle deterioration. Therefore, we further considered the roles of potential risk factors for frailty: age, sex, ethnicity, nutritional status, polypharmacy, educational level, cognitive function, marital status, living status, drinking and smoking status, regular exercise, and self-reported health. We found that more marked TSH suppression was associated with lower hand-grip strength, but only in men. Serum TSH concentrations are considered the most reliable indicator of thyroid function abnormalities, and TSH analysis stands as the primary means of studying thyroid function (28). In contrast, free T4 assays often fail to be reliable due to variable TBG and albumin levels (29–31). This may explain why only low TSH concentrations, not free T4 or free T3, were associated with low grip strength in this study. However, these findings need to be confirmed in a large cohort study using methods that take into account the effects of confounding factors.

Grip strength is a measure of the maximum static force that the hand can exert on a dynamometer and is a reliable index of overall muscle strength (32, 33). Low grip strength is associated with comorbidities such as hypertension, diabetes, cardiovascular disease, stroke, and chronic obstructive

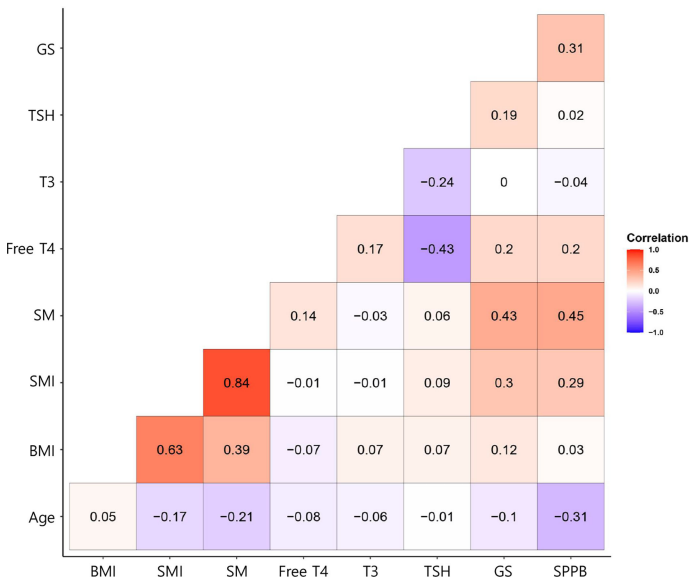

FIGURE 3 | Correlation coefficients for the relationships between explanatory variables and the response variable for the participants with thyroid cancer. In the corrgram, the depth of shading at the correlation matrices indicates the magnitude of the correlation. Positive and negative correlations are represented in red and blue, respectively.

pulmonary disease, as well as high all-cause mortality (34–39). Therefore, grip strength has been suggested to represent a "biomarker of aging" across the lifespan (40, 41). Importantly, low grip strength is a clinical marker of poor mobility and a better predictor of clinical outcomes than low muscle mass (42, 43). Moreover, muscle strength is not solely dependent on muscle mass, and the relationship between muscle strength and mass is not always linear (44, 45). Therefore, careful consideration of the importance of muscle strength *per se* and muscle mass is necessary in research studies and in the clinic to better understand the effects of particular factors on muscle health. In the present study, we have shown that a low-TSH concentration is associated with low grip strength in older patients with DTC. Although we believe that TSH may regulate the biogenesis and molecular function of skeletal muscle cells (14), further experimental studies are required to define the effects of TSH on muscle physiology and pathology in the context of aging.

The present study had several limitations. First, because most of the participants were elderly, there are likely to be various factors that would have affected their muscle metabolism and function; therefore, it was not possible to assess the pure relationship between TSH and muscle function. Given the potential limitations of the observational studies and the marginally statistically significant association, it is difficult to determine between TSH and muscle parameters using the conclusion of this study. Moreover, our study population was exclusively South Korean, and we cannot be certain that our results apply to other populations. Furthermore, we excluded the participants with liver cirrhosis, renal failure, stroke sequelae, myocardial infarction, or angina in the current study, but other confounders, such as respiratory disease, autoimmune disease, uncontrolled diabetes, low calcium intake and vitamin D level, sex hormone level, and statin use, should also be excluded to enhance statistical significance. In addition, we studied a relatively small number of patients in a single institution. Therefore, further studies should be conducted using a larger sample size and over a wider area.

In conclusion, we have shown that a low TSH concentration is associated with low grip strength, especially in individuals of <70 years of age and in men. Therefore, clinicians should be aware of the adverse effects of TSH suppression on muscle function in patients with DTC who undergo TSH suppression therapy, especially if they are male and under 70 years old.

SUPPLEMENTAL METHODS

Statistical analysis

To identify factors affecting TSH concentration, logistic regression analysis was performed using age, sex, SMI, free T4, T3, sarcopenia, grip strength, SPPB score, gait speed, and skeletal muscle mass as explanatory variables. Because low-TSH and high-TSH represent a binary response variable, we used the Logit-model. Finally, Akaike's information criterion and multicollinearity among the explanatory variables were considered in the selection of the final model.

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 Chungnam National University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception or design: H-SY and MS. Acquisition, analysis, or interpretation of data: JCL, B-SS., YMK, Y-RK, YEK, and JHL. Drafting the work or revising: JCL and H-SY. Final approval of the manuscript: H-SY. All authors contributed to the article and approved the submitted version.

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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.2021.769074/full#supplementary-material

Supplementary Table 1 | Clinical characteristics of the study sample (N = 134). Data are mean ± SD or number (%). TSH, thyroid stimulating hormone; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; SPPB, short physical performance battery.

Supplementary Table 2 | Clinical characteristics of the study sample, categorized according to serum FT4 concentration (N = 134). Data are mean ± SD or number (%). The chi-square or *t*-tests were used to compare the groups, as appropriate. Significant differences between the FT4 groups are highlighted in bold. TSH, thyroid stimulating hormone; SPPB, short physical performance battery.

Supplementary Table 3 | Clinical characteristics of the study sample, categorized according to serum T3 concentration (N = 134). Data are mean ± SD or number (%). The chi-square or *t*-tests were used to compare the groups, as appropriate. TSH, thyroid stimulating hormone; SPPB, short physical performance battery.

REFERENCES

1. Yi KH, Park YJ, Koong SS, Kim JH, Na DG, Ryu JS, et al. Revised Korean Thyroid Association Management Guidelines for Patients With Thyroid Nodules and Thyroid Cancer. *Korean J Otorhinolaryngol Head Neck Surg* (2011) 54:8–36. doi: 10.3342/kjorl-hns.2011.54.1.8
2. American Thyroid Association (ATA) Guidelines Taskforce on Thyroid Nodules and Differentiated Thyroid Cancer, Cooper DS, Doherty GM, Haugen BR, Kloos RT, Lee SL, et al. Revised American Thyroid Association Management Guidelines for Patients With Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* (2009) 19:1167–214. doi: 10.1089/thy.2009.0110
3. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, et al. 2015 American Thyroid Association Management Guidelines for Adult Patients With Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* (2016) 26:1–133. doi: 10.1089/thy.2015.0020
4. Cooper DS, Specker B, Ho M, Sperling M, Ladenson PW, Ross DS, et al. Thyrotropin Suppression and Disease Progression in Patients With Differentiated Thyroid Cancer: Results From the National Thyroid Cancer Treatment Cooperative Registry. *Thyroid* (1998) 8:737–44. doi: 10.1089/thy.1998.8.737
5. McGriff NJ, Csako G, Gourgiotis L, Lori CG, Pucino F, Sarlis NJ. Effects of Thyroid Hormone Suppression Therapy on Adverse Clinical Outcomes in Thyroid Cancer. *Ann Med* (2002) 34:554–64. doi: 10.1080/078538902321117760
6. Pujol P, Daures JP, Nsakala N, Baldet L, Bringer J, Jaffiol C. Degree of Thyrotropin Suppression as a Prognostic Determinant in Differentiated Thyroid Cancer. *J Clin Endocrinol Metab* (1996) 81:4318–23. doi: 10.1210/jcem.81.12.8954034
7. Grani G, Ramundo V, Verrienti A, Sponziello M, Durante C. Thyroid Hormone Therapy in Differentiated Thyroid Cancer. *Endocrine* (2019) 66:43–50. doi: 10.1007/s12020-019-02051-3
8. Berger MJ, Doherty TJ. Sarcopenia: Prevalence, Mechanisms, and Functional Consequences. *Interdiscip Top Gerontol* (2010) 37:94–114. doi: 10.1159/000319997
9. Xu LB, Zhang HH, Shi MM, Huang ZX, Zhang WT, Chen XD, et al. Metabolic Syndrome-Related Sarcopenia Is Associated With Worse Prognosis in Patients With Gastric Cancer: A Prospective Study. *Eur J Surg Oncol* (2020) 46:2262–9. doi: 10.1016/j.ejso.2020.07.032
10. Lee SJ, Kim NC. Association Between Sarcopenia and Metabolic Syndrome in Cancer Survivors. *Cancer Nurs* (2017) 40:479–87. doi: 10.1097/NCC.0000000000000454
11. Nishiyama A, Staub Y, Suga Y, Fujita M, Tanimoto A, Ohtsubo K, et al. Sarcopenia may Influence the Prognosis in Advanced Thyroid Cancer Patients Treated With Molecular Targeted Therapy. *In Vivo* (2021) 35:401–10. doi: 10.21873/invivo.12271
12. Jodar E, Munoz-Torres M, Escobar-Jimenez F, Quesada-Charneco M, Lund del Castillo JD. Bone Loss in Hyperthyroid Patients and in Former Hyperthyroid Patients Controlled on Medical Therapy: Influence of Aetiology and Menopause. *Clin Endocrinol* (1997) 47:279–85. doi: 10.1046/j.1365-2265.1997.2261041.x
13. Tauchmanova L, Nuzzo V, Del Puente A, Fonderico F, Esposito-Del Puente A, Padulla S, et al. Reduced Bone Mass Detected by Bone Quantitative Ultrasonometry and DEXA in Pre- and Postmenopausal Women With Endogenous Subclinical Hyperthyroidism. *Maturitas* (2004) 48:299–306. doi: 10.1016/j.maturitas.2004.02.017
14. Ohn JH, Han SK, Park DJ, Park KS, Park YJ. Expression of Thyroid Stimulating Hormone Receptor mRNA in Mouse C2C12 Skeletal Muscle Cells. *Endocrinol Metab* (2013) 28:119–24. doi: 10.3803/EnM.2013.28.2.119
15. Moon MK, Kang GH, Kim HH, Han SK, Koo YD, Cho SW, et al. Thyroid Stimulating Hormone Improves Insulin Sensitivity in Skeletal Muscle Cells *via* cAMP/PKA/CREB Pathway-Dependent Upregulation of Insulin Receptor Substrate-1 Expression. *Mol Cell Endocrinol* (2016) 436:50–8. doi: 10.1016/j.mce.2016.07.018
16. Abe E, Marians RC, Yu W, Wu XB, Ando T, Li Y, et al. TSH Is a Negative Regulator of Skeletal Remodeling. *Cell* (2003) 115:151–62. doi: 10.1016/S0092-8674(03)00771-2
17. Oh JH, Song S, Rhee H, Lee SH, Kim DY, Choe JC, et al. Normal Reference Plots for the Bioelectrical Impedance Vector in Healthy Korean Adults. *J Korean Med Sci* (2019) 34:e198. doi: 10.3346/jkms.2019.34.e198
18. Jang IY, Jung HW, Lee CK, Yu SS, Lee YS, Lee E. Comparisons of Predictive Values of Sarcopenia With Different Muscle Mass Indices in Korean Rural Older Adults: A Longitudinal Analysis of the Aging Study of PyeongChang Rural Area. *Clin Interv Aging* (2018) 13:91–9. doi: 10.2147/CIA.S155619
19. Roberts HC, Denison HJ, Martin HJ, Patel HP, Syddall H, Cooper C, et al. A Review of the Measurement of Grip Strength in Clinical and Epidemiological Studies: Towards a Standardised Approach. *Age Ageing* (2011) 40:423–9. doi: 10.1093/ageing/afr051
20. Peel NM, Kuys SS, Klein K. Gait Speed as a Measure in Geriatric Assessment in Clinical Settings: A Systematic Review. *J Gerontol A Biol Sci Med Sci* (2013) 68:39–46. doi: 10.1093/gerona/gls174
21. Jung HW, Roh H, Cho Y, Jeong J, Shin YS, Lim JY, et al. Validation of a Multi-Sensor-Based Kiosk for Short Physical Performance Battery. *J Am Geriar Soc* (2019) 67:2605–9. doi: 10.1111/jgs.16135
22. Chen LK, Woo J, Assantachai P, Auyeung TW, Chou MY, Iijima K, et al. Asian Working Group for Sarcopenia: 2019 Consensus Update on Sarcopenia Diagnosis and Treatment. *J Am Med Dir Assoc* (2020) 21:300–7. doi: 10.1016/j.jamda.2019.12.012
23. Wang LY, Smith AW, Palmer FL, Tuttle RM, Mahrous A, Nixon IJ, et al. Thyrotropin Suppression Increases the Risk of Osteoporosis Without Decreasing Recurrence in ATA Low- and Intermediate-Risk Patients With Differentiated Thyroid Carcinoma. *Thyroid* (2015) 25:300–7. doi: 10.1089/thy.2014.0287
24. Flynn RW, Bonellie SR, Jung RT, MacDonald TM, Morris AD, Leese GP. Serum Thyroid-Stimulating Hormone Concentration and Morbidity From Cardiovascular Disease and Fractures in Patients on Long-Term Thyroxine Therapy. *J Clin Endocrinol Metab* (2010) 95:186–93. doi: 10.1210/jc.2009-1625
25. Cappola AR, Fried LP, Arnold AM, Danese MD, Kuller LH, Burke GL, et al. Thyroid Status, Cardiovascular Risk, and Mortality in Older Adults. *JAMA* (2006) 295:1033–41. doi: 10.1001/jama.295.9.1033
26. Heemstra KA, Hamdy NA, Romijn JA, Smit JW. The Effects of Thyrotropin-Suppressive Therapy on Bone Metabolism in Patients With Well-Differentiated Thyroid Carcinoma. *Thyroid* (2006) 16:583–91. doi: 10.1089/thy.2006.16.583
27. Kim JH, Hwang Bo Y, Hong ES, Ohn JH, Kim CH, Kim HW, et al. Investigation of Sarcopenia and Its Association With Cardiometabolic Risk Factors in Elderly Subjects. *J Korean Geriatr Soc* (2010) 14:121–30. doi: 10.4235/jkgs.2010.14.3.121
28. Andersen S, Bruun NH, Pedersen KM, Laurberg P. Biologic Variation is Important for Interpretation of Thyroid Function Tests. *Thyroid* (2003) 13(11):1069–78. doi: 10.1089/105072503770867237
29. Stockigt JR. Free Thyroid Hormone Measurement. *A Crit Appraisal Endocrinol Metab Clin North Am* (2001) 30(2):265–89. doi: 10.1016/S0889-8529(05)70187-0
30. Chakravarthy V, Ejaz S. Thyroxine-Binding Globulin Deficiency. In: *StatPearls*. Treasure Island (FL: StatPearls Publishing (2021).
31. Khoo S, Lyons G, McGowan A, Gurnell M, Oddy S, Visser WE, et al. Familial Dysalbuminaemic Hyperthyroxinaemia Interferes With Current Free Thyroid Hormone Immunoassay Methods. *Eur J Endocrinol* (2020) 182(6):533–8. doi: 10.1530/EJE-19-1021
32. Bohannon RW. Muscle Strength: Clinical and Prognostic Value of Hand-Grip Dynamometry. *Curr Opin Clin Nutr Metab Care* (2015) 18(5):465–70. doi: 10.1097/MCO.0000000000000202
33. Chen LK, Liu LK, Woo J, Assantachai P, Auyeung TW, Bahyah KS, et al. Sarcopenia in Asia: Consensus Report of the Asian Working Group for Sarcopenia. *J Am Med Dir Assoc* (2014) 15(2):95–101. doi: 10.1016/j.jamda.2013.11.025
34. Leong DP, Teo KK, Rangarajan S, Lopez-Jaramillo P, Avezum A Jr, Orlandini A, et al. Prospective Urban Rural Epidemiology (PURE) Study Investigators. Prognostic Value of Grip Strength: Findings From the Prospective Urban Rural Epidemiology (PURE) Study. *Lancet* (2015) 386(9990):266–73. doi: 10.1016/S0140-6736(14)62000-6
35. Gale CR, Martyn CN, Cooper C, Sayer AA. Grip Strength, Body Composition, and Mortality. *Int J Epidemiol* (2006) 36(1):228–35. doi: 10.1093/ije/dyl224

36. Bohannon RW. Hand-Grip Dynamometry Predicts Future Outcomes in Aging Adults. *J Geriatr Phys Ther* (2008) 31(1):3–10. doi: 10.1519/00139143-200831010-00002
37. Cooper R, Kuh D, Hardy R. Mortality Review GroupFALCon and HALCyon Study Teams. Objectively Measured Physical Capability Levels and Mortality: Systematic Review and Meta-Analysis. *BMJ* (2010) 341:c4467. doi: 10.1136/bmj.c4467
38. Peterson MD, Zhang P, Choksi P, Markides KS, Al Snih S. Muscle Weakness Thresholds for Prediction of Diabetes in Adults. *Sports Med* (2016) 46(5):619–28. doi: 10.1007/s40279-015-0463-z
39. Waschki B, Kirsten A, Holz O, Muller KC, Meyer T, Watz H, et al. Physical Activity is the Strongest Predictor of All Cause Mortality in Patients With COPD: A Prospective Cohort Study. *Chest* (2011) 140(2):331–42. doi: 10.1378/chest.10-2521
40. Syddall H, Cooper C, Martin F, Briggs R, Aihie Sayer A. Is Grip Strength a Useful Single Marker of Frailty? *Age Ageing* (2003) 32(6):650–6. doi: 10.1093/ageing/afg111
41. Sayer AA, Kirkwood TB. Grip Strength and Mortality: A Biomarker of Ageing? *Lancet* (2015) 386(9990):226–7. doi: 10.1016/S0140-6736(14)62349-7
42. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, et al. Sarcopenia: European Consensus on Definition and Diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age Ageing* (2010) 39(4):412–23. doi: 10.1093/ageing/afq034
43. Lauretani F, Russo CR, Bandinelli S, Bartali B, Cavazzini C, Di Iorio A, et al. Ageassociated Changes in Skeletal Muscles and Their Effect on Mobility:an Operational Diagnosis of Sarcopenia. *J Appl Physiol (1985)* (2003) 95(5):1851–60. doi: 10.1152/japplphysiol.00246.2003
44. Goodpaster BH, Park SW, Harris TB, Kritchevsky SB, Nevitt M, Schwartz AV, et al. The Loss of Skeletal Muscle Strength, Mass, and Quality in Older Adults: The Health, Aging and Body Composition Study. *J Gerontol A Biol Sci Med Sci* (2006) 61(10):1059–64. doi: 10.1093/gerona/61.10.1059
45. Janssen I, Baumgartner RN, Ross R, Rosenberg IH, Roubenoff R. Skeletal Muscle Cutpoints Associated With Elevated Physical Disability Risk in Oldermen and Women. *Am J Epidemiol* (2004) 159(4):413–21. doi: 10.1093/aje/kwh058

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Hormonal Regulation of the MHC Class I Gene in Thyroid Cells: Role of the Promoter "Tissue-Specific" Region

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In previous studies we have demonstrated that the expression of the Major Histocompatibility Complex (MHC) class I gene in thyrocytes is controlled by several hormones, growth factors, and drugs. These substances mainly act on two regions of the MHC class I promoter a "tissue-specific" region (–800 to –676 bp) and a "hormone/cytokines-sensitive" region (–500 to –68 bp). In a previous study, we have shown that the role of the "tissue-specific" region in the MHC class I gene expression is dominant compared to that of the "hormone/cytokines-sensitive" region. In the present report we further investigate the dominant role of the "tissue-specific" region evaluating the effect of thyroid stimulating hormone (TSH), methimazole (MMI), phenylmethimazole (C10), glucose and thymosin-α1. By performing experiments of electrophoretic mobility shift assays (EMSAs) we show that TSH, MMI and C10, which inhibit MHC class I expression, act on the "tissue-specific" region increasing the formation of a silencer complex. Glucose and thymosin-α1, which stimulate MHC class I expression, act decreasing the formation of this complex. We further show that the silencer complex is formed by two distinct members of the transcription factors families activator protein-1 (AP-1) and nuclear factor-kB (NF-kB), c-jun and p65, respectively. These observations are important in order to understand the regulation of MHC class I gene expression in thyroid cells and its involvement in the development of thyroid autoimmunity.

Keywords: major histocompatibility complex class I (MHC-1), NF-kB, AP-1, thyroid, c-jun, p65, hormonal regulation

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INTRODUCTION

Several studies have demonstrated that Major histocompatibility complex (MHC) class I as well as class II overexpression on non-immune cells are important in the development of autoimmune diseases and thyroid autoimmunity (1–9). Thus, there is an increased expression of MHC class I and II molecules in pancreatic β islet cells of patients with type 1 diabetes mellitus, in muscle biopsies of patients with inflammatory myopathies, and in thyrocytes from patients with autoimmune thyroid

diseases (3, 5, 7). MHC class I overexpression is an early feature of the experimental-induced thyroiditis and this suggests its pathogenetic role in the autoimmune process (10, 11). Furthermore, MHC class I expression is necessary for the induction of systemic lupus erythematosus (SLE) and type 1 diabetes mellitus in experimental models of these diseases (1, 2). Indeed, down-regulation or absence of MHC class I expression is considered the hallmark of tissue immune privilege (4, 12). Regarding the thyroid, previous studies have demonstrated that hormones and growth factors that regulate thyroid cell growth and function, such as thyroid stimulating hormone (TSH), hydrocortisone, insulin and insulin-like growth factor-I (IGF-I) decrease MHC class I expression in a coordinate and specific way (7, 13, 14). Of note is that MHC class I expression is also decreased by iodide, phorbol esters, transforming growth factor (TGF)-β1, and methimazole (MMI), whereas it is increased by α-and γ-interferons (IFNs), thymosin-α1, and high levels of glucose (15–21). The specific regulation of MHC class I gene by these substances is particularly intriguing since the level of expression of MHC class I molecules differs between tissues, with the highest expression in lymphoid tissues and lower expression in some tissues as endocrine glands, skeletal muscle, myocardium, or gastric mucosa (22, 23). We believe that the levels of MHC class I molecules are lower in tissues that have potential autoantigens. Regarding the thyroid, we have hypothesized that the decrease of MHC class I molecules on the surface of thyrocytes by several hormones and factors, may be an important mechanism to preserve thyroid self-tolerance and prevent autoimmune thyroid disease. Indeed, the same hormones and factors increase the transcription of thyroid specific genes that can act as potential autoantigens (7, 16, 17). These data assume a particular importance considering the possibility that innate immune activation can lead to an autoimmune response. Pivotal studies have demonstrated that thyroid cells have functional pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and retinoic acid-inducible gene (RIG)-like receptors, that respond to various pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (8, 24). Therefore, it has been hypothesized that the presence of PAMPs or DAMPs into thyroid cells can trigger an innate immune response, and eventually make thyrocytes to behave as antigen-presenting cells and to initiate an autoimmune response (25, 26). Experiments conducted in FRTL-5 cells have shown that the release of genomic ds DNA by injury activate several genes involved in the immune response including the MHC class I gene (26). We think that this mechanism is also true for other tissues expressing normally low levels of MHC class I molecules. Indeed, in patients with type 1 diabetes MHC class I overexpression is associated with pancreatic islets infiltration by cytotoxic T lymphocytes specific for autoantigens (27). Moreover, some further studies suggest that MHC class I overexpression is the trigger of immune-mediated myopathies (28, 29).

Given these data, we believe it is noteworthy that we know the regulation of MHC class I expression in thyroid cells to better understand the pathogenesis of autoimmune thyroid diseases and to detect how hormones and drugs can modulate the gene expression.

Previous studies have defined the 5' flanking region of the PD1 gene a swine classical MHC Class I gene whose properties are maintained when it is transfected into cells from different species (13–15, 17–20, 24, 30–32). It has got two main regions that control the expression of MHC class I gene in a particular cell (**Figure 1**). The "tissue-specific" region, with overlapping enhancer and silencer elements, is -800 to -676 bp from the start of transcription; it sets the constitutive level of Class I expression in each tissue. The "hormone/cytokines-sensitive" region," -500 to -68 bp, is responsible for the regulation of class I expression within each tissue and is modulated by the hormones, growth factors, drugs and cytokines mentioned above (6, 16). **Table 1** shows the effects of different hormones, growth factors, cytokines and drugs on the MHC class I promoter activity in the thyroid cell line FRTL-5, as described in previous studies (13–15, 17–20, 30). For all these compounds,

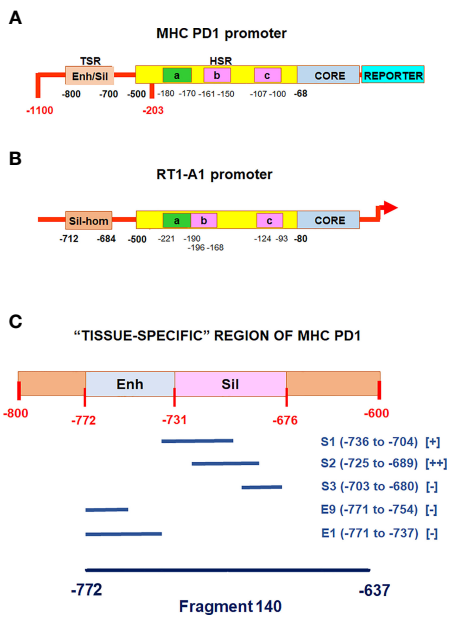

FIGURE 1 | Diagrammatic representation of the MHC class I gene promoters. **(A)**, diagrammatic representation of the pig MHC class I PD1 promoter (6, 16). TSR: "tissue-Specific" Region including an overlapping enhancer and silencer (enh/sil) elements; HSR: "hormones/cytokines-sensitive" region including: the enhancer A (a), the interferon response element (b), the CRE-like element (c), the core promoter containing CAAT and TATAA-like elements (CORE); REPORTER: indicate the reporter gene used in previous studies for functional analysis by CAT or luciferase assays. **(B)**, diagrammatic representation of the rat MHC class I RT1-A1 gene promoter (Ensembl gene accession number: ENSRNOG00000038999). Sil-hom: a region homologous to the PD1 silencer of the "tissue-Specific" region; a: enhancer A, b: an overlapping interferon response element, c: enhancer B containing a CRE-like element, CORE: a region containing CAAT and TATAA elements; the arrow indicates the start of transcription. **(C)**, detailed diagram of the "tissue -specific" region of the MHC class I PD1 promoter, the relative positions of the oligonucleotides used as competitors in are indicated, as well as that of the 140 fragment used as labeled probe. [+] and [-] indicate presence or absence of competition, respectively.

TABLE 1 | Effects of the indicated compounds on the MHC class I promoter activity.

Compound	p (-1100)	p (-203)	Reference
TSH	Decrease	Decrease	(13)
Hydrocortisone	Decrease	Decrease	(14)
Insulin/IGF-1	Decrease	Increase	(30)
α-and γ-Interferons	Increase	Increase	(14, 18)
Iodide	Decrease	Decrease	(15)
TGF-β1	Decrease	Decrease	(19)
Thymosin-α1	Increase	Increase	(20)
Glucose	Increase	Increase	(18)
Methimazole	Decrease	Decrease	(17)
Phenylmethimazole	Decrease	Decrease	(17)

p(-1100) and p(-203) indicate a reporter vector containing respectively the full length (-1100 bp) or the deleted mutant (-203 bp) of the MHC class I promoter PD1. Data are from previous studies (13–15, 17–20, 30).

except insulin and IGF-1, there is a similar effect both in the full length promoter -1100 bp from the start of transcription and in the deleted mutant -203 bp from the start of transcription, which lacks the "tissue-specific" region but retains the "hormone/cytokines-sensitive" region (see **Figure 1A**). Experiments performed with reporter vectors containing mutations of one of the responsive elements of the "hormone/cytokines-sensitive" region [the enhancer A, the Interferon response element or the cyclic-AMP response (CRE)-like element], showed a loss of the effect of the related compounds and allowed the identification of this region (13–15, 17–20, 30). Furthermore, the disparate response of insulin and IGF-1 between the full length promoter and the deleted mutant p(-203) bp, have shown a dominant role of the upstream "tissue-specific" region in regulating the MHC class I gene transcription (30). The functional relationship between these two regulatory regions has been confirmed by the observation that insulin and IGF-1 lose their inhibitory effect on the activity of a full length MHC class I promoter that lacks the enhancer A element. Furthermore, electrophoretic mobility shift assays (EMSAs) have shown that both the "tissue-specific" region and the "hormone/cytokines-sensitive" region (particularly the enhancer A and the interferon response elements) interact with different members of the same family of transcription factors, nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) (13–20, 30). In detail, the silencer element of the "tissue-specific" region interacts with the p65 subunit of NF-kB and c-jun, whereas the enhancer A element binds a protein complex named Mod-1 consisting of the p50 subunit of NF-kB and fra-2.

As shown in **Table 1**, the MHC class I gene expression is decreased by TSH, MMI and C10, whereas it is increased by glucose and thymosin-α1. These compounds act on the "hormone/cytokines-sensitive" region of the MHC class I promoter. In more detail, TSH, MMI and C10 induce the formation of specific protein/DNA complexes with a silencer sequence between -127 and -90 bp containing a CRE-like site (13, 17), whereas glucose and thymosin-α1 induce the formation of a protein/DNA complex, named Mod-1, with the Enhancer A sequence between -180 and -170 bp (18, 20). Since we have previously observed that the upstream "tissue-specific" region has got a dominant role in the regulation of MHC class I gene expression by insulin and IGF-1 (30), we have planned to evaluate the effects of TSH, MMI, C10, glucose and thymosin-α1 on this region.

In the present manuscript we illustrate that TSH, MMI, C10, glucose and thymosin-α1 act on the "tissue-specific" region of the MHC class I promoter modifying the formation of the silencer complex made up of two distinct members of the transcription factors families AP-1 and NF-kB, c-jun and p65 respectively. The effects of the aforementioned compounds on the silencer complex formation is consistent with those produced on the MHC class I gene expression. This observation further suggests that in the FRTL-5 cells the "tissue-specific" region acts as a dominant regulatory element of MHC class I promoter.

MATERIALS AND METHODS

Materials

C10 was a gift from Intherthyr Research Corporation (Marietta, OH, USA) (17). Thymosin-α1 was kindly provided by SciClone Pharmaceuticals Inc (Foster City, CA, USA). [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]-dCTP (3000 Ci/mmol) were from Perkin Elmer Italia (Monza, Italy). Heat-treated, mycoplasma-free calf serum was from Life Technologies Europe (Monza, Italy). For EMSAs we used antibodies against the p65 (sc-8008) and p50 (sc-8414) subunits of NF-kB, c-jun (sc74543), and normal mouse IgG (sc-2025) as negative control (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The oligonucleotides S1, S2, S3, E1 and E9 were kindly provided by Dr. Dinah S. Singer (National Cancer Institute, NIH, Bethesda, MD, USA). Chromatin immunoprecipitation was carried out by using the following antibodies: NF-kB p65 (L8F6) mouse mAb, NF-kB p105/50 (D4P4D) rabbit mAb, c-Jun (60A8) rabbit mAb and normal rabbit or mouse IgG as negative control (Cell Signaling Technology Inc., Danvers, MA, USA). The source of all other materials was the Sigma Aldrich Co. (St. Louis, MO, USA), unless otherwise specified.

Cell Culture

The F1 subclone of FRTL-5 rat thyroid cells (American Type Culture Collection, CRL-8305) was a gift from the Interthyr Research Foundation (Marietta, OH, USA). Cells were grown in 6H5% medium consisting of Coon's modified Ham's F-12

medium supplemented with 5% calf serum, 2 mM glutamine, 1 mM nonessential amino acids, and a six-hormone (6H) mixture: 1 mU/mL bovine TSH, 10 µg/mL insulin, 0.4 ng/mL cortisol, 5 mg/mL transferrin, 10 ng/mL glycyl-L-histidyl-L-lysine acetate, and 10 ng/mL somatostatin. These cells were diploid, between the 5th and 25th passage, and had all of the functional properties described previously (13–15, 17–21, 24, 30, 33–38). Fresh 6H5% medium was added to the cells every 2 to 3 days, and they were passaged every 7 days. In individual experiments, cells were grown to 60% confluence in 6H5% medium and then shifted to the appropriate treatment condition as previously reported (17, 18, 30). For the insulin treatment, cells were shifted to a three-hormone (3H) medium (i.e., with no TSH, insulin, or hydrocortisone) with only 0.2% calf serum (3H0.2%) ± insulin 10 µg/ml for 7 days. For the treatment with MMI, C10 and TSH, cells were shifted to a five-hormone (5H) medium (i.e., without TSH) with 5% calf serum (5H5% medium) for 7 days to become quiescent, and then treated with MMI 5 mM, C10 0.5 mM, or TSH 0.1 nM for 36 hours. In the experiments performed with thymosin-α1 and glucose, cells were shifted to 5H5% medium for 7 days to become quiescent, then they were cultured in 6H5% medium ± thymosin-α1 1 µg/mL for 12 hours, or 6H0.2% medium ± glucose 30 mM for 48 hours. The latter low serum medium was chosen to avoid a potential effect by variable concentrations of the glucose contained in the calf serum.

Cell Extracts

Cellular extracts were prepared by a modification of described methods (39). In brief, cells were washed twice in cold PBS, pH 7.4, scraped, and centrifuged (500xg). The cell pellet was resuspended in 2 volumes of Dignam buffer C [25% glycerol, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES-KOH), pH 7.9, 1.5 mM MgCl2, 0.42 M NaCl, 0.5 mM dithiothreitol, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 0.5 mM phenylmethylsulfonyl fluoride]. The final NaCl concentration was adjusted based on cell pellet volume to 0.42 M. Cells were lysed by repeated cycles of freezing and thawing. The extracts were centrifuged (100,000xg) at 4°C for 20 minutes. The supernatant was recovered, aliquoted and stored at –70°C.

EMSAs

The generation and preparation of the -140 bp probe and of the S1, S2, S3, E1 and E9 oligonucleotides were previously described (30, 31). Their sequences are reported in **Table 2**. The -140 bp probe was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA), then purified on an 8% native polyacrylamide gel. EMSAs were performed as previously described (14, 30). Binding reactions in low salts and no detergent included 1.5 fmol [^{32}P]DNA, 3 µg cell extract and 3 µg poly(dI-dC) in 10 mM Tris-Cl, pH 7.9, 1 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM ethylendiaminotetraacetic acid (EDTA), and 5% glycerol in a 20 µl total volume. Incubations were performed at room temperature for 30 min. Where indicated unlabeled oligonucleotides or antibodies were added to the binding reaction and incubated with the extracts for 20 minutes prior the addition of labeled DNA. Following incubations, reaction mixtures were electrophoresed on 4.5% native polyacrylamide gels at 160 V in 0.5x TBE at room temperature. Gels were dried and autoradiographed. Binding activity was quantified by optical densitometry using a STORM 860 Imager (Molecular Dynamics, GE Healthcare Italy, Milan, Italy) and data are shown in **Table 3**.

TABLE 2 | Sequences (5'-3') of the MHC class I PD1 promoter 140 fragment and of oligonucleotides S1, S2, E1 and E9 used as competitors in the EMSAs.

140 fragment (-772 -637)
GGTCCACATTCAAAATAACCTTTGAGAAATTACCATAATGATAGCATCCAAAATT
ATCTGAAAAGGTTATTAAAAATACATGTCCTACATGTGTGCGGGGCTTTTACATT
TCATAGATGTCAGCCACCAAAAGGAG
S1 oligonucleotide (-740 -700)
C*GCG*AATGATAGCATCCAAAATTATCTGAAAAGGTTA*GCGC*
S2 oligonucleotide (-727 -687)
*GG*CCAAAATTATCTGAAAAGGTTATTAAAAATACATGTC*GG*
E1 oligonucleotide (-772 -733)
GGTCCACATTCAAAATAACCTTTGAGAAATTACCAT*CGC*G
E9 oligonucleotide (-772 -746)
GGTCCACATTCAAAATAAC*AGGAGCGC*

In blue the region -772 -732 spanning the enhancer element, in red the region -731 -676 spanning the silencer element. In italic and underlined are mutated nucleotides. See also ref. 31.

RNA Isolation and Northern Analysis

RNA was prepared using a RNeasy Mini kits (Qiagen Inc., Valencia, CA, USA). Twenty µg of the different RNA samples were run on denaturing agarose gels, capillary blotted on Nytran membranes (Schleicher & Schuell-Whatman, Florrham Park, NJ, USA), UV cross-linked, and hybridized by using QuickHyb Hybridization Solution (Stratagene, La Jolla, CA, USA), following the manufacturer protocol. The probes were labeled with [α-32P]-dCTP using Ladderman Labeling kits (Takara Mirus Bio, Madison, WI, USA). The MHC class I probe was a 1.0 kb HpaI fragment of the MHC class-I pH 7 clone spanning the entire cDNA insert (40). The β-actin probe was as described previously (40). Quantitation was performed using the STORM 860 Imager (Molecular Dynamics).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed using SimpleChIP enzymatic chromatin IP kit (Cell Signaling Technology Inc.) following the manufacturer

TABLE 3 | Densitometric analysis of the EMSAs shown in **Figure 2A** and **Figures 3A, B, C**.

Treatment	Band intensity (arbitrary unit)
Control 3H0.2%	100
+ Insulin	289 ± 7.2*
Control 5H5%	100
+ MMI	267 ± 8.4*
+ C10	269 ± 6.6*
+ TSH	233 ± 9.1*
Control 6H5%	100
+ Thymosin-α1	58 ± 5.0*
Control 6H0.2%	100
+ Glucose	44 ± 6.9*

*The intensity of each relative control is set to 100. Data are means ± S.D from three independent experiments. *p < 0.05 compared to relevant control.*

instructions. In brief, FRTL-5 cells cultured in 3H0.2% ± insulin 10 μg/ml for 7 days, as described above, were crosslinked for 10 min at room temperature by 1% formaldehyde. Nuclear DNA was digested by Micrococcal nuclease and nuclei were lysed by ultrasonic homogenizer. The complexes were purified by ChIP grade protein G agarose and the crosslinks were reversed incubating the samples with 200 mM NaCl and 120 μg of Proteinase K for 2 hours at 65°C. Chromatin immunoprecipitation was performed with the antibodies indicated above, DNA was purified and analyzed using real-time quantitative PCR (qPCR).

qPCR Analysis

qPCR was performed using SimpleChIP Universal qPCR master mix (Cell Signaling Technology Inc.) and a primer pair (forward: 5'-TCAAGGCCAGCTTGGTCTAC-3'; reverse: 5'-CAGCAGCCCAGCAGCCTC-3') flanking a region of the rat MHC class I gene RT1.A1 (Ensembl gene accession number: ENSRNOG00000038999) homologous to the silencer element of the "tissue-specific" region of the PD1 promoter used in the EMSAs. Each sample was run, in triplicate, in a QuantStudio 7 PRO Real-Time PCR System (Applied Biosystem, ThermoFisher Scientific, Waltham, MA, USA) and the immunoprecipitation efficiency was calculated using the percent input method.

Other Assays

Protein concentrations were determined using BCA protein assay kits (Pierce Biotechnology Inc., Rockford, IL, USA), with crystalline BSA as standard.

Statistical Analysis

All experiments were repeated at least three times with independent batches of cells. The quantitative data obtained by optical densitometry were evaluated as means ± S.D. The significance between experimental values was determined by unpaired two-tailed t-test or two-way ANOVA, with $p < 0.05$ or better when the data from all of the experiments were considered.

RESULTS

EMSAs Indicate That the Functional Effects of TSH, Glucose, Thymosin α-1, MMI and C10 on MHC Class I Gene Expression Correlate With Changes in Binding of DNA/Protein Complexes to the "Tissue-Specific" Region of the PD1 Promoter

We have performed EMSA experiments using a radiolabeled probe with a sequence encompassing the MHC class I promoter between -772 to -637 bp, termed 140 fragment because of its total length (including nucleotides from restriction sites on either end). This fragment encloses the overlapping enhancer and silencer elements of the "tissue-specific" region previously described (31, 32). As previously showed (30), the addition of insulin 10 μg/ml to the FRTL-5 cells maintained in a 3H medium culture (i.e., no TSH, insulin, or hydrocortisone and containing only 0.2% calf serum) increased the formation of a slowing complex (**Figure 2A** lane 3, arrow; **Table 3**) similar to a previous identified silencer of the promoter transcription (31, 32). This treatment decreased the MHC class I RNA level (**Figure 2B**) as already observed in previous studies (41).

The identification of this complex with the silencer element previously described (30, 31) was confirmed by competition experiments. Indeed, the preincubation of the cellular extracts with unlabeled double-strand oligonucleotides, spanning the functional silencer element previously identified and named S1 and S2 (30, 31) (**Figure 1C**) inhibited its formation (**Figure 2C** lanes 2 and 3 *vs.* 1, respectively). This inhibition was specific since two oligonucleotides, corresponding to the enhancer portion of the "tissue-specific" regions (**Figure 1C**), named E1 and E9 (31) did not decrease the complex (**Figure 2C** lanes 4 and 5 *vs.* 1, respectively). It must be emphasized that high levels of this silencer complex are associated with low levels of MHC class I expression (6, 30–32). EMSAs experiments performed by using extracts from cells treated with TSH, MMI, C10, glucose and thymosin-α1, confirmed the consistency between the silencer formation and the expression of the MHC class I gene (**Figure 3**). Indeed, cellular extracts from cells cultured in 5H medium (i.e. without TSH) and 5% calf serum treated with compounds that cause a decrease of MHC class I expression, as MMI 5 mM, or C10 0.5 mM, or TSH 0.1 nM, showed an increase of the silencer complex (**Figure 3A** lanes 2, 3, 4 *vs.* 1, respectively; **Table 3**). Conversely, cellular extracts from cells treated with compounds that cause an increase of MHC class I expression, as thymosin-α1 1 μg/mL or glucose 30 mM, showed a decrease of the silencer complex (**Figure 3B** lane 2 *vs.* 1 and **Figure 3C** lane 2 *vs.* 1, respectively; **Table 3**). The choice of treatment conditions is based on our previous experiments (17, 18, 20) and are detailed in the *Materials and Methods* section. **Figure 3D** shows the effect of these treatments on the MHC class I RNA level, which confirm our previous studies

Cell extracts from FRTL-5 cells maintained in 6H5% medium were then preincubated with antibodies against the p65 subunit of NF-kB and c-jun. This experiment has shown a decrease of the silencer complex (**Figure 4**, lanes 2 and 3 respectively), whereas no effect was seen using antibodies against the p50 subunit of NF-kB (**Figure 4**, lane 4 *vs.* 1). This decrease indicates an involvement of c-jun and p65 in the formation of the silencer complex as previously observed using extracts from FRTL-5 cells treated with IGF-1 or insulin (30).

ChIP Assay Confirms the Binding of c-jun and the p65 Subunit of NF-kB to the "Tissue-Specific" Region of the Rat MHC Class I Promoter

EMSAs, although very useful for analyzing the interaction between transcription factors and specific DNA sequences, it has the limit of carrying out the reaction in a test tube. Therefore, we have evaluated *in vivo* using ChIP assay the interaction between c-jun and the p65 subunit of NF-kB with a region of the promoter of the rat MHC class I (RT1-A1 gene) homologous to the silencer element of the "tissue-specific" region of the MHC

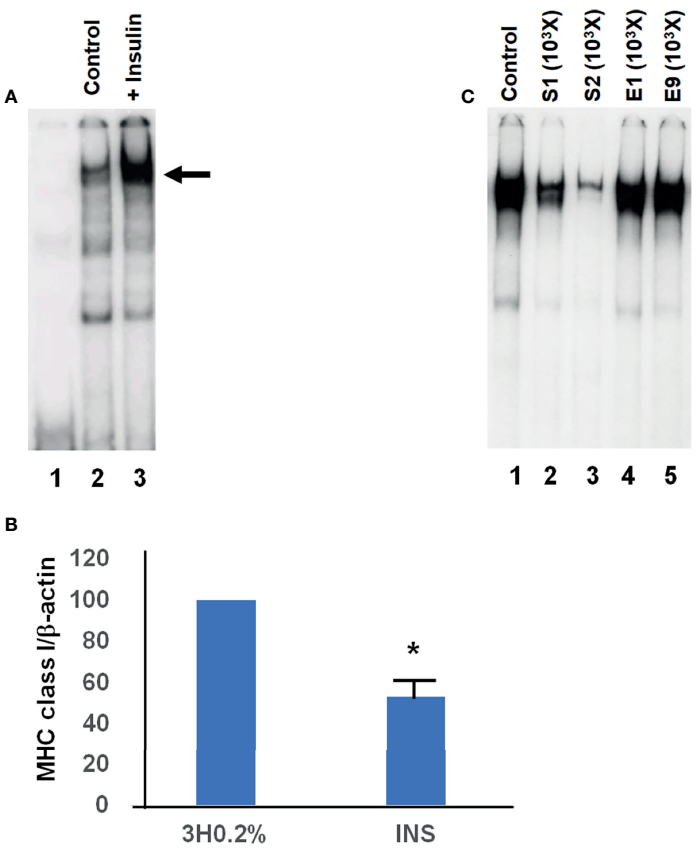

FIGURE 2 | Representative EMSAs showing the effect of insulin on the formation of the silencer element. **(A)**, EMSAs were performed, as detailed in Material and Methods, using cellular extracts from FRTL-5 and the –140 bp probe that spans the region between –772 to –637 bp from the transcription start site (this fragment encloses the overlapping enhancer and silencer elements of the "tissue-specific" region). Lane 1, radioactive probe alone; lane 2 is the incubation of radioactive probe with extracts from control cells (3H 0.2% medium); lane 3 is the incubation of radioactive probe with extracts from cells in 3H 0.2% medium + insulin 10 µg/ml. The arrow indicates the protein-DNA complex identified as the silencer element. **(B)**, effect of insulin treatment on MHC class I RNA levels. FRTL-5 cells were grown and treated as in the EMSAs shown in (A). Data are means MHC class-I/β-actin ± S.D. expressed as percentages of control from three separate experiments, *$p < 0.05$ compared to control. INS, cells in 3H 0.2% medium + insulin 10 µg/ml. **(C)**, EMSAs were performed, as detailed in Material and Methods, using cellular extracts from FRTL-5 and the –140 bp probe that spans the region between –772 to –637 bp from the transcription start site. Lane 1 is the incubation of radioactive probe with extracts from cells maintained in 3H 0.2% medium + insulin 10 µg/ml; lanes 2 to 5 show the effect of the unlabeled oligonucleotides S1, S2, E1 and E9 (1000 fold excess) on the complex formation. Data are from a representative experiment repeated three times with similar results.

class I PD1 promoter. The cross-linked chromatin complex was obtained from FRTL-5 cells treated with 3H 0.2% ± insulin 10 µg/ml for 7 days and immunoprecipitation was performed using antibodies against the rat c-jun and the rat p65 subunit of NF-kB. The purified DNA was then analyzed by qPCR using primers flanking the rat homologous silencer element. As shown in **Figure 5** the experiment confirmed the binding of the transcription factors c-jun and the p65 subunit of NF-kB with this region and the treatment with insulin increased the protein-DNA interaction as observed with EMSAs. No effect was seen in using antibodies against the rat p50 subunit of NF-kB.

DISCUSSION

Several studies performed in the past have demonstrated the effects of various hormones, growth factors, cytokines and drugs on MHC class I gene expression. They have also shown that these compounds act on a region between -500 to -68 bp, therefore called "hormone/cytokines-sensitive" region. This region has got several enhancer and silencer elements as illustrated in **Figure 1**. In more details, those studies have shown that some compounds such as TSH, MMI and C10 act inducing the formation of two protein–DNA complexes on a region that includes the constitutive 38 bp silencer of the MHC class-I promoter, which contains a CRE-like sequence (11, 15). Other compounds as hydrocortisone, insulin/IGF-1, TGF-β1, thym and glucose act regulating the binding of the Mod-1 complex with the enhancer A element (12, 16–18, 22). In most cases, an increase in Mod-1 binding stimulates the gene transcription, while a decrease inhibits it. However, we have previously observed (22) that although insulin or IGF-1 makes Mod-1 binding increase it leads the transcription of the MHC class I gene to be reduced. This discrepancy was explained by the observation that insulin

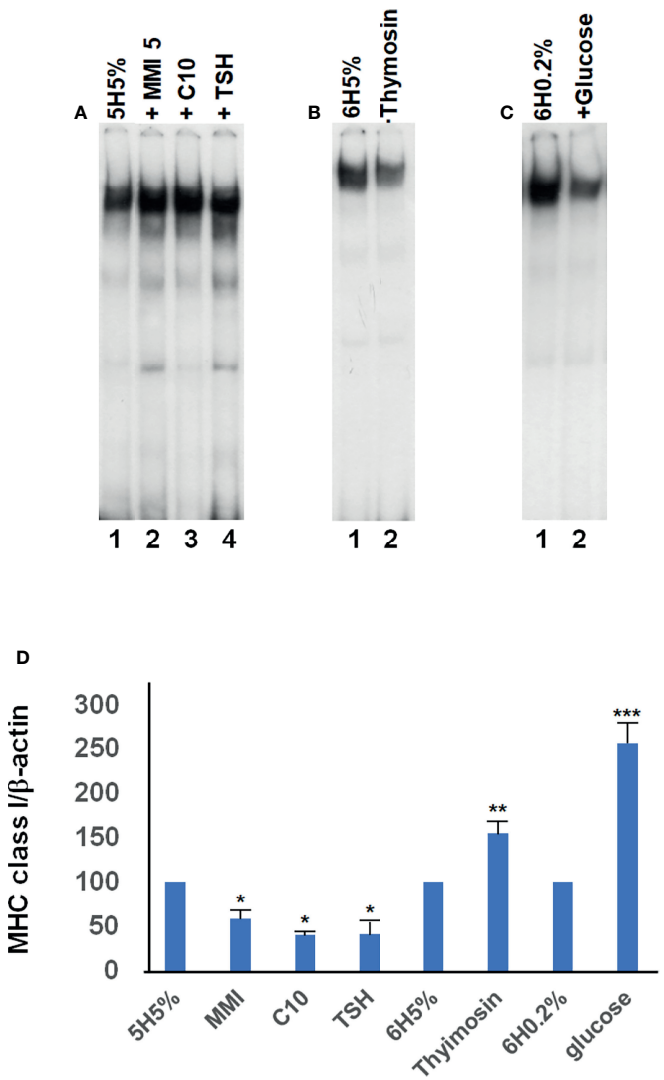

FIGURE 3 | Representative EMSAs showing the effect of different compounds on the formation of the silencer element. EMSAs were performed, as detailed in Material and Methods, using cellular extracts from FRTL-5 and the –140 bp probe that spans the region between –772 to –637 bp from the transcription start site (this fragment encloses the overlapping enhancer and silencer elements of the "tissue-specific" region). **(A)**, Lane 1 is the incubation of the radioactive probe with extracts from control cells (5H 5% medium, i.e. without TSH); lanes 2 to 4 show the effect of MMI 5 mM, C10 0.5 mM, and TSH 0.1 nM, respectively, on the complex formation after 36 hours of treatment. **(B)**, lane 1 is the incubation of radioactive probe with extracts from control cells (6H 5% medium); lanes 2 show the effect of thymosin-α1 1 μg/mL on the complex formation after 12 hours of treatment. **(C)**, lane 1 is the incubation of radioactive probe with extracts from control cells (6H 0.2% medium); lanes 2 show the effect of glucose 30 mM on the complex formation after 48 hours of treatment. Data are from a representative experiment repeated three times with similar results. Differences are statistically significant, $p < 0.05$. **(D)**, effect of the treatments performed in EMSAs on the MHC class I RNA levels. FRTL-5 cells were grown and prepared as detailed in Materials and Methods. Data are means MHC class-I/β-actin ± S.D. expressed as percentages of control from three separate experiments, *$p < 0.05$ compared to the relative control 5H5%; **$p < 0.05$ compared to the relative control 6H5%; ***$p < 0.05$ compared to the relative control 6H0.2%.

and IGF-1 also increased the binding of a protein complex to a silencer element located upstream on the promoter in the "tissue-specific" region. From these results, which have highlighted a dominant role of this region in the MHC class I gene regulation, we were prompted to evaluate the effects of other factors on it.

As a first attempt we have chosen to evaluate the effect of either compounds that decrease the MHC class I gene expression, such as TSH, MMI and C10, or compounds that increase it, such as thymosin-α1 and glucose. TSH was chosen since it is the main regulator of thyroid growth and function. MMI is a drug widely used to treat hyperthyroidism that, beside an antithyroid effect, also has anti-inflammatory and immunosuppressive effects (42–44). C10, a phenyl derivative of MMI (17), is a more potent anti-inflammatory agent both *in vitro* and *in vivo* (17, 45). Thymosin-α1 is a drug used to stimulate the immune response (46) and one of its mechanisms of action is the increase of MHC class I expression (20, 47). The increased expression of MHC class I molecules on thyroid cells

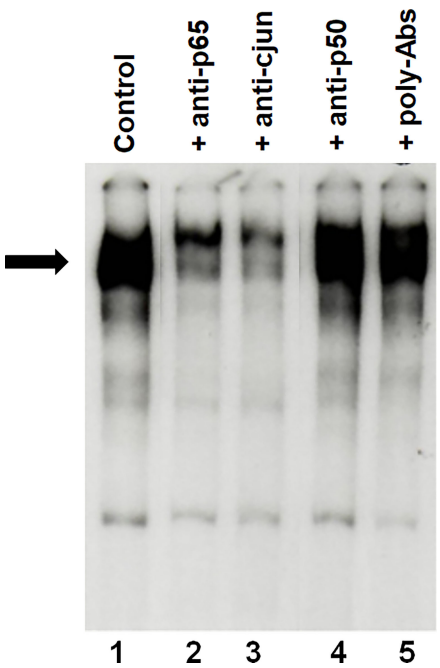

FIGURE 4 | Representative EMSAs showing the ability of antisera specific for the p65 subunit of NF-kB or c-jun to inhibit the formation of the silencer complex. EMSAs were performed, as detailed in Material and Methods, using cellular extracts from FRTL-5 and the –140 bp probe that spans the region between –772 to –637 bp from the transcription start site. Lane 1 is the incubation of the radioactive probe with extracts from control cells (6H 5%); lanes 2 to 6 are the incubation of the radioactive probe with extracts from control cells after preincubation with antibodies directed against the p65 subunits of NF-kB, c-jun and the p50 subunit of NF-kB respectively; lane 5 refers to preincubation with normal polyclonal mouse IgGs as negative control. Data are from a representative experiment repeated three times with similar results. Differences are statistically significant, $p < 0.05$.

induced by the high glucose levels can be involved in the higher incidence of thyroid autoimmunity associated with hyperglycemia (48, 49). Furthermore, an increased expression of MHC class I molecules in endothelial cells has been associated with coronary artery disease (50) and this can be related with the vascular complications induced by hyperglycemia.

In the present study we have demonstrated that in addition to insulin and IGF-1, even TSH, MMI, C10, thymosin-α1 and glucose regulate the silencer complex in the "tissue-specific" region and their effects on this site are in tune with the effects they have on the downstream "hormone/cytokines-sensitive" region. These data further suggest that in FRTL-5 cells the "tissue-specific" region acts as a dominant regulatory element. They also show that for most of the compounds tested the two regulatory regions of the MHC class I promoter act in concert with each other and the only exception observed so far is for insulin and IGF-1. The functional relationship between the "tissue-specific" region and the "hormone/cytokines-sensitive" region has been observed in a previous study where we evaluated the effect of insulin and IGF-1 on a reporter vector containing the full length of the MHC class I promoter with a deletion of the enhancer A element (30). The deletion of the enhancer A site resulted in the loss of the insulin/IGF-1 effect on the promoter activity. A weakness of the present work is the lack of information on the effects of other compounds involved in the autoimmune process. Further studies need to be performed in order to find whether other compounds, particularly cytokines, have a disparate response between these two regulatory regions. Moreover, further studies should evaluate the regulation of MHC class I by hormones, growth factors and cytokines in other tissues beside thyroid.

In the present study, we have also noticed that in FRTL-5 cells the transcription factors involved in the upstream silencer complex formation are the p65 subunit of NF-kB and the AP-1 family member c-jun. It must be underlined that the two primary regions that regulate MHC class I transcription in thyroid cells, that are the "tissue-specific" region and the "hormone/cytokines-sensitive" region, interact with different members of the same family of transcription factors. Indeed, the upstream silencer interact with the p65 subunit of NF-kB and c-jun, whereas the downstream enhancer A element binds either the complex Mod-1 (formed by the heterodimer p50/fra-2) or the classic NF-kB dimer p50/p65, which has an opposite effect compared to Mod-1 (14, 15). Thus, it is possible that a regulatory effect in one subunit can affect the other and alter the way in which these factors regulate the promoter. Further studies are needed to deepen our knowledge on the functional interactions between these transcription factors and the promoter regions, as well as to evaluate if other transcription factors are involved.

Of particular interest is also the observation that MHC class I expression in thyroid cells is controlled by several hormones and growth factors. This hormonal regulation is considered important for the suppression of autoimmunity during hormonally induced changes in thyroid cell function, which results in an enhanced expression of potential thyroid autoantigens, such as thyroglobulin, thyroid peroxidase, and the TSH receptor (7, 8). Indeed, several observations suggest that the overexpression of HLA molecules on nonimmune cells has an important role in the pathogenesis of autoimmune diseases. It has been hypothesized that several insults to nonimmune cells of target tissues (such as viral infections, dsRNA, ds DNA or tissue injury) can activate the innate immune response through PRRs such as TLRs and RIG-like receptors, and induce the secretion of α-, β- or γ-IFN. This would result in an overexpression of MHC molecules and cytokines by the target cells, which would then recruit and activate lymphocytes and hence initiate an autoimmune response (7, 8, 24–26, 51). Therefore, we think it is very important to know of the mechanisms that regulate the MHC class I gene as well as the compounds that can modify them. Indeed, it is of remarkable importance to underline is the selective regulation of the distinct members of NF-kB and AP-1 by several hormones and growth factors. The hormonal regulation of the NF-kB dimers in thyroid cells is not restricted to the MHC gene. Studies performed in the FRTL-5 cells have shown that TSH can modify the composition of the NF-kB dimers activated by TNF-α. In the absence of TSH,

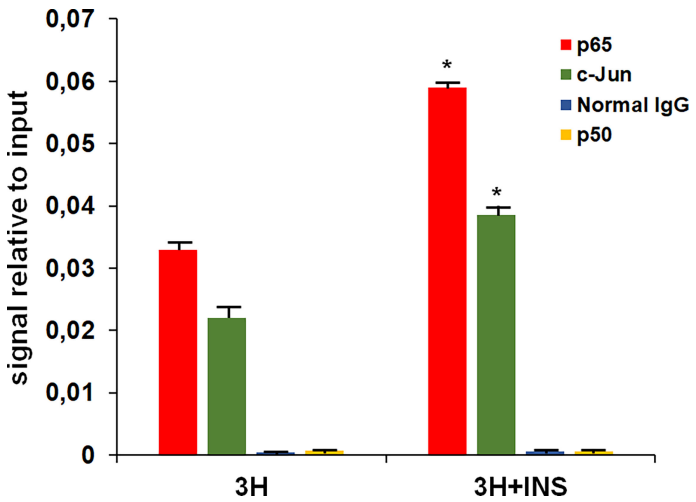

FIGURE 5 | qPCR analysis of purified DNA from ChIP performed using antibodies against the p65 subunit of NF-kB and c-jun. 3H, control cells (3H 0.2% medium); 3H + INS (3H 0.2% medium + insulin 10 μg/ml). Normal rabbit or mouse IgG were used as negative control in the ChIP assay. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin. Data are means ± SD for three independent experiments. *$p < 0.05$ *versus* relevant control.

TNF-α treatment activates only the p50 homodimers, whereas in the presence of TSH there is also the activation of the p50/p65 heterodimers, which results in the modification of the IL-6 gene expression (52).

The data presented herein suggest that the "tissue-specific" region represents the primary regulator of the MHC class I gene transcription in thyroid cells. They further suggest that some drugs such as MMI, its derivative C10 and thymosin-α1 regulate the binding of c-jun and the p65 subunit of NF-kB to this region. This can have an impact on the therapy of autoimmune and inflammatory diseases as well as on the treatment of cancer. Indeed, we speculate that the effectiveness of MMI in the treatment of autoimmune thyroid diseases is not only due to its antithyroid effect but also to its ability to suppress the inflammatory and immune processes. For this reason, we and other groups have studied MMI derivatives characterized by a high anti-inflammatory and immunosuppressive potency and a low or no effect on thyroid function (17, 45, 53, 54). C10 is one of these derivatives and several studies suggest its potential use in severe inflammatory diseases (45). On the other hand, the information obtained on the mechanism of action of thymosin-α1 are important for its use in cancer immunotherapy and to stimulate the research of new drugs with the same mechanism of action, considering that the loss of MHC class I expression is a feature of several tumors including papillary thyroid cancer (55–59).

The present study, as well as most of the studies discussed here, has been performed in the FRTL-5 cells. They are a non-transformed rat thyroid cell line in continuous culture that represents a well-defined and reproducible in-vitro model of thyroid function (13–15, 17–21, 24, 30, 33–38). The reliability of the FRTL-5 cells as a model to study MHC gene regulation has been validated by studies conducted in animal models and in human tissues (1, 7, 8, 54, 60, 61).

In summary, our data show that the "tissue-specific" region of the MHC class I promoter is the target of several hormones and growth factors that regulate the gene expression. Future research directions should be performed to deepen the knowledge about MHC class I promoter activity in thyroid and in other tissues, either nonimmune and immune and to understand its role in autoimmune diseases and cancer.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

CG and GN designed and drafted the manuscript. The experimental procedures and data analysis were performed by CG, SV, FV, IB, AG, and GN. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Mozes E, Kohn LD, Hakim F, Singer DS. Resistance of MHC Class I-Deficient Mice to Experimental Systemic Lupus Erythematosus. *Science* (1993) 261:91–3. doi: 10.1126/science.8316860
2. Serreze DV, Chapman HD, Varnum DS, Gerling I, Leiter EH, Shultz LD. Initiation of Autoimmune Diabetes in NOD/Lt Mice Is MHC Class I-Dependent. *J Immunol* (1997) 158:3978–86.
3. Richardson SJ, Rodriguez-Calvo T, Gerling IC, Mathews CE, Kaddis JS, Russell MA, et al. Islet Cell Hyperexpression of HLA Class I Antigens: A Defining Feature in Type 1 Diabetes. *Diabetologia* (2016) 59:2448–58. doi: 10.1007/s00125-016-4067-4
4. Ito T, Meyer KC, Ito N, Paus R. Immune Privilege and the Skin. *Curr Dir Autoimmun* (2008) 10:27–52. doi: 10.1159/000131412
5. Gono T, Katsumata Y, Kawaguchi Y, Soejima M, Wakasugi D, Miyawaki M, et al. Selective Expression of MHC Class I in the Affected Muscle of a Patient With Idiopathic Inflammatory Myopathy. *Clin Rheumatol* (2009) 28:873–6. doi: 10.1007/s10067-009-1172-5
6. René C, Lozano C, Eliaou J-F. Expression of Classical HLA Class I Molecules: Regulation and Clinical Impacts. *HLA* (2016) 87:338–49. doi: 10.1111/tan.12787
7. Kohn LD, Napolitano G, Singer DS, Molteni M, Scorza R, Shimojo N, et al. Graves' Disease: A Host Defense Mechanism Gone Awry. *Int Rev Immunol* (2000) 19:633–64. doi: 10.3109/08830180009088516
8. Luo Y, Yoshihara A, Oda K, Ishido Y, Suzuki K. Excessive Cytosolic DNA Fragments as a Potential Trigger of Graves' Disease: An Encrypted Message Sent by Animal Models. *Front Endocrinol* (2016) 7:144. doi: 10.3389/fendo.2016.00144
9. Gianfran C, Pisapia L, Picascia S, Strazzullo M, Del Pozzo G. Expression Level of Risk Genes of MHC Class II Is a Susceptibility Factor for Autoimmunity: New Insights. *J Autoimmun* (2018) 89:1–10. doi: 10.1016/j.jaut.2017.12.016
10. Verma S, Hutchings P, Guo J, McLachlan S, Rapoport B, Cooke A. Role of MHC Class I Expression and CD8(+) T Cells in the Evolution of Iodine-Induced Thyroiditis in NOD-H2(h4) and NOD Mice. *Eur J Immunol* (2000) 30:1191–202. doi: 10.1002/(SICI)1521-4141(200004)30:4<1191::AID-IMMU1191>3.0.CO;2-L
11. Weider T, Richardson SJ, Morgan NG, Paulsen TH, Dahl-Jørgensen K, Hammerstad SS. Upregulation of HLA Class I and Antiviral Tissue Responses in Hashimoto's Thyroiditis. *Thyroid* (2020) 30:432–42. doi: 10.1089/thy.2019.0607
12. Gilhar A, Laufer-Britva R, Keren A, Paus R. Frontiers in Alopecia Areata Pathobiology Research. *J Allergy Clin Immunol* (2019) 144:1478–89. doi: 10.1016/j.jaci.2019.08.035
13. Saji M, Shong M, Napolitano G, Palmer LA, Taniguchi SI, Ohmori M, et al. Regulation of Major Histocompatibility Complex Class-I Gene Expression in Thyroid Cells: Role of the cAMP Response Element-Like Sequence. *J Biol Chem* (1997) 272:20096–107. doi: 10.1074/jbc.272.32.20096
14. Giuliani C, Saji M, Napolitano G, Palmer LA, Taniguchi SI, Shong M, et al. Hormonal Modulation of Major Histocompatibility Complex Class I Gene Expression Involves an Enhancer A-Binding Complex Consisting of Fra-2 and the P50 Subunit of NF-Kappa B. *J Biol Chem* (1995) 270:11453–62. doi: 10.1074/jbc.270.19.11453
15. Taniguchi S-I, Shong M, Giuliani C, Napolitano G, Saji M, Montani V, et al. Iodide Suppression of Major Histocompatibility Class I Gene Expression in Thyroid Cells Involves Enhancer A and the Transcription Factor NF-Kb. *Mol Endocrinol* (1998) 12:19–33. doi: 10.1210/mend.12.1.0052
16. Giuliani C, Bucci I, Napolitano G. The Role of the Transcription Factor Nuclear Factor-Kappa B in Thyroid Autoimmunity and Cancer. *Front Endocrinol* (2018) 9:471. doi: 10.3389/fendo.2018.00471
17. Giuliani C, Bucci I, Montani V, Singer DS, Monaco F, Kohn LD, et al. Regulation of Major Histocompatibility Complex Gene Expression in Thyroid Epithelial Cells by Methimazole and Phenylmethimazole. *J Endocrinol* (2010) 204:57–66. doi: 10.1677/JOE-09-0172
18. Napolitano G, Bucci I, Giuliani C, Massafra C, Di Petta C, Devangelio E, et al. High Glucose Levels Increase Major Histocompatibility Complex Class I Gene Expression in Thyroid Cells and Amplify Interferon-γ Action. *Endocrinology* (2002) 143:1008–17. doi: 10.1210/endo.143.3.8674
19. Napolitano G, Montani V, Giuliani C, Di Vincenzo S, Bucci I, Todisco V, et al. Transforming Growth Factor-β1 Down-Regulation of Major Histocompatibility Complex Class I in Thyrocytes: Coordinate Regulation of Two Separate Elements by Thyroid-Specific as Well as Ubiquitous Transcription Factors. *Mol Endocrinol* (2000) 14:486–505. doi: 10.1210/mend.14.4.0454
20. Giuliani C, Napolitano G, Mastino AS, Di Vincenzo S, D'Agostini C, Grelli S, et al. Thymosin-α1 Regulates MHC Class-I Expression in FRTL-5 Cells at a Transcriptional Level. *Eur J Immunol* (2000) 30:778–86. doi: 10.1002/1521-4141(200003)30:3<778::AID-IMMU778>3.0.CO,2-I
21. Grassadonia A, Tinari N, Fiorentino B, Suzuki K, Nakazato M, De Tursi M, et al. The 90K Protein Increases Major Histocompatibility Complex Class I Expression and Is Regulated by Hormones, Gamma-Interferon, and Double-Strand Polynucleotides. *Endocrinology* (2004) 145:4728–36. doi: 10.1210/en.2004-0506
22. Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The Detailed Distribution of HLA-A, B, C Antigens in Normal Human Organs. *Transplantation* (1984) 38:287–92. doi: 10.1097/00007890-198409000-00018
23. Kotekar AS, Weissman JD, Gegonne A, Cohen H, Singer DS. Histone Modifications, But Not Nucleosomal Positioning, Correlate With Major Histocompatibility Complex Class I Promoter Activity in Different Tissues In Vivo. *Mol Cell Biol* (2008) 28:7323–36. doi: 10.1128/MCB.00889-08
24. Harii N, Lewis CJ, Vasko V, McCall K, Benavides-Peralta U, Sun X, et al. Thyrocytes Express a Functional Toll-Like Receptor 3: Overexpression can be Induced by Viral Infection and Reversed by Phenylmethimazole and Is Associated With Hashimoto's Autoimmune Thyroiditis. *Mol Endocrinol* (2005) 19:1231–50. doi: 10.1210/me.2004-0100
25. Kawashima A, Yamazaki K, Hara T, Akama T, Yoshihara A, Sue M, et al. Demonstration of Innate Immune Responses in the Thyroid Gland: Potential to Sense Danger and a Possible Trigger for Autoimmune Reactions. *Thyroid* (2013) 23:477–87. doi: 10.1089/thy.2011.0480
26. Kawashima A, Tanigawa K, Akama T, Wu H, Sue M, Yoshihara A, et al. Fragments of Genomic DNA Released by Injured Cells Activate Innate Immunity and Suppress Endocrine Function in the Thyroid. *Endocrinology* (2011) 152:1702–12. doi: 10.1210/en.2010-1132
27. Newby BN, Mathews CE. Type I Interferon Is a Catastrophic Feature of the Diabetic Islet Microenvironment. *Front Endocrinol* (2017) 8:232. doi: 10.3389/fendo.2017.00232
28. Luo YB, Mastaglia FL. Dermatomyositis, Polymyositis and Immune-Mediated Necrotising Myopathies. *Biochim Biophys Acta* (2015) 1852:622–32. doi: 10.1016/j.bbadis.2014.05.034
29. Bhattarai S, Ghannam K, Krause S, Benveniste O, Marg A, de Bruin G, et al. The Immunoproteasomes Are Key to Regulate Myokines and MHC Class I Expression in Idiopathic Inflammatory Myopathies. *J Autoimmun* (2016) 75:118–29. doi: 10.1016/j.jaut.2016.08.004
30. Giuliani C, Saji M, Bucci I, Fiore G, Liberatore M, Singer DS, et al. Transcriptional Regulation of Major Histocompatibility Complex Class I Gene by Insulin and IGF-I in FRTL-5 Thyroid Cells. *J Endocrinol* (2006) 189:605–15. doi: 10.1677/joe.1.06486
31. Weissman JD, Singer DS. A Complex Regulatory DNA Element Associated With a Major Histocompatibility Complex Class I Gene Consists of Both a Silencer and an Enhancer. *Mol Cell Biol* (1991) 11:4217–27. doi: 10.1128/mcb.11.8.4217
32. Murphy C, Nikodem D, Howcroft K, Weissman JD, Singer DS. Active Repression of Major Histocompatibility Complex Class I Genes in Human Neuroblastoma Cell Line. *J Biol Chem* (1996) 271:30992–9. doi: 10.1074/jbc.271.48.30992
33. Törnquist K, Sukumaran P, Kemppainen K, Löf C, Viitanen T. Canonical Transient Receptor Potential Channel 2 (TRPC2): Old Name-New Games. Importance in Regulating of Rat Thyroid Cell Physiology. *Pflugers Arch* (2014) 466:2025–34. doi: 10.1007/s00424-014-1509-z
34. Wen G, Ringseis R, Eder K. Endoplasmic Reticulum Stress Inhibits Expression of Genes Involved in Thyroid Hormone Synthesis and Their Key Transcriptional Regulators in FRTL-5 Thyrocytes. *PloS One* (2017) 12(11): e0187561. doi: 10.1371/journal.pone.0187561
35. Ambesi Impiombato FSinventor; Interthyr Research Foundation Inc, assignee. United States Patent US 4608341: USA patent (1986).
36. Lin R, Hogen V, Cannon S, Marion KM, Fenton MS, Hershman JM. Stability of Recombinant Human Thyrotropin Potency Based on Bioassay in FRTL-5 Cells. *Thyroid* (2010) 20:1139–43. doi: 10.1089/thy.2009.0408

37. Giuliani C, Bucci I, Di Santo S, Rossi C, Grassadonia A, Mariotti M, et al. Resveratrol Inhibits Sodium/Iodide Symporter Gene Expression and Function in Rat Thyroid Cells. *PloS One* (2014) 9(9):e107936. doi: 10.1371/journal.pone.0107936
38. Giuliani C, Iezzi M, Ciolli L, Hysi A, Bucci I, Di Santo S, et al. Resveratrol has Anti-Thyroid Effects Both *In Vitro* and In Vivo. *Food Chem Toxicol* (2017) 107:237–47. doi: 10.1016/j.fct.2017.06.044
39. Dignam J, Lebovitz R, Roeder R. Accurate Transcription Initiation by RNA Polymerase II in a Soluble Extract From Isolated Mammalian Nuclei. *Nucleic Acids Res* (1983) 11:1475–89. doi: 10.1093/nar/11.5.1475
40. Saji M, Moriarty J, Ban T, Kohn LD, Singer DS. Hormonal Regulation of Major Histocompatibility Complex Class I Genes in Rat Thyroid FRTL-5 Cells: Thyroid-Stimulating Hormone Induces a cAMP-Mediated Decrease in Class I Expression. *Proc Natl Acad Sci USA*. (1992) 89:1944–19488. doi: 10.1073/pnas.89.5.1944
41. Saji M, Moriarty J, Ban T, Singer DS, Kohn LD. Major Histocompatibility Complex Class I Gene Expression in Rat Thyroid Cells Is Regulated by Hormones, Methimazole, and Iodide as Well as Interferon. *J Clin Endocrinol Metab* (1992) 75:871–8. doi: 10.1210/jcem.75.3.1381373
42. Mozes E, Zinger H, Kohn LD, Singer DS. Spontaneous Autoimmune Disease in (NZB X NZW) F1 Mice is Ameliorated by Treatment With Methimazole. *J Clin Immunol* (1998) 18:106–13. doi: 10.1023/a:1023242732212
43. Wang P, Sun SH, Silver PB, Chan CC, Agarwal RK, Wiggert B, et al. Methimazole Protects From Experimental Autoimmune Uveitis (EAU) by Inhibiting Antigen Presenting Cell Function and Reducing Antigen Priming. *J Leukoc Biol* (2003) 73:57–64. doi: 10.1189/jlb.0102047
44. Starosz A, Stożek K, Moniuszko M, Grubczak K, Bossowski A. Evaluating the Role of Circulating Dendritic Cells in Methimazole-Treated Pediatric Graves' Disease Patients. *Genes* (2021) 12:164. doi: 10.3390/genes12020164
45. Giuliani C, Bucci I, Napolitano G. Phenylmethimazole Is a Candidate Drug for the Treatment of Severe Forms of Coronavirus Disease 2019 (COVID-19) as Well as Other Virus-Induced "Cytokines Storm". *Med Hypotheses* (2021) 146:110473. doi: 10.1016/j.mehy.2020.110473
46. Dominari A, Hathaway Iii D, Pandav K, Matos W, Biswas S, Reddy G, et al. Thymosin Alpha 1: A Comprehensive Review of the Literature. *World J Virol* (2020) 9:67–78. doi: 10.5501/wjv.v9.i5.67
47. Garaci E, Favalli C, Pica F, Sinibaldi Vallebona P, Palamara AT, Matteucci C, et al. Thymosin Alpha 1: From Bench to Bedside. *Ann N Y Acad Sci* (2007) 1112:225–34. doi: 10.1196/annals.1415.044
48. Vitacolonna E, Lapolla A, Di Nenno B, Passante A, Bucci I, Giuliani C, et al. Gestational Diabetes and Thyroid Autoimmunity. *Int J Endocrinol* (2012) 2012:867415. doi: 10.1155/2012/867415
49. Sarfo-Kantanka O, Sarfo FS, Ansah EO, Yorke E, Akpalu J, Nkum BC, et al. Frequency and Determinants of Thyroid Autoimmunity in Ghanaian Type 2 Diabetes Patients: A Case-Control Study. *BMC Endocr Disord* (2017) 17(1):2. doi: 10.1186/s12902-016-0152-4
50. Foglieni C, Maisano F, Dreas L, Giazzon A, Ruotolo G, Ferrero E, et al. Mild Inflammatory Activation of Mammary Arteries in Patients With Acute Coronary Syndromes. *Am J Physiol Heart Circ Physiol* (2008) 294:H2831–7. doi: 10.1152/ajpheart.91428.2007
51. Pisetsky DS. The Role of Innate Immunity in the Induction of Autoimmunity. *Autoimmun Rev* (2008) 8:69–72. doi: 10.1016/j.autrev.2008.07.028
52. Kikumori T, Kambe F, Nagaya T, Funahashi H, Seo H. Thyrotropin Modifies Activation of Nuclear Factor kB by Tumour Necrosis Factor α in Rat Thyroid Cell Line. *Biochem J* (2001) 354:573–9. doi: 10.1042/bj3540573
53. Alapati A, Deosarkar SP, Lanier OL, Qi C, Carlson GE, Burdick MM, et al. Simple Modifications to Methimazole That Enhance Its Inhibitory Effect on Tumor Necrosis Factor-α-Induced Vascular Cell Adhesion Molecule-1 Expression by Human Endothelial Cells. *Eur J Pharmacol* (2015) 751:59–66. doi: 10.1016/j.ejphar.2015.01.032
54. Noori MS, O'Brien JD, Champa ZJ, Deosarkar SP, Lanier OL, Qi C, et al. Phenylmethimazole and a Thiazole Derivative of Phenylmethimazole Inhibit IL-6 Expression by Triple Negative Breast Cancer Cells. *Eur J Pharmacol* (2017) 803:130–7. doi: 10.1016/j.ejphar.2017.03.049
55. Angell TE, Lechner MG, Jang JK, LoPresti JS, Epstein AL. MHC Class I Loss Is a Frequent Mechanism of Immune Escape in Papillary Thyroid Cancer That Is Reversed by Interferon and Selumetinib Treatment In Vitro. *Clin Cancer Res* (2014) 20:6034–44. doi: 10.1158/1078-0432.CCR-14-0879
56. Han LT, Hu JQ, Ma B, Wen D, Zhang T-T, Lu Z-W, et al. IL-17A Increases MHC Class I Expression and Promotes T Cell Activation in Papillary Thyroid Cancer Patients With Coexistent Hashimoto's Thyroiditis. *Diagn Pathol* (2019) 14:52. doi: 10.1186/s13000-019-0832-2
57. Lu ZW, Hu JQ, Liu WL, Wen D, Wei WJ, Wang YL, et al. IL-10 Restores MHC Class I Expression and Interferes With Immunity in Papillary Thyroid Cancer With Hashimoto Thyroiditis. *Endocrinology* (2020) 161(10):bqaa062. doi: 10.1210/endocr/bqaa062
58. Zitvogel L, Perreault C, Finn OJ, Kroemer G. Beneficial Autoimmunity Improves Cancer Prognosis. *Nat Rev Clin Oncol* (2021) 18:591–602. doi: 10.1038/s41571-021-00508-x
59. Jongsma MLM, Neefjes J, Spaapen RM. Playing Hide and Seek: Tumor Cells in Control of MHC Class I Antigen Presentation. *Mol Immunol* (2021) 136:36–44. doi: 10.1016/j.molimm.2021.05.009
60. Schuppert F, Taniguchi S, Schröder S, Dralle H, von zur Mühlen A, Kohn LD. *In Vivo* and *In Vitro* Evidence for Iodide Regulation of Major Histocompatibility Complex Class I and Class II Expression in Graves' Disease. *J Clin Endocrinol Metab* (1996) 81:3622–8. doi: 10.1210/jcem.81.10.8855812
61. Schuppert F, Ehrenthal D, Frilling A, Suzuki K, Napolitano G, Kohn LD. Increased Major Histocompatibility Complex (MHC) Expression in Nontoxic Goiters Is Associated With Iodide Depletion, Enhanced Ability of the Follicular Thyroglobulin to Increase MHC Gene Expression, and Thyroid Autoantibodies. *J Clin Endocrinol Metab* (2000) 85:858–67. doi: 10.1210/jcem.85.2.6394

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REVIEW
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Thyroid Autoimmunity in Female Infertility and Assisted Reproductive Technology Outcome

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The regulation of the female reproductive system is one of the most relevant actions of thyroid hormones. Adequate thyroid hormones production is essential for normal menstrual function and fertility as well as for the successful maintenance of pregnancy. The relationship between reproductive failure and thyroid disorders is particularly relevant and attracts attention worldwide. Thyroid autoimmunity (TAI), defined by the presence of circulating antithyroid antibodies targeting thyroid peroxidase (TPOAb) and thyroglobulin (TgAb), is prevalent among women of reproductive age and is the most frequent cause of thyroid dysfunction. Several studies addressed the association between TAI, thyroid function, and fertility as well as pregnancy outcome after spontaneous or assisted conception. Infertility, miscarriages, and fetal-maternal complications are described in overt autoimmune hypothyroidism. More debatable is the role of mild thyroid dysfunction, mainly subclinical hypothyroidism (SCH), and TAI in the absence of thyroid dysfunction in infertility and reproductive outcome. Assisted reproductive technology (ART) has become an integral element of care for infertility. Women with TAI undergoing ART are of particular interest since they carry a higher risk of developing hypothyroidism after the ovarian stimulation but whether TAI, in absence of thyroid dysfunction, adversely affects ART outcome is still controversial. Likewise, the role of levothyroxine (LT4) in improving fertility and the success of ART in euthyroid women with TAI is unclear. This review discusses the role of TAI, in the absence of thyroid dysfunction, in infertility and in ART outcome.

Keywords: thyroid autoimmunity, female infertility, assisted conception, assisted reproduction technology, pregnancy outcome, miscarriage, thyroid peroxidase antibodies, thyroglobulin antibodies

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INTRODUCTION

The definition of infertility is the failure to achieve clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (1). Female factors (ovulatory disorders, endometriosis, pelvic adhesions, tubal blockage/abnormalities, and hyperprolactinemia) account for 35%, male factors for 30%, and combined factors for 20%. In 15% of the cases the cause remains unknown (idiopathic or unexplained infertility). The prevalence of infertility varies worldwide and is estimated to affect between 8 and 12% of reproductive-aged couples (2). Multiple elements can

contribute to infertility affecting the female, the male, or both partners and many studies have been dedicated to identifying treatable risk factors that contribute to infertility. Among these risk factors are the presence of thyroid dysfunction and/or TAI. Therefore, screening for TSH and TPOAb is generally part of the initial work-up of infertile women. Indeed, thyroid hormones (TH) regulate the hypothalamic-pituitary-ovarian axis. A role of TH on luteinizing hormone (LH) secretion, on granulosa cell functions, LH/hCG receptor expression, and follicle development has been demonstrated (3). Therefore, ovulatory dysfunction and infertility are common in untreated thyroid dysfunction (4). Moreover, TH plays a critical role in implantation and early fetal development through actions on the placenta and endometrium (5). If adequate TH is necessary to avoid reducing the chances of conception and implantation, the same is true for the maintenance and outcome of pregnancy as well as for fetal brain development. In pregnancy significant adaptations of thyroid function are required, and the maternal thyroid must increase the hormone production by approximately 50% (6, 7). These pregnancy related adaptations cannot be accomplished in women with undiagnosed or uncontrolled hypothyroidism. The adaptation is even more difficult in assisted conception since the controlled ovarian stimulation protocols of ART anticipate the strain on thyroid function observed only after conception in spontaneous pregnancy (8). Thyroid disorders are more prevalent in females and are frequently encountered as a new diagnosis in women seeking pregnancy or during pregnancy. TAI is characterized by the presence of circulating antithyroid antibodies targeting thyroid peroxidase (TPOAb) and thyroglobulin (TgAb). Indeed, also circulating antibodies targeting TSH receptor, that are hallmarks of Graves' disease, have significant implications in reproductive age women and in pregnancy but their discussion is beyond the scope of this work and has been reviewed elsewhere (9). TAI is the leading cause of thyroid dysfunction and comprises a spectrum of conditions ranging from euthyroid thyroiditis to subclinical or overt hypothyroidism and, less frequently, to transient thyrotoxicosis. There is evidence that TAI-related thyroid dysfunction (mainly overt and subclinical hypothyroidism) adversely affects conception and pregnancy outcomes (10). Rather, it is unclear what impact TAI has with normal thyroid function in infertility, and in the outcome of pregnancy especially in women undergoing ART; clinical studies as well as meta-analyses report discrepant results (11). Despite a multitude of studies, a clear pathophysiological link connecting TAI and reproductive failure has not been identified. It is challenging to distinguish the effect of TAI per se from the changes it can induce in thyroid function, that is, TSH levels at the high end of the normal range or compatible with subclinical and overt hypothyroidism. When looking at studies addressing the role of TAI-related thyroid dysfunction in reproductive failure it must be remembered that the serum TSH level cut-off used to define normal thyroid function in pregnancy has been changed over time. The upper limit of serum TSH was set at 2.5 mIU/L according to the American Thyroid Association (ATA) guidelines published in 2011 (12). In the newest guidelines published in 2017, the need for trimester specific and local population TSH reference is strongly reaffirmed and, if this is not available, the upper limit of TSH in the first trimester is set at 4.0 mIU/L (13). Recently, guidelines specifically addressing thyroid dysfunction prior to or during ART have been published by the European Thyroid Association (ETA) (14). In searching a thyroid function independent pathogenic role of TAI in reproductive failure, several studies have been dedicated to potential immune-dependent direct effect on the ovary, on the endometrium, and on the feto-maternal unit. The findings are interesting but merely speculative and need confirmation (15). There remain unanswered questions on the relationship between TAI and reproductive outcome; several fields of research are open (11, 16). Increasing knowledge on the pathophysiological link between TAI and reproductive failure would improve the management and treatment of infertile women with TAI. The treatment of infertile euthyroid women with TAI remains the greatest areas of uncertainty, some studies demonstrate that LT4 reduces the risk of adverse pregnancy outcome while others do not (17). In this work we reviewed the epidemiological, clinical, and pathophysiological data addressing the relationship of TAI, without thyroid dysfunction, with infertility and ART outcome.

PREVALENCE OF THYROID AUTOIMMUNITY IN INFERTILE PATIENTS

The prevalence of TAI differs with races, age, iodine supply, and smoking, and is estimated as high as 5–16% in women aged 20–45 years in Europe (18, 19). Many studies have investigated the prevalence of TAI in infertile women. The studies are heterogeneous for design (frequently cross-sectional or retrospective), sample size, subjects (women with different causes of infertility or with a selected cause) and controls (unselected, age matched fertile, healthy non-pregnant) and for different methods of autoantibodies assay. In studies published until the early 2000s the prevalence of TAI in infertile women ranged from 6.8 to 14.5% but without significant differences compared to controls (20, 21). Only in two studies the prevalence was higher than the control population in women with anovulation (26%), in women with idiopathic infertility, and, mostly, with endometriosis (30%) compared to the unselected population (22, 23). However pooling the result of the studies the prevalence of TAI among women attending infertility clinics has been estimated higher compared to the general population with a relative risk of 2.1 (24). In more recent studies a prevalence of TAI in infertile women between 13% and 19% has been reported (25–27). In a large observational cohort study of 19,213 women with a history of miscarriage or infertility trying for a pregnancy, TPOAb was found in 9.5% of asymptomatic women (28). TAI was found in 5.9% of women at the first intra-uterine insemination and in 25% of women undergoing ART (17, 29). The secondary analysis of data from two multicenter, randomized, controlled trials reported that 8.6% infertile women had TPOAb (30). In one study not showing significant

difference of TAI prevalence between the whole infertile population and the controls, higher prevalence was observed in women with female causes and, the highest, in women with endometriosis (29%) (31). This association was not confirmed in a subsequent study showing TPOAb in 14.9% of women with endometriosis and in 22.2% of the control group (32). An increased prevalence of TAI has also been reported in women with polycystic ovary syndrome (PCOS). In a prospective study TPOAb/TgAb or hypoechoic pattern at thyroid ultrasound was significantly higher in PCOS women compared to controls (26.9 vs. 8.3% and 42.3% vs. 6.5% respectively). PCOS patients had a higher mean TSH level and a higher incidence of TSH levels above the upper limit of normal (22). Again these findings were not confirmed in a recent study on 210 women with PCOS and 343 age matched controls: no differences were found in the prevalence of TPOAb and/or hypohecoic pattern at thyroid ultrasound between patient and controls (4.8% and 7.6% and 9.3% and 12.3%, respectively) but subjects with TAI showed significantly higher adiposity and insulin resistance index than those without (33). Finally, in a meta-analysis pooling four studies, TAI was more prevalent in euthyroid women with idiopathic infertility with an odds ratio of 1.5 (34). It is worth remembering that in most of the cited studies TAI is defined based on the presence of TPOAb. In a prospective study both TPOAb and TgAb were present in 8% of the cases but isolated TgAb prevalence was close to that of isolated TPOAb (4% vs. 5%). Interestingly women with TgAb had significantly higher serum TSH levels compared with women without TAI. The study also showed a higher prevalence of TAI in infertile patients (35). In 436 women attending a fertility center, TPOAb and TgAb were detected in 10.6% and 9.2%, respectively, overlap was found in 4.6% (36). Therefore, up to 5% of TAI women can be missed if only TPOAb is measured. On the other hand, it is well known that circulating thyroid autoantibodies could wane in pregnancy or be absent, outside pregnancy, in the so-called "seronegative chronic autoimmune thyroiditis" (37). **Table 1** summarizes some of the studies on the prevalence of TAI in infertile women.

In conclusion, a higher prevalence of TAI cannot be demonstrated in all infertile women, but it is plausible in women with PCOS and with unexplained infertility. In the case of PCOS TAI might further affect fertility, being also associated to higher TSH levels and to a worse metabolic phenotype.

PATHOPHYSIOLOGY

A clear pathophysiological link connecting TAI to infertility and to pregnancy outcome after spontaneous conception or ART has not been identified. There are several hypotheses and potential points of action have been proposed. Thyroid hormone-dependent as well as thyroid hormone-independent immunological effect of TAI on the ovary, on the uterus, and on fetoplacental unit have all been implicated. Moreover, TAI could represent a peripheral marker of a general immune imbalance affecting fertilization, implantation, and pregnancy maintenance (15–17). The most relevant hypotheses are briefly illustrated.

Thyroid Antibodies Are Directly Pathogenic to the Reproductive Organs

In searching a potential target of TAI in infertility and ART outcome, antithyroid antibodies direct binding and damage to the reproductive organs has been investigated. Interestingly it has

TABLE 1 | Prevalence of thyroid autoimmunity in infertile women.

Author, year	Patients' characteristics	Hallmark of TAI	Percentage of TAI+		Conclusion
Karakan, 2013 (25)	253 women undergoing ART for male/female causes of infertility	TPOAb, TgAb	13.4%		No difference in causes of infertility between TAI+ and TAI-
Dhillon-Smith, 2020 (28)	19,350 women, miscarriage/infertility	TPOAb	9.5%		Higher prevalence of TAI in obese women
Hamad, 2021 (29)	584 women undergoing ART for female/male/ combined infertility	TPOAb, TgAb	25.3%		TAI more prevalent in women with combined infertility factors
			Percentage of TAI+		
			Patients	Controls	
Poppe, 2002 (31)	438 women with various causes of infertility, 100 age-matched healthy parous controls	TPOAb	14%	8%	Higher prevalence of TAI in women with female causes (the highest in endometriosis)
Petta, 2007 (32)	148 women with endometriosis 158 without	TPOAb, TgAb	14.9%	22.2%	No difference in TAI prevalence
Janssen, 2004 (22)	175 patients with PCOS, 168 age-matched without PCOS	TPOAb, TgAb Hypoechoic pattern at T-US	26%. 42.3%	8.3% 6.5%	Threefold higher prevalence of TAI in patients with PCOS
Kim, 2020 (33)	210 women with PCOS, 343 age-matched controls	TPOAb Hypoechoic pattern at T-US	4.8% 9.3%	7.6% 12.3%	No difference in TAI prevalence
Unuane, 2013 (35)	356 women, female infertility, 458 not consulting for infertility/male infertility	TPOAb, TgAb	19%	13%	Higher prevalence of TAI in infertile women

TAI, thyroid autoimmunity; ART, assisted reproductive technology; TPOAb, thyroperoxidase antibody; TgAb, thyroglobulin antibody; PCOS, polycystic ovary syndrome; T-US, thyroid ultrasound.

been demonstrated that antithyroid antibodies can pass through the blood–follicle barrier during the maturation period. In a study of 31 women undergoing IVF, TPOAb and TgAb were measurable in the follicular fluid, on the day of oocyte retrieval, in 14 patients with TAI and in none of the negative control. The follicular fluid concentrations of antithyroid antibodies were approximately half with respect to those in the serum thus indicating that they pass the blood-follicle barrier and reach concentration proportional to their blood levels. In this study oocyte fertilization, good quality embryos, and pregnancy rates were lower in women with TAI than in negative controls, while early miscarriage rate was higher. This "follicle hypothesis" suggests that presence of antithyroid antibodies may create a cytotoxic environment that damages the maturing oocyte reducing its quality and fertilization potential (38). A recent study confirmed the association of antithyroid antibodies in follicular fluid and ART outcome. In 52 women undergoing ART a statistically significant correlation was found between the levels of TPOAb and TgAb in serum and follicular fluid. Pregnancy rates per initiated cycle and per embryo transfer cycle were lower in TAI women thus suggesting a negative effect on the post-implantation embryo development (39). Although only in three patients, thyroid peroxidase has been demonstrated, by immunocytochemistry, in the granulosa cumulus cells of the human ovarian follicle, thereby supporting the hypothesis that TPOAb could target their antigen directly at the level of the ovary (40). In an autoimmune thyroiditis animal model antithyroid antibodies were evidenced on the surface of pre-implantation embryos (41). Also, it has been shown that human anti-zona pellucida antibodies recognize antigens within the murine thyroid tissue. It can be speculated that the zona pellucida, which has an important functional role in the interaction between the oocyte and the sperm cell and in pre-implantation, may be a target for antithyroid antibodies (42). Although the evidence is limited, a role of a hostile immune environment at the level of the ovary, with TPO as the direct antigen, has been proposed as one of the non-thyroid hormone dependent mechanisms at least in the early stage of autoimmune process in infertility (16). These antibodies may generate an inflammatory response that alters the milieu of the maturing oocyte affecting ovarian reserve and embryo quality.

Thyroid Autoantibodies Are an Epiphenomenon of a Generalized Immune Dysfunction

An alternative pathogenetic hypothesis considers that TAI might be a marker of both generalized humoral and cellular immune dysfunction. Polyclonal lymphocyte B activation is more frequent in TAI and is associated with the increased levels of non-organ-specific antibodies, such as antinuclear antibodies (ANA), anti-dsDNA, anti-ssDNA, and antiphospholipid antibodies (aPL). Indeed, most of these autoantibodies are associated with reproductive failure; aPL can cross-react with trophoblast–placental tissue thus reducing trophoblast viability, syncytialization and invasion (43). An increased rate of recurrent miscarriage is reported in patients with poly-autoimmune disorders as compared with patients affected by isolated TAI (44). An increased levels of antibodies against laminin-1 (LN-1), that have been associated with reproductive failure, have been demonstrated in serum and follicular fluid of infertile women with TAI. The serum levels of these antibodies were inversely correlated with oocyte count, along with a significantly reduced implantation rate and pregnancy rate (45). The concurrent presence of anti-ovarian autoantibodies, hallmarks of premature ovarian insufficiency (POF) obviously can affect the immune homeostasis of oocytes and impair ovarian reserve. POF can occur in isolation but is often associated with other autoimmune conditions. Hypothyroidism, TAI, and Graves' disease are the most seen associated disorders (46). Lymphocyte T cells, T helper (Th)1/Th2 balance, and regulatory T (Treg) cells play an important role in pregnancy; an altered cellular immune status and increased secretion of inflammatory cytokines is considered to contribute to adverse pregnancy outcome such as miscarriage and pre-term birth (PBT) (47). IL-2 and INF-γ, produced by Th1 cells, play important roles for the induction of implantation failure and abortion while the proinflammatory cytokine IL-17 is involved in the pathogenesis of abortion and PTB (48). Animal models have shown that an increased incidence of fetal loss and enhanced Th1 cell proliferation in Tg-immunized mice (49). Also natural killer (NK) cells dysregulation in the peripheral blood, with a prevalence of cytotoxic over immunoregulatory is closely related to reproductive failures, including recurrent miscarriage (RM) and infertility (50). Indeed, phenotypic and functional analysis on peripheral blood mononuclear cells from healthy donors and from TAI patients showed Th1 oriented changes of innate immunity, elevated NK, and NKT-like cells ratios, and enhanced natural cytotoxicity in TAI positive euthyroid women (51). Proinflammatory cytokine such as IL-2 and IL-17 as well as interferon gamma have been shown increased in the serum and/or in the follicular fluid of patients with TAI along with a quantitative and qualitative changes in endometrial T cells with reduction of secretion of IL-4 and IL 10 (52–54). Also, an increase in the percentage of cytotoxic NK cells in women with TAI was associated with reproductive failures (51). These data, although merely descriptive, suggest that cellular immune dysfunction, with a proinflammatory Th1 immune response and excessive activation of NK cells and NKT-like cells might induce the occurrence of infertility, miscarriage, and PTB in women with TAI (15).

Thyroid Antibodies Cause a Relative Hypothyroidism

The natural history of chronic autoimmune thyroiditis is a progressive decline in the functional capacity of the thyroid gland, leading to subclinical and, ultimately, to overt hypothyroidism. A decreased functional capacity of the thyroid gland may become apparent during early pregnancy since this condition constitutes a state of extra demand of thyroid hormone production. Indeed, it is well known that TAI increases the risk of developing subclinical or overt hypothyroidism during pregnancy. In the first half of spontaneous pregnancy women

with TPOAb had an increased prevalence of subclinical and overt hypothyroidism compared to controls (20.1 vs. 2.4% and 3.3 vs. 0.1%, respectively) (55). In women undergoing ART the ovarian stimulation anticipates the extra demand of thyroid hormone, which occurs only after conception during spontaneous pregnancy, and therefore TSH levels can increase significantly above 2.5 mIU/L before pregnancy in the presence of TAI (8). In early pregnancy the human chorionic gonadotropin (hCG), through its high homology with TSH, stimulates the thyroid, thus helping to maximize thyroid hormone secretion (6). An inappropriate thyroidal response to hCG stimulation has been considered an early marker of abnormal thyroid functional capacity in women with TAI. A study gathering data from two large population-based prospective cohorts showed that there was a positive association of hCG with FT4 as well as a negative association of hCG with TSH in TPOAb-negative women but not in TPOAb-positive women during early pregnancy. Women with TPOAb and lower FT4 than expected had a higher risk of premature delivery. This study therefore links the adverse pregnancy outcome to changes in thyroid function in TAI (56). It is believed that women with TAI may not benefit from the stimulation due to hCG aimed to meet the increased demand of thyroid hormones in early pregnancy. A recent study showed that TgAb also interferes with the thyroidal response to hCG. In 822 women at 7-20 weeks of gestation, when TSH was within the pregnancy-specific reference range, high concentrations of TPOAb and TgAb attenuated the FT4 stimulation and TSH suppression induced by hCG. This effect was more pronounced when both TPOAb and TgAb were present (57). Whether these data can be extrapolated for the dose of hCG used for the ovulation induction remains unknown and deserves further investigation (11). The antithyroid antibodies-induced impaired response of thyroid to hCG is not in contrast with their action on the ovary. Indeed, a two-stage mechanism has been recently proposed: at early stages of autoimmunity, when TPOAb levels are low and thyroid function is normal, the main effect of the antibodies is the creation of a hostile immune environment at the level of the ovary; as the autoimmune process progresses, the impaired response to hCG also becomes apparent (16).

THYROID AUTOIMMUNITY AND ASSISTED REPRODUCTIVE TECHNOLOGY OUTCOME

Several studies addressed the effect of TAI on pregnancy outcomes in euthyroid women who had conceived spontaneously (58). Euthyroid women with TAI carried twofold risk of miscarriage and were found to have slightly higher age and TSH levels (59). A triple or double risk of miscarriage or preterm birth, respectively, has also been reported in women with TPOAb (60). In a recent study the presence of TPOAb was also associated with preterm birth; the association did not appear to be related to differences in thyroid function, although the highest risk of preterm birth was observed in women with TPOAb and TSH >4.0 mIU/L. No association of TgAb positivity with preterm birth was found (61). In infertile women the presence of TPOAb increased the risk of miscarriage while a preconceptional TSH ≥ 2.5 mIU/L did not (30). Likewise, there has been intense interest in whether TAI may affect the success of the infertility treatments. Whenever possible primary approaches to female infertility are targeted to the identified cause and include ovulation induction for women with ovulatory dysfunction; endoscopic or surgical procedures to treat tubal obstruction or endometriosis. Intrauterine insemination (IUI) with donor or partner sperm is the first line treatment in couples with unexplained infertility, cervical factor infertility, and male infertility. When all these approaches fail ART is indicated. ART comprises both *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Although the two main indicators of ART success are the proportion of clinically pregnant women and the proportion of miscarried women, there are several parameters used to evaluate each step of the *in vitro* and *in vivo* ART outcomes starting from the ovarian response to the controlled hyperstimulation (COH), and the number of oocytes retrieved (NOR) and going further to the fertilization rate (FR) and to the implantation rate (IR). The most relevant outcome is the pregnancy rate (PR), and, better, the clinical pregnancy rate (CPR). Pregnancy indeed can be defined as either biochemical (i.e., a transient rise in hCG concentration) or clinical (the formation of a gestational sac and fetal heartbeat) and the outcome can be defined as live birth rate (LBR), ending with a miscarriage, miscarriage rate (MR), or with a pre-term birth (PTBR). Also, infant/neonatal complications are of interest, among these, above all, low birth weight (LBW).

Ovarian Reserve in Women With Thyroid Autoimmunity

The term ovarian reserve (OR), that is, the complete follicle pool and, mostly, the functional ovarian reserve (the number of maturing growing follicles after recruitment), expresses the woman's reproductive potential. Several studies addressed the effect of TAI on OR by evaluating its predictive markers such as high basal follicle stimulating hormone (FSH) levels, antral follicle count (AFC), and anti-Müllerian hormone concentrations (AMH). In 30 adolescent patients with TAI, AMH levels were comparable to controls. Serum AMH was negatively correlated with TSH but not with TPOAb or TgAb levels (62). Also in a case-control study 30 adolescent girls, newly diagnosed with TAI, had normal ovarian reserve based on measurements of AMH, inhibin B, FSH, LH/FSH ratio, estradiol, and AFC (63). In 775 reproductive age women, without any thyroid or ovarian dysfunction, those with AMH levels in the lower quartile for age had higher levels of TPOAb at baseline while there was no statistically significant difference in thyroid hormones compared to women with AMH in the higher quartiles. Over a 12-year follow up FT4 was decreased in all quartiles whereas TPOAb increased only in women with AMH in the low quartile (64). In a study involving 108 euthyroid TAI patients with regular menstrual cycles, either treated or not with

LT4, lower AMH levels were found in patients compared to the age-matched healthy women (65). OR is one of the most important determinants of downstream ART outcomes since it can affect the ovarian response to the COH protocols. Indeed, in 288 infertile women (55 euthyroid with TAI and 233 without) undergoing their first ART, higher levels of AMH were associated with better COH outcome as reflected by the estradiol/recombinant FSH ratio (E2/rFSH) and by the number of oocytes reaching metaphase II (M II oocytes). Women with TAI had lower AMH and higher FSH levels compared to TAI negative, but a poorer COH outcome was observed also in women with TAI and higher AMH levels. Thus, although diminished OR impairs COH outcome independently from TAI, the latter reduces ovarian response in women with preserved OR (66). The same group had previously also shown the effect of thyroid function on the above-mentioned markers of COH. In 262 women undergoing ART, those with TAI and TSH levels below 2.5 mIU/L had better ovarian response demonstrated by higher serum estradiol levels, higher E2/rFSH ratio, and a greater number of M II oocytes compared with those with TSH >2.5 mIU/L (67). In a study involving 1044 infertile women eligible for IUI/IVF, TSH levels, TPOAb positivity, and TgAb positivity were comparable between patients with variable ovarian reserve according to AMH levels. However, after patients with known causes of diminished ovarian reserve (i.e., genetic) were excluded, TPOAb positivity was higher in patients with low ovarian reserve thus indicating an association of TPOAb with idiopathic low ovarian reserve in unexplained infertility (68). In a study group of 436 women seeking fertility treatment, thyroid function or TPOAb positivity was not associated with AFC, while TgAb positivity was associated with a higher AFC. However, among women with diminished ovarian reserve or unexplained infertility, TPOAb and not TgAb, as well as lower FT3, were associated with a lower AFC (36). On the contrary in a large study including 5000 women no differences were found in the prevalence of TAI as well as of overt or subclinical hypothyroidism in women with low, normal, or high ovarian reserve expressed by age-specific AMH values (69). Also in 225 infertile women TSH <3.0 mIU/L was associated with significantly higher AMH compared to those with TSH ≥3.0 mIU/L after adjustment for thyroid autoimmunity and age, thus suggesting that thyroid function, even in the euthyroid range, has a more significant impact than TAI in the ovarian reserve (70).

In Vitro and *In Vivo* ART Outcome in Women With Thyroid Autoimmunity

Multiple clinical studies, systematic reviews, and metanalyses have evaluated the impact of TAI in ART *in vitro* and *in vivo* outcomes with conflicting conclusions. Indeed, the studies differ for sample size, design, causes of infertility, and type of ART, TSH level cut-off used to define euthyroidism as well as for the endpoints. Embryo quality was assessed in 431 embryos in euthyroid women with low ovarian reserve undergoing IVF. Comparable embryo quality was observed between women with TSH low-normal or high normal TSH (cut off 2.5 mIU/L) but impaired embryo quality was observed in women with TPOAb and TSH ≤ 2.5 mIU/L. Increasing TSH affected embryo quality and a trend toward the same effect was observed in women with TPOAb (71). Also, in a retrospective cohort study of 123 euthyroid women with or without TAI undergoing ART, embryo quality was significantly impaired in women with at least one autoantibody while no differences were found in AMH, FSH, and in the number of oocytes picked up and fertilized as well as in implantation rate and in pregnancy rate (72). Several prospective cohort studies releveled lower LBR and increased MR in euthyroid women with TPOAb undergoing IVF/ICSI. In 234 euthyroid women undergoing the first cycle of ART no differences in pregnancy rate were observed but the TAI women had threefold higher risk of miscarriage (73). No differences in pregnancy rate were observed between 412 TPOAb negative women and the 72 TPOAb positive that had been randomly assigned to receive LT4 or placebo. Nevertheless, a twofold risk of miscarriage was observed in euthyroid TPOAb positive women not subjected to LT4 treatment. The risk of miscarriage was not reduced by LT4 treatment in TPOAb positive women. This links the miscarriage to an abnormal immune response rather than to subsequent mild thyroid failure (74). Also, in a prospective study involving 590 infertile hypothyroid women treated with LT4, to maintain TSH levels below 2.5 μIU/mL, higher TPOAb titer was one of the risk factors for miscarriage, after IUI and IVF despite appropriate treatment (75). Among 194 euthyroid women undergoing IVF, the 60 with TPOAb/Tg Ab showed lower clinical pregnancy and live birth rate when confronted with controls. The same parameters were improved in 30 antithyroid antibodies-positive women treated with prednisone (76). On the contrary no effect on pregnancy outcome was observed in several retrospective studies published in the last 20 years. In 416 euthyroid women no differences in pregnancy and delivery rates were observed between women with and without TPOAb. However, women with TPOAb who failed to become pregnant or miscarried had high-normal TSH compared to the ones who delivered and compared to women without TPOAb (77). In a study enrolling 2406 women, analysis of cumulative delivery did not show differences between TAI positive and negative women after 6 IVF/ICSI cycles (78). Also, in more recent studies no impact of TPOAb and TgAb was observed in the MR and on the *in vitro* ART outcome such as NOR, FR, and embryo quality (26, 79, 80). In 584 women undergoing IVF/ICSI with TSH between 0.45 and 4.5 μIU/ml, NOR, FR, and CPR did not significantly differ between TAI positive and TAI negative. Subgroup analysis for only primary infertility patients showed a statistically significant lower CPR in TAI positive compared to TAI negative (29).

Also looking at meta-analysis studies conflicting results are reported. A meta-analysis of prospective studies reporting data on 1098 subfertile women undergoing IVF reported high miscarriage risk in euthyroid TAI women undergoing IVF but did not find significant difference in FR, CPR, and delivery rates (81). Comparable results were reported in a further study showing that TAI increased the risk of MR while it did not affect NOR, implantation, and clinical pregnancy rate. The effect

persisted after meta-regression analysis of age and TSH serum levels although the authors do not exclude possible modifying effects of these two variables (82). A more recent metanalysis including 14 studies performed on euthyroid TAI women did not show differences in *in vitro* and *in vivo* ART outcomes (CPR, MR, LBR per cycle, number of embryos transferred, NOR) neither for maternal age nor TSH levels when compared to women without TAI (83). Thus, heterogeneous results are reported and, also, the risk for adverse outcome is reported lower in the more recent metanalysis studies than in the previous ones (11). The explanation for the heterogeneity of these results can be found in the study design, in the TSH threshold used to define euthyroidism and as well as in the type of ART treatment and ovulation induction protocols. The effect of preconception TSH levels in the ART outcomes has been the subject of a recent meta-analysis. The study reported that when the TSH cut-off value for SCH was set at 2.5 mIU/L, no significant differences were observed in ART-related outcomes between SCH patients and normal women. On the contrary there was an increased risk of MR in women with SCH when a TSH cut-off value of 3.5–5.0 mIU/L was used (84). Regarding the studies on ART procedure, some have shown that the ICSI outcome was not affected by TAI. Indeed, in the last years ICSI has become the most popular insemination method worldwide. In a meta-analysis of studies including 1855 ICSI cycles, women with and without TAI, not differing for age, had comparable CPR, MR, and LBR. The authors propose that the presence of TAI may become a new indication for ICSI, independently of the cause of infertility, as it may overcome the detrimental impact of autoimmunity on fertilization and embryo quality (85). Indeed, previous observations reported that ICSI, which requires no interaction between the sperm cell and the zona pellucida, resulted in similar PR in women with or without TAI undergoing ART for male infertility (86). If this is true different outcomes are expected between IVF/ICSI and IUI. In a retrospective cohort study, enrolling 3143 patients, no significant different outcomes (PR, MR, LBR) were observed after IUI in euthyroid women with and without TAI also when comparing subgroups according to TSH level (TSH ≥2.5 mIU/L vs. TSH <2.5 mIU/L) (87). This latter finding was confirmed in a study enrolling 726 euthyroid women undergoing IUI for unexplained infertility. In this study, cycle characteristics and pregnancy outcomes (CPR, MR, LBR) of patients with serum TSH levels between 0.3-2.5 mIU/L and 2.5-4.5 mIU/L were compared and no statistically significant differences could be detected (88). In conclusion, although ART is an ideal model to analyze the effect of TAI on each stage of the reproductive process, compared to spontaneous conception, the multitude of variables present in the studies (age, causes of infertility, protocols for ovarian stimulation, the type of ART treatment, thyroid antibody levels, thyroid function) make it difficult to attribute to TAI per se a role on adverse outcomes and also to identify the TSH level threshold at which a negative impact on ART is expected, keeping in mind that TSH above 2.5 mIU/L is easily encountered after ovarian stimulation and even more so in the presence of TAI (78, 84). In **Table 2** are illustrated the above-mentioned studies addressing the effect of TAI on the prediction markers of ovarian reserve/response as well as on the pregnancy outcome.

IMPLICATIONS FOR SCREENING AND MANAGEMENT IN CLINICAL PRACTICE

Universal thyroid screening in pregnancy fulfills most criteria for a beneficial and cost-effective screening program, aimed to reduce fetal and maternal complications, but it is still a matter of debate (89). Despite the discordant findings of the studies regarding the association of antithyroid antibodies with infertility and adverse outcome after ART, infertile women constitute a selected group of patients for whom specific recommendations for screening and management come from the guidelines. According to ATA, women with infertility or with a history of miscarriage are "at high risk" of developing thyroid dysfunction and for them serum TSH concentration is recommended as soon pregnancy is confirmed with reflex TPOAb measurement if TSH is >2.5-10 mIU/L. Women undergoing ART, instead, fulfill the criteria for TSH universal screening (13). As per the very recent ETA guidelines, specifically referring to ART, TSH and TPOAb should be measured in women seeking medical advice for infertility; "the TgAb can be added systematically according to the local regulatory authority rules" while it should be measured when TSH levels are higher than 2.5 mIU/L and TPOAb are absent (14). Thyroid ultrasound, which is useful to calculate the gland volume as well as to detect features compatible with autoimmune thyroiditis, is not mentioned in ATA guidelines while is recommended by ETA if TSH is >2.5 mIU/L and TPOAb is negative. Regarding the treatment, there is not debate about the need to start LT4 in women with overt hypothyroidism who undergo ART or become pregnant spontaneously independently from their thyroid antibodies status. For ATA the decision to treat women with TSH >2.5 and 10 mIU/L is based on TSH level and on the TPOAb status. Treatment is recommended in TPOAb-positive women when TSH is above 4 mUI/mL (if trimester specific ranges are not available) while it can be considered in TPOAb positive women with TSH >2.5 < 4 mUI/L and in TPOAb negative women with TSH >4 < 10 mUI/L. According to ATA, for euthyroid women undergoing ART LT4 treatment, at low starting dose of 25-50 μg "may be considered given its potential benefits in comparison to its minimal risk". Indeed the 2.5 mUI/L TSH threshold still has value in women undergoing ART since ATA states that it is prudent to recommend treatment for "any TSH elevation over 2.5 mUI/L" before the procedure. On the contrary, also in case of ART, for some authors, a TSH level of 4 mIU/L is the cut-off for treatment since LT4 seems to increase LBR only when TSH is >4.0 mIU/L and also taking into account potential harmful effects of overtreatment (17, 90). The ETA guidelines recommend that women with serum TSH >4.0 mIU/L planning ART should be treated with LT4 independently of their TPOAb status, **Table 3**. In women with TAI and TSH levels >2.5<4 mIU/L, LT4 treatment at low starting dose of 25-50 μg LT4, before ovarian stimulation, could be "initiated in a case-by-

TABLE 2 | Studies addressing the effect of TAI on ovarian reserve/response and on assisted reproductive technology (ART) outcome.

Author, year	Patients' characteristics	Study outcomes	Thyroid function	Main conclusion
Polyzos, 2015 (69)	5000 women, infertility work up/other reasons	TAI prevalence in women with variable OR	EU/SCH/OH	No difference of TAI in women with variable OR
Chen, 2017 (68)	1044 women eligible for IUI/IVF	TAI prevalence in women with variable OR	EU	Idiopathic low OR associated with TAI
Korevar, 2018 (36)	436 women (46 TAI+) infertility work up	Association of TAI with OR	EU	TAI associated with lower AFC in women with unexplained infertility or diminished OR
Weghofer, 2016 (70)	225 women (25 TAI+) infertility work up	Association of TAI/TSH with OR	EU	TSH <3.0 mIU/L associated with higher OR
Magri, 2015 (66)	288 women (55 TAI+), IVF	Ovarian response to COH	EU	Poorer ovarian response in TAI
Weghofer, 2016 (70)	98 women (17 TAI+) with low OR, IVF	EQ	EU	Poorer embryo quality in TAI
Andrisani, 2018 (72)	123 (29 TAI+), IVF/ICSI	EQ, IR, PR	EU	Poorer EQ in TAI, no differences in IR and PR
Poppe, 2003 (73)	234 women (32 TAI+), IVF/ICSI	LBR, CPR, MR	EU	No difference in CPR in TAI, higher MR in TAI
Negro, 2005 (74)	484 (72 TAI+), IVF/ICSI	PR, MR	EU	No difference in PR, higher MR in TAI
Negro, 2007 (77)	416women (42 TAI+), IVF/ICSI	NOR, LBR, CPR, MR	EU	No effect on CPR
Tan, 2014 (86)	835 women (110 TAI+), ICSI	CPR, MR, PTB	EU	No effect of TAI
Litwicka, 2015 (76)	194 (60 TAI+), IVF	NOR, LBR, CPR, MR,	EU	Lower LBR, higher MR in TAI
Lukasuk, 2015 (26)	573 (114 TAI+), ICSI	NOR, LBR, PR, MR,	EU	No effect on PR, LBR, MR, lower NOR in TAI
Unuane, 2016 (78)	2406 women (33 TAI+), ICSI	LBR	EU	No effect of TAI
Unuane, 2017 (87)	3143 women (187 TAI+), IUI	LBR, CPR, MR	EU	No effect of TAI
Poppe, 2020 (79)	279 women, ART, IVF/ICSI	NOR, FR, EQ	EU/SCH	No effect of TAI/SCH
Hamad, 2021 (29)	584 women (148 TAI+), IVF/ICSI	CPR	EU	No effect of TAI

TAI, thyroid autoimmunity; OR, ovarian reserve; AFC, antral follicle count; EQ, embryo quality; NOR, number of oocytes retrieved; IR, implantation rate; CPR, clinical pregnancy rate; LBR, live birth rate; MR, miscarriage rate; EU, euthyroidism; SCH, subclinical hypothyroidism; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

case manner" such as in women with recurrent miscarriage, in women over 35 years of age, and with ovarian causes of infertility (14). According to ETA, LT4 treatment of TAI women with TSH levels >2.5<4 mIU/L could optimize ovarian reserve and improve embryo quality while there is no definitive evidence of benefits on pregnancy outcome. Several studies have addressed the benefit of LT4 on ART outcome in euthyroid women with TAI. In one randomized clinical trial (RCT) previously mentioned, pregnancy rate was not affected either by presence of TPOAb or by treatment with LT4. Euthyroid women (TSH ≤ 2.5 mIU/L) with TAI had high MR but LT4 treatment did not improve the delivery rate (74). These findings were supported by a recent large RCT. Treatment with LT4, 25-μg/d or 50-μg/d according to the TSH ≤2.5 or >2.5 mIU/L, respectively, did not reduce MR or increase CPR and LBR among 600 Chinese euthyroid women with TAI undergoing IVF. The study excluded women at high risk of miscarriage, and women positive for antinuclear and anticardiolipin antibodies, or lupus anticoagulant to eliminate other autoimmune confounding factors. Moreover, although in a limited number of patients, no benefits of therapy were observed in women with TSH>4 mIU/L (91). This study supports the conservative approach expressed by the recent guidelines (14). Nevertheless, in a meta-analysis, also including some studies, LT4 treatment had no significant impact on live births in women with SCH or TAI undergoing IVF; however, it decreased miscarriage rates. It is worth remembering that in some studies patients characterized by TPO-positivity and TSH levels 4.0 or 4.5 mIU/L were mixed

TABLE 3 | Recommendations for women with infertility and/or undergoing assisted reproductive technology (ART) according to the ETA 2021 guidelines (14).

Test/Treatment	ETA 2021
TSH screening	Women seeking care for infertility
Recommended TSH upper limits	4.0 mIU/L or ULRR*
TPOAb measurement	Women seeking care for infertility
TgAb measurement	If TSH>2.5 mIU/L and TPOAb negative
Thyroid ultrasound	If TSH>2.5 mIU/L and TPOAb negative
L-T4 treatment in women undergoing ART	Recommended in overt hypothyroidism
	Recommended if TSH is >4 mIU/L with/without TAI
	Suggested in TAI if TSH is >2.5< 4 mIU/L on a case-by-case basis
Fertilization preferred method in TAI	ICSI suggested

TSH, thyroid stimulating hormone; ART, assisted reproductive technology; ULRR, Upper limit reference range. TAI, thyroid autoimmunity. TPOAb, thyroperoxidase antibody; TgAb, thyroglobulin antibody; L-T4, levothyroxine; OS, ovarian stimulation ICSI, intracytoplasmic sperm injection. ART, assisted reproductive technology.
**If ULRR is >4.0 mUI/L.*

in data synthesis (92). The TABLET study, a randomized, placebo-controlled trial, investigated the effect of a fixed dose of LT4 50 μg daily, on LBR in TPOAb positive euthyroid women. This very large study included women with a history of recurrent miscarriage or receiving treatment for infertility. Prespecified subgroup analyses were completed for the primary outcome according to maternal age, the baseline TSH level (≤2.5 mIU/L or >2.5 mIU/L), and infertility treatment. LT4 treatment did not result in a higher LBR compared with placebo (93). The studies not showing a benefit of LT4 support the hypothesis that the increased risk of miscarriage in TAI is linked to an abnormal immune response rather than to mild thyroid failure. In this regard ETA guidelines suggest ICSI as the preferred fertilization method in women with TAI to overcome potential negative effects of antithyroid antibodies on oocytes and embryos (14). Currently no treatments have been shown to reduce thyroid autoimmunity and this aspect continues to be studied (94). In a previous study the use of glucocorticoids demonstrated an increase in pregnancy rates with treatment compared with placebo (76). In a more recent study it has been demonstrated that glucocorticoid may improve the pregnancy outcomes of ART women with antithyroid antibodies positive, but does not reduce the risk of miscarriage (95). Selenium supplementation decreases thyroid autoantibodies concentration during pregnancy but with no benefit on fetal or maternal outcomes (96).

CONCLUSIONS

Overt thyroid dysfunction leads to menstrual disturbances, fertility problems, and pregnancy complications. Also, thyroid autoimmunity, even in the setting of euthyroidism, might adversely affect fertility and reproductive outcome by creating a cytotoxic environment that damages the maturing oocyte reducing its quality and fertilization potential and by inducing a subtle dysfunction during early pregnancy. Thyroid autoimmunity is associated with some causes of infertility (ovarian and idiopathic) and with some adverse pregnancy outcome, such as miscarriage, after assisted conception. Women with TAI undergoing ART are an ideal model to analyze the effect of thyroid autoimmunity/dysfunction on each stage of the reproductive process, compared to spontaneous conception. A better understanding of the pathophysiology may have an impact on the therapeutic approach. Although many questions remain unanswered, there is no doubt that it is justified for women seeking medical advice for infertility screening for thyroid dysfunction and autoimmunity. The decision for treatment should be based on the current evidence and recommendations but it cannot omit an individualized clinical judgement on the cause of the infertility as well as on the obstetric history of the women.

AUTHOR CONTRIBUTIONS

IB: substantial contributions to the conception and design of the work; reviewing the literature; drafting the work; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. CG, GDD, GF, and GN: contributions to the design of the work; revising the work critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, et al. The International Glossary on Infertility and Fertility Care, 2017. *Fertil Steril* (2017) 108(3):393–406. doi: 10.1016/j.fertnstert.2017.06.005
2. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA. National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. *PloS Med* (2012) 9(12): e1001356. doi: 10.1371/journal.pmed.1001356
3. Vissenberg R, Manders VD, Mastenbroek S, Fliers E, Afink GB, Ris-Stalpers C, et al. Pathophysiological Aspects of Thyroid Hormone Disorders/Thyroid Peroxidase Autoantibodies and Reproduction. *Hum Reprod Update* (2015) 21 (3):378–87. doi: 10.1093/humupd/dmv004
4. Krassas GE, Poppe K, Glinoer D. Thyroid Function and Human Reproductive Health. *Endocr Rev* (2010) 31(5):702–55. doi: 10.1210/er.2009-0041
5. Oki N, Matsuo H, Nakago S, Murakoshi H, Laoag-Fernandez JB, Maruo T. Effects of 3,5,3'-Triiodothyronine on the Invasive Potential and the Expression of Integrins and Matrix Metalloproteinases in Cultured Early Placental Extravillous Trophoblasts. *J Clin Endocrinol Metab* (2004) 89 (10):5213–21. doi: 10.1210/jc.2004-0352
6. Glinoer D. The Regulation of Thyroid Function in Pregnancy: Pathways of Endocrine Adaptation From Physiology to Pathology. *Endocr Rev* (1997) 18 (3):404–33. doi: 10.1210/edrv.18.3.0300
7. de Escobar GM, Obregon MJ, del Rey FE. Maternal Thyroid Hormones Early in Pregnancy and Fetal Brain Development. *Best Pract Res Clin Endocrinol Metab* (2004) 18(2):225–48. doi: 10.1016/j.beem.2004.03.012
8. Mintziori G, Goulis DG, Toulis KA, Venetis CA, Kolibianakis EM, Tarlatzis BC. Thyroid Function During Ovarian Stimulation: A Systematic Review. *Fertil Steril* (2011) 96(3):780–5. doi: 10.1016/j.fertnstert.2011.06.020
9. Bucci I, Giuliani C, Napolitano G. Thyroid-Stimulating Hormone Receptor Antibodies in Pregnancy: Clinical Relevance. *Front Endocrinol (Lausanne)* (2017) 8:137. doi: 10.3389/fendo.2017.00137
10. Pearce EN. Thyroid Disorders During Pregnancy and Postpartum. *Best Pract Res Clin Obstet Gynaecol* (2015) 29(5):700–6. doi: 10.1016/j.bpobgyn.2015.04.007
11. Poppe K. MANAGEMENT OF ENDOCRINE DISEASE: Thyroid and Female Infertility: More Questions Than Answers?! *Eur J Endocrinol* (2021) 184(4): R123–R35. doi: 10.1530/EJE-20-1284
12. Stagnaro-Green A, Abalovich M, Alexander E, Azizi F, Mestman J, Negro R, et al. Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and Postpartum. *Thyroid* (2011) 21(10):1081–125. doi: 10.1089/thy.2011.0087
13. Alexander EK, Pearce EN, Brent GA, Brown RS, Chen H, Dosiou C, et al. 2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum. *Thyroid* (2017) 27(3):315–89. doi: 10.1089/thy.2016.0457

14. Poppe K, Bisschop P, Fugazzola L, Minziori G, Unuane D, Weghofer A. 2021 European Thyroid Association Guideline on Thyroid Disorders Prior to and During Assisted Reproduction. *Eur Thyroid J* (2021) 9(6):281–95. doi: 10.1159/000512790
15. Zhu Q, Xu QH, Xie T, Wang LL, Liu H, Muyayalo KP, et al. Recent Insights Into the Impact of Immune Dysfunction on Reproduction in Autoimmune Thyroiditis. *Clin Immunol* (2021) 224:108663. doi: 10.1016/j.clim.2020.108663
16. Dosiou C. Thyroid and Fertility: Recent Advances. *Thyroid* (2020) 30(4):479–86. doi: 10.1089/thy.2019.0382
17. Unuane D, Velkeniers B. Impact of Thyroid Disease on Fertility and Assisted Conception. *Best Pract Res Clin Endocrinol Metab* (2020) 34(4):101378. doi: 10.1016/j.beem.2020.101378
18. Valdes S, Maldonado-Araque C, Lago-Sampedro A, Lillo JA, Garcia-Fuentes E, Perez-Valero V, et al. Population-Based National Prevalence of Thyroid Dysfunction in Spain and Associated Factors: Di@bet.es Study. *Thyroid* (2017) 27(2):156–66. doi: 10.1089/thy.2016.0353
19. Ragusa F, Fallahi P, Elia G, Gonnella D, Paparo SR, Giusti C, et al. Hashimotos' Thyroiditis: Epidemiology, Pathogenesis, Clinic and Therapy. *Best Pract Res Clin Endocrinol Metab* (2019) 33(6):101367. doi: 10.1016/j.beem.2019.101367
20. Roussev RG, Kaider BD, Price DE, Coulam CB. Laboratory Evaluation of Women Experiencing Reproductive Failure. *Am J Reprod Immunol* (1996) 35 (4):415–20. doi: 10.1111/j.1600-0897.1996.tb00503.x
21. Kutteh WH, Yetman DL, Carr AC, Beck LA, Scott RT. Increased Prevalence of Antithyroid Antibodies Identified in Women With Recurrent Pregnancy Loss But Not in Women Undergoing Assisted Reproduction. *Fertil Steril* (1999) 71 (5):843–8. doi: 10.1016/S0015-0282(99)00091-6
22. Janssen OE, Mehlmauer N, Hahn S, Offner AH, Gartner R. High Prevalence of Autoimmune Thyroiditis in Patients With Polycystic Ovary Syndrome. *Eur J Endocrinol* (2004) 150(3):363–9. doi: 10.1530/eje.0.1500363
23. Kaider AS, Kaider BD, Janowicz PB, Roussev RG. Immunodiagnostic Evaluation in Women With Reproductive Failure. *Am J Reprod Immunol* (1999) 42(6):335–46. doi: 10.1111/j.1600-0897.1999.tb00110.x
24. Poppe K, Velkeniers B, Glinoer D. Thyroid Disease and Female Reproduction. *Clin Endocrinol (Oxf)* (2007) 66(3):309–21. doi: 10.1111/j.1365-2265.2007.02752.x
25. Karacan M, Alwaeely F, Cebi Z, Berberoglugil M, Batukan M, Ulug M, et al. Effect of Antithyroid Antibodies on ICSI Outcome in Antiphospholipid Antibody-Negative Euthyroid Women. *Reprod BioMed Online* (2013) 27 (4):376–80. doi: 10.1016/j.rbmo.2013.07.002
26. Lukaszuk K, Kunicki M, Kulwikowska P, Liss J, Pastuszek E, Jaszczolt M, et al. The Impact of the Presence of Antithyroid Antibodies on Pregnancy Outcome Following Intracytoplasmatic Sperm Injection-ICSI and Embryo Transfer in Women With Normal Thyreotropine Levels. *J Endocrinol Invest* (2015) 38 (12):1335–43. doi: 10.1007/s40618-015-0377-5
27. Sakar MN, Unal A, Atay AE, Zebitay AG, Verit FF, Demir S, et al. Is There an Effect of Thyroid Autoimmunity on the Outcomes of Assisted Reproduction? *J Obstet Gynaecol* (2016) 36(2):213–7. doi: 10.3109/01443615.2015.1049253
28. Dhillon-Smith RK, Tobias A, Smith PP, Middleton LJ, Sunner KK, Baker K, et al. The Prevalence of Thyroid Dysfunction and Autoimmunity in Women With History of Miscarriage or Subfertility. *J Clin Endocrinol Metab* (2020) 105(8):2667–77. doi: 10.1210/clinem/dgaa302
29. Hamad A, Alhalabi N, Nmr N, Abbas F, Al-Hammami H, Ibrahim N, et al. Impact of Thyroid Autoimmunity in Euthyroid Women on the Outcomes of *In Vitro* Fertilization. *Ann Med Surg (Lond)* (2021) 67:102473. doi: 10.1016/j.amsu.2021.102473
30. Seungdamrong A, Steiner AZ, Gracia CR, Legro RS, Diamond MP, Coutifaris C, et al. Preconceptional Antithyroid Peroxidase Antibodies, But Not Thyroid-Stimulating Hormone, are Associated With Decreased Live Birth Rates in Infertile Women. *Fertil Steril* (2017) 108(5):843–50. doi: 10.1016/j.fertnstert.2017.08.026
31. Poppe K, Glinoer D, Van Steirteghem A, Tournaye H, Devroey P, Schiettecatte J, et al. Thyroid Dysfunction and Autoimmunity in Infertile Women. *Thyroid* (2002) 12(11):997–1001. doi: 10.1089/105072502320908330
32. Petta CA, Arruda MS, Zantut-Wittmann DE, Benetti-Pinto CL. Thyroid Autoimmunity and Thyroid Dysfunction in Women With Endometriosis. *Hum Reprod* (2007) 22(10):2693–7. doi: 10.1093/humrep/dem267
33. Kim JJ, Yoon JW, Kim MJ, Kim SM, Hwang KR, Choi YM. Thyroid Autoimmunity Markers in Women With Polycystic Ovary Syndrome and Controls. *Hum Fertil (Camb)* (2020) 25(1):128–34. doi: 10.1080/14647273.2019.1709668
34. van den Boogaard E, Vissenberg R, Land JA, van Wely M, Ven der Post JA, Goddijn M, et al. Significance of (Sub)Clinical Thyroid Dysfunction and Thyroid Autoimmunity Before Conception and in Early Pregnancy: A Systematic Review. *Hum Reprod Update* (2016) 22(4):532–3. doi: 10.1093/humupd/dmw003
35. Unuane D, Velkeniers B, Anckaert E, Schiettecatte J, Tournaye H, Haentjens P, et al. Thyroglobulin Autoantibodies: Is There Any Added Value in the Detection of Thyroid Autoimmunity in Women Consulting for Fertility Treatment? *Thyroid* (2013) 23(8):1022–8. doi: 10.1089/thy.2012.0562
36. Korevaar TIM, Minguez-Alarcon L, Messerlian C, de Poortere RA, Williams PL, Broeren MA, et al. Association of Thyroid Function and Autoimmunity With Ovarian Reserve in Women Seeking Infertility Care. *Thyroid* (2018) 28 (10):1349–58. doi: 10.1089/thy.2017.0582
37. Rotondi M, Chiovato L, Pacini F, Bartalena L, Vitti P. Management of Subclinical Hypothyroidism in Pregnancy: A Comment From the Italian Society of Endocrinology and the Italian Thyroid Association to the 2017 American Thyroid Association Guidelines-"The Italian Way". *Thyroid* (2018) 28(5):551–5. doi: 10.1089/thy.2017.0424
38. Monteleone P, Parrini D, Faviana P, Carletti E, Casarosa E, Uccelli A, et al. Female Infertility Related to Thyroid Autoimmunity: The Ovarian Follicle Hypothesis. *Am J Reprod Immunol* (2011) 66(2):108–14. doi: 10.1111/j.1600-0897.2010.00961.x
39. Medenica S, Garalejic E, Arsic B, Medjo B, Bojovic Jovic D, Abazovic D, et al. Follicular Fluid Thyroid Autoantibodies, Thyrotropin, Free Thyroxine Levels and Assisted Reproductive Technology Outcome. *PloS One* (2018) 13(10): e0206652. doi: 10.1371/journal.pone.0206652
40. Monteleone P, Faviana P, Artini PG. Thyroid Peroxidase Identified in Human Granulosa Cells: Another Piece to the Thyroid-Ovary Puzzle? *Gynecol Endocrinol* (2017) 33(7):574–6. doi: 10.1080/09513590.2017.1296424
41. Lee YL, Ng HP, Lau KS, Liu WM, WS O, Yeung WS, et al. Increased Fetal Abortion Rate in Autoimmune Thyroid Disease is Related to Circulating TPO Autoantibodies in an Autoimmune Thyroiditis Animal Model. *Fertil Steril* (2009) 91(5 Suppl):2104–9. doi: 10.1016/j.fertnstert.2008.07.1704
42. Kelkar RL, Meherji PK, Kadam SS, Gupta SK, Nandedkar TD. Circulating Auto-Antibodies Against the Zona Pellucida and Thyroid Microsomal Antigen in Women With Premature Ovarian Failure. *J Reprod Immunol* (2005) 66(1):53–67. doi: 10.1016/j.jri.2005.02.003
43. Deroux A, Dumestre-Perard C, Dunand-Faure C, Bouillet L, Hoffmann P. Female Infertility and Serum Auto-Antibodies: A Systematic Review. *Clin Rev Allergy Immunol* (2017) 53(1):78–86. doi: 10.1007/s12016-016-8586-z
44. Cellini M, Santaguida MG, Stramazzo I, Capriello S, Brusca N, Antonelli A, et al. Recurrent Pregnancy Loss in Women With Hashimoto's Thyroiditis With Concurrent Non-Endocrine Autoimmune Disorders. *Thyroid* (2020) 30 (3):457–62. doi: 10.1089/thy.2019.0456
45. Caccavo D, Pellegrino NM, Nardelli C, Vergine S, Leone L, Marolla A, et al. Anti-Laminin-1 Antibodies in Serum and Follicular Fluid of Women With Hashimoto's Thyroiditis Undergoing *In Vitro* Fertilization. *Int J Immunopathol Pharmacol* (2016) 29(2):280–7. doi: 10.1177/0394632015627281
46. Szeliga A, Calik-Ksepka A, Maciejewska-Jeske M, Grymowicz M, Smolarczyk K, Kostrzak A, et al. Autoimmune Diseases in Patients With Premature Ovarian Insufficiency-Our Current State of Knowledge. *Int J Mol Sci* (2021) 22 (5):2594–605. doi: 10.3390/ijms22052594
47. Ghaebi M, Nouri M, Ghasemzadeh A, Farzadi L, Jadidi-Niaragh F, Ahmadi M, et al. Immune Regulatory Network in Successful Pregnancy and Reproductive Failures. *BioMed Pharmacother* (2017) 88:61–73. doi: 10.1016/j.biopha.2017.01.016
48. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy. *Am J Reprod Immunol* (2010) 63(6):601–10. doi: 10.1111/j.1600-0897.2010.00852.x
49. Imaizumi M, Pritsker A, Kita M, Ahmad L, Unger P, Davies T. Pregnancy and Murine Thyroiditis: Thyroglobulin Immunization Leads to Fetal Loss in Specific Allogeneic Pregnancies. *Endocrinology* (2001) 142(2):823–9. doi: 10.1210/endo.142.2.7966

50. Seshadri S, Sunkara SK. Natural Killer Cells in Female Infertility and Recurrent Miscarriage: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2014) 20(3):429–38. doi: 10.1093/humupd/dmt056
51. Miko E, Meggyes M, Doba K, Farkas N, Bogar B, Barakonyi A, et al. Characteristics of Peripheral Blood NK and NKT-Like Cells in Euthyroid and Subclinical Hypothyroid Women With Thyroid Autoimmunity Experiencing Reproductive Failure. *J Reprod Immunol* (2017) 124:62–70. doi: 10.1016/j.jri.2017.09.008
52. Turhan Iyidir O, Konca Degertekin C, Sonmez C, Atak Yucel A, Erdem M, Akturk M, et al. The Effect of Thyroid Autoimmunity on T-Cell Responses in Early Pregnancy. *J Reprod Immunol* (2015) 110:61–6. doi: 10.1016/j.jri.2015.04.002
53. Lu H, Huang Y, Xin H, Hao C, Cui Y. The Expression of Cytokines IFN-Gamma, IL-4, IL-17A, and TGF-Beta1 in Peripheral Blood and Follicular Fluid of Patients Testing Positive for Anti-Thyroid Autoantibodies and its Influence on *In Vitro* Fertilization and Embryo Transfer Pregnancy Outcomes. *Gynecol Endocrinol* (2018) 34(11):933–9. doi: 10.1080/09513590.2018.1459546
54. Twig G, Shina A, Amital H, Shoenfeld Y. Pathogenesis of Infertility and Recurrent Pregnancy Loss in Thyroid Autoimmunity. *J Autoimmun* (2012) 38 (2-3):J275–J81. doi: 10.1016/j.jaut.2011.11.014
55. Medici M, de Rijke YB, Peeters RP, Visser W, de Muinck Keizer-Schrama SM, Jaddoe VV, et al. Maternal Early Pregnancy and Newborn Thyroid Hormone Parameters: The Generation R Study. *J Clin Endocrinol Metab* (2012) 97 (2):646–52. doi: 10.1210/jc.2011-2398
56. Korevaar TI, Steegers EA, Pop VJ, Broeren MA, Chaker L, de Rijke YB, et al. Thyroid Autoimmunity Impairs the Thyroidal Response to Human Chorionic Gonadotropin: Two Population-Based Prospective Cohort Studies. *J Clin Endocrinol Metab* (2017) 102(1):69–77. doi: 10.1210/jc.2016-2942
57. Hou Y, Liu A, Li J, Wang H, Yang Y, Li Y, et al. Different Thyroidal Responses to Human Chorionic Gonadotropin Under Different Thyroid Peroxidase Antibody and/or Thyroglobulin Antibody Positivity Conditions During the First Half of Pregnancy. *Thyroid* (2019) 29(4):577–85. doi: 10.1089/thy.2018.0097
58. Dhillon-Smith RK, Coomarasamy A. TPO Antibody Positivity and Adverse Pregnancy Outcomes. *Best Pract Res Clin Endocrinol Metab* (2020) 34 (4):101433. doi: 10.1016/j.beem.2020.101433
59. Chen L, Hu R. Thyroid Autoimmunity and Miscarriage: A Meta-Analysis. *Clin Endocrinol (Oxf)* (2011) 74(4):513–9. doi: 10.1111/j.1365-2265.2010.03974.x
60. Thangaratinam S, Tan A, Knox E, Kilby MD, Franklyn J, Coomarasamy A. Association Between Thyroid Autoantibodies and Miscarriage and Preterm Birth: Meta-Analysis of Evidence. *Bmj* (2011) 342:d2616. doi: 10.1136/bmj.d2616
61. The Consortium on Thyroid and Pregnancy—Study Group on Preterm Birth. Association of Thyroid Function Test Abnormalities and Thyroid Autoimmunity With Preterm Birth: A Systematic Review and Meta-Analysis. *JAMA* (2019) 322(7):632–41. doi: 10.1001/jama.2019.10931
62. Ozalp Akin E, Aycan Z. Evaluation of the Ovarian Reserve in Adolescents With Hashimoto's Thyroiditis Using Serum Anti-Mullerian Hormone Levels. *J Clin Res Pediatr Endocrinol* (2018) 10(4):331–5. doi: 10.4274/jcrpe.0047
63. Pirgon O, Sivrice C, Demirtas H, Dundar B. Assessment of Ovarian Reserve in Euthyroid Adolescents With Hashimoto Thyroiditis. *Gynecol Endocrinol* (2016) 32(4):306–10. doi: 10.3109/09513590.2015.1116510
64. Bahri S, Tehrani FR, Amouzgar A, Rahmati M, Tohidi M, Vasheghani M, et al. Overtime Trend of Thyroid Hormones and Thyroid Autoimmunity and Ovarian Reserve: A Longitudinal Population Study With a 12-Year Follow Up. *BMC Endocr Disord* (2019) 19(1):47. doi: 10.1186/s12902-019-0370-7
65. Ozturk Unsal I, Hepsen S, Akhanli P, Calapkulu M, Sencar ME, Yalcindag A, et al. Evaluation of Serum Anti-Mullerian Hormone Levels in Women With Hashimoto Thyroiditis in the Reproductive Age. *Turk J Med Sci* (2021) 51 (2):716–21. doi: 10.3906/sag-2012-177
66. Magri F, Schena L, Capelli V, Gaiti M, Zerbini F, Brambilla E, et al. Anti-Mullerian Hormone as a Predictor of Ovarian Reserve in ART Protocols: The Hidden Role of Thyroid Autoimmunity. *Reprod Biol Endocrinol* (2015) 13 (1):106. doi: 10.1186/s12958-015-0103-3
67. Magri F, Capelli V, Gaiti M, Brambilla E, Montesion L, Rotondi M, et al. Impaired Outcome of Controlled Ovarian Hyperstimulation in Women With Thyroid Autoimmune Disease. *Thyroid* (2013) 23(10):1312–8. doi: 10.1089/thy.2013.0022
68. Chen CW, Huang YL, Tzeng CR, Huang RL, Chen CH. Idiopathic Low Ovarian Reserve Is Associated With More Frequent Positive Thyroid Peroxidase Antibodies. *Thyroid* (2017) 27(9):1194–200. doi: 10.1089/thy.2017.0139
69. Polyzos NP, Sakkas E, Vaiarelli A, Poppe K, Camus M, Tournaye H. Thyroid Autoimmunity, Hypothyroidism and Ovarian Reserve: A Cross-Sectional Study of 5000 Women Based on Age-Specific AMH Values. *Hum Reprod* (2015) 30(7):1690–6. doi: 10.1093/humrep/dev089
70. Weghofer A, Barad DH, Darmon S, Kushnir VA, Gleicher N. What Affects Functional Ovarian Reserve, Thyroid Function or Thyroid Autoimmunity? *Reprod Biol Endocrinol* (2016) 14(1):26. doi: 10.1186/s12958-016-0162-0
71. Weghofer A, Himaya E, Kushnir VA, Barad DH, Gleicher N. The Impact of Thyroid Function and Thyroid Autoimmunity on Embryo Quality in Women With Low Functional Ovarian Reserve: A Case-Control Study. *Reprod Biol Endocrinol* (2015) 13:43. doi: 10.1186/s12958-015-0041-0
72. Andrisani A, Sabbadin C, Marin L, Ragazzi E, Dessole F, Armanini D, et al. The Influence of Thyroid Autoimmunity on Embryo Quality in Women Undergoing Assisted Reproductive Technology. *Gynecol Endocrinol* (2018) 34 (9):752–5. doi: 10.1080/09513590.2018.1442427
73. Poppe K, Glinoer D, Tournaye H, Devroey P, van Steirteghem A, Kaufman L, et al. Assisted Reproduction and Thyroid Autoimmunity: An Unfortunate Combination? *J Clin Endocrinol Metab* (2003) 88(9):4149–52. doi: 10.1210/jc.2003-030268
74. Negro R, Mangieri T, Coppola L, Presicce G, Casavola EC, Gismondi R, et al. Levothyroxine Treatment in Thyroid Peroxidase Antibody-Positive Women Undergoing Assisted Reproduction Technologies: A Prospective Study. *Hum Reprod* (2005) 20(6):1529–33. doi: 10.1093/humrep/deh843
75. Inagaki Y, Takeshima K, Nishi M, Ariyasu H, Doi A, Kurimoto C, et al. The Influence of Thyroid Autoimmunity on Pregnancy Outcome in Infertile Women: A Prospective Study. *Endocr J* (2020) 67(8):859–68. doi: 10.1507/endocrj.EJ19-0604
76. Litwicka K, Arrivi C, Varricchio MT, Mencacci C, Greco E. In Women With Thyroid Autoimmunity, Does Low-Dose Prednisolone Administration, Compared With No Adjuvant Therapy, Improve *In Vitro* Fertilization Clinical Results? *J Obstet Gynaecol Res* (2015) 41(5):722–8. doi: 10.1111/jog.12615
77. Negro R, Formoso G, Coppola L, Presicce G, Mangieri T, Pezzarossa A, et al. Euthyroid Women With Autoimmune Disease Undergoing Assisted Reproduction Technologies: The Role of Autoimmunity and Thyroid Function. *J Endocrinol Invest* (2007) 30(1):3–8. doi: 10.1007/BF03347388
78. Unuane D, Velkeniers B, Deridder S, Bravenboer B, Tournaye H, De Brucker M. Impact of Thyroid Autoimmunity on Cumulative Delivery Rates in *In Vitro* Fertilization/Intracytoplasmic Sperm Injection Patients. *Fertil Steril* (2016) 106(1):144–50. doi: 10.1016/j.fertnstert.2016.03.011
79. Poppe K, Autin C, Veltri F, Sitoris G, Kleynen P, Praet JP, et al. Thyroid Disorders and *In Vitro* Outcomes of Assisted Reproductive Technology: An Unfortunate Combination? *Thyroid* (2020) 30(8):1177–85. doi: 10.1089/thy.2019.0567
80. Chen X, Mo ML, Huang CY, Diao LH, Li GG, Li YY, et al. Association of Serum Autoantibodies With Pregnancy Outcome of Patients Undergoing First IVF/ICSI Treatment: A Prospective Cohort Study. *J Reprod Immunol* (2017) 122:14–20. doi: 10.1016/j.jri.2017.08.002
81. Toulis KA, Goulis DG, Venetis CA, Kolibianakis EM, Negro R, Tarlatzis BC, et al. Risk of Spontaneous Miscarriage in Euthyroid Women With Thyroid Autoimmunity Undergoing IVF: A Meta-Analysis. *Eur J Endocrinol* (2010) 162(4):643–52. doi: 10.1530/EJE-09-0850
82. Busnelli A, Paffoni A, Fedele L, Somigliana E. The Impact of Thyroid Autoimmunity on IVF/ICSI Outcome: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2016) 22(6):775–90. doi: 10.1093/humupd/dmw019
83. Venables A, Wong W, Way M, Homer HA. Thyroid Autoimmunity and IVF/ICSI Outcomes in Euthyroid Women: A Systematic Review and Meta-Analysis. *Reprod Biol Endocrinol* (2020) 18(1):120. doi: 10.1186/s12958-020-00671-3
84. Zhao T, Chen BM, Zhao XM, Shan ZY. Meta-Analysis of ART Outcomes in Women With Different Preconception TSH Levels. *Reprod Biol Endocrinol* (2018) 16(1):111. doi: 10.1186/s12958-018-0424-0

85. Poppe K, Autin C, Veltri F, Kleynen P, Grabczan L, Rozenberg S, et al. Thyroid Autoimmunity and Intracytoplasmic Sperm Injection Outcome: A Systematic Review and Meta-Analysis. *J Clin Endocrinol Metab* (2018) 103 (5):1755–66. doi: 10.1210/jc.2017-02633
86. Tan S, Dieterle S, Pechlavanis S, Janssen OE, Fuhrer D. Thyroid Autoantibodies Per Se Do Not Impair Intracytoplasmic Sperm Injection Outcome in Euthyroid Healthy Women. *Eur J Endocrinol* (2014) 170(4):495–500. doi: 10.1530/eje-13-0790
87. Unuane D, Velkeniers B, Bravenboer B, Drakopoulos P, Tournaye H, Parra J, et al. Impact of Thyroid Autoimmunity in Euthyroid Women on Live Birth Rate After IUI. *Hum Reprod* (2017) 32(4):915–22. doi: 10.1093/humrep/dex033
88. Karakis LS, Kiyak H, Okmen B, Ozdemir C, Turkgeldi E. Impact of Preconceptional Serum Thyroid Stimulating Hormone Values Ranging Between 2.5 and 4.5 mIU/L on Live Birth Rates Following Ovulation Induction and Intrauterine Insemination Treatment for Unexplained Infertility. *BMC Womens Health* (2021) 21(1):162. doi: 10.1186/s12905-021-01299-0
89. Taylor PN, Zouras S, Min T, Nagarahaj K, Lazarus JH, Okosieme O. Thyroid Screening in Early Pregnancy: Pros and Cons. *Front Endocrinol (Lausanne)* (2018) 9:626. doi: 10.3389/fendo.2018.00626
90. Velkeniers B, Van Meerhaeghe A, Poppe K, Unuane D, Tournaye H, Haentjens P. Levothyroxine Treatment and Pregnancy Outcome in Women With Subclinical Hypothyroidism Undergoing Assisted Reproduction Technologies: Systematic Review and Meta-Analysis of RCTs. *Hum Reprod Update* (2013) 19(3):251–8. doi: 10.1093/humupd/dms052
91. Wang H, Gao H, Chi H, Zeng L, Xiao W, Wang Y, et al. Effect of Levothyroxine on Miscarriage Among Women With Normal Thyroid Function and Thyroid Autoimmunity Undergoing *In Vitro* Fertilization and Embryo Transfer: A Randomized Clinical Trial. *Jama* (2017) 318 (22):2190–8. doi: 10.1001/jama.2017.18249
92. Rao M, Zeng Z, Zhao S, Tang L. Effect of Levothyroxine Supplementation on Pregnancy Outcomes in Women With Subclinical Hypothyroidism and Thyroid Autoimmuneity Undergoing *In Vitro* Fertilization/Intracytoplasmic Sperm Injection: An Updated Meta-Analysis of Randomized Controlled Trials. *Reprod Biol Endocrinol* (2018) 16(1):92. doi: 10.1186/s12958-018-0410-6
93. Dhillon-Smith RK, Middleton LJ, Sunner KK, Cheed V, Baker K, Farrell-Carver S, et al. Levothyroxine in Women With Thyroid Peroxidase Antibodies Before Conception. *N Engl J Med* (2019) 380(14):1316–25. doi: 10.1056/NEJMoa1812537
94. De Leo S, Pearce EN. Autoimmune Thyroid Disease During Pregnancy. *Lancet Diabetes Endocrinol* (2018) 6(7):575–86. doi: 10.1016/s2213-8587(17)30402-3
95. Zhou G, Zhou M, Duan X, Li W. Glucocorticoid Supplementation Improves Reproductive Outcomes in Infertile Women With Antithyroid Autoimmunity Undergoing ART: A Meta-Analysis. *Med (Baltimore)* (2021) 100(16):e25554. doi: 10.1097/MD.0000000000025554
96. Mantovani G, Isidori AM, Moretti C, Di Dato C, Greco E, Ciolli P, et al. Selenium Supplementation in the Management of Thyroid Autoimmunity During Pregnancy: Results of the "SERENA Study", a Randomized, Double-Blind, Placebo-Controlled Trial. *Endocrine* (2019) 66(3):542–50. doi: 10.1007/s12020-019-01958-1

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