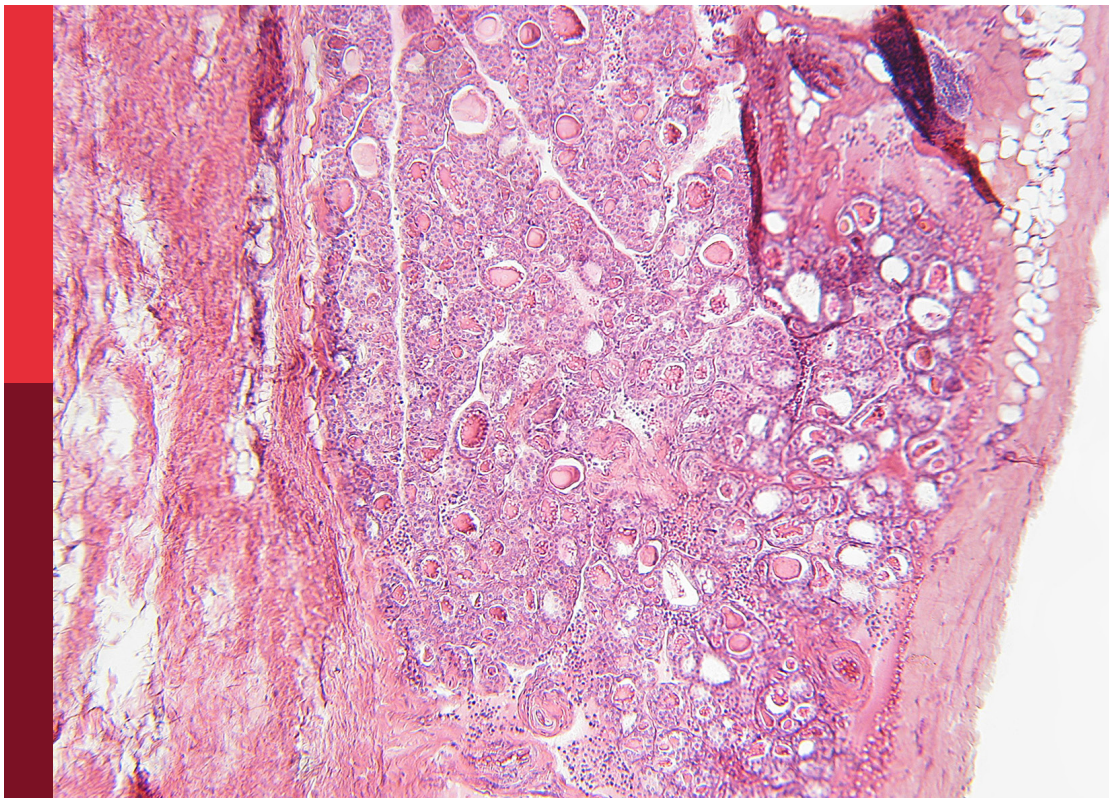
frontiers | Research Topics

Environmental exposures and thyroid health

Edited by
Maaike Van Gerwen, Janete Maria Cerutti
and Catherine Fiona Sinclair

Published in
Frontiers in Endocrinology



ISSN 1664-8714
ISBN 978-2-83251-767-3
DOI 10.3389/978-2-83251-767-3

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Environmental exposures and thyroid health

Topic editors

Maaike Van Gerwen — Icahn School of Medicine at Mount Sinai, United States
Janete Maria Cerutti — Federal University of São Paulo, Brazil
Catherine Fiona Sinclair — Icahn School of Medicine at Mount Sinai, United States

Citation

Van Gerwen, M., Cerutti, J. M., Sinclair, C. F., eds. (2023). *Environmental exposures and thyroid health*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-767-3

Table of contents

frontiers | Frontiers in Endocrinology

TYPE Editorial
PUBLISHED 13 February 2023
DOI 10.3389/fendo.2023.1154547

OPEN ACCESS

EDITED AND REVIEWED BY
Jeff M P Holly,
University of Bristol,
United Kingdom

*CORRESPONDENCE
Maaike van Gerwen
maaike.vangerwen@mountsinai.org

SPECIALTY SECTION
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 30 January 2023
ACCEPTED 06 February 2023
PUBLISHED 13 February 2023

CITATION
van Gerwen M, Cerutti JM and Sinclair CF (2023) Editorial: Environmental exposures and thyroid health.
Front. Endocrinol. 14:1154547.
doi: 10.3389/fendo.2023.1154547

Editorial: Environmental exposures and thyroid health

Maaike van Gerwen[1,2]*, Janete Maria Cerutti[3] and Catherine Fiona Sinclair[1,4]

[1]Department of Otolaryngology-Head and Neck Surgery, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [2]Institute for Translational Epidemiology, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [3]Genetic Bases of Thyroid Tumor Laboratory, Division of Genetics, Department of Morphology and Genetics, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil, [4]Department of Surgery, Monash University, Melbourne, VIC, Australia

KEYWORDS

thyroid, environment, endocrine disruptors, thyroid cancer, thyroid function, pollutants

Editorial on the Research Topic
Environmental exposures and thyroid health

A variety of environmental exposures have been suggested to explain, at least in part, the increasing trends of both thyroid cancer and thyroid disease over recent decades. Thyroid cancer incidence has increased on average 3.6% per year (95% confidence interval (CI): 3.2%- 3.9%) between 1974 and 2013 in the United States (US). (1) This increase has primarily been due to an increase in papillary thyroid cancer diagnoses, which increased on average by 4.4% per year (95% CI: 4.0%- 4.7%). (1) From 2013, average subcentimeter thyroid cancer incidence rates started to decline by -3.7% per year (95% CI: -8.7%- 1.7%), while the average incidence of thyroid cancers measuring more than one centimeter increased at +2.0% per year (95% CI: 1.1%- 2.9%). (2) These increasing papillary thyroid cancer incidence rates have also been reported in other countries suggesting a worldwide phenomenon. (3)

In addition to carcinoma, studies have also shown increasing trends in autoimmune thyroid diseases. A study published in 1972 reported an increasing trend in Hashimoto's thyroiditis, also known as chronic lymphocytic thyroiditis or autoimmune thyroiditis, from 6.5 per 100,000 persons in 1935-1944 to 69.0 per 100,000 persons in 1965-1967 in Minnesota (US). (4) Retrospective review of medical records in an Italian region showed that Hashimoto's thyroiditis became 10 times more common between 1990 and 2005. (5) An increasing annual frequency of Hashimoto's thyroiditis was found at a Sicilian cytological unit (Italy) between 1988 and 2007. (6) These global increasing trends in thyroid cancer and thyroid disease, which cannot be solely explained by increased access, use and quality of diagnostic tools, indicate that modifiable risk factors including exposure to environmental exposures may play a causative role (7). (8, 9) The articles published in this research topic highlight the variety of risk factors and exposures associated with thyroid health.

Medical conditions as endogenous risk factors

Diabetes is the most commonly diagnosed endocrine disorder. (9) Although diabetes and thyroid dysfunction are closely linked conditions, diabetes has not been consistently linked to thyroid cancer. A pooled analysis of five prospective US studies (n= 674,491) showed no association between a self-reported history of diabetes and thyroid cancer risk (HR: 1.08 (95% C: 0.83- 1.40)). (10) However, the recent meta-analysis by Dong et al showed that patients with diabetes had a 1.3-fold increased risk of thyroid cancer (95% CI: 1.22- 1.44) compared to non-diabetes patients; this positive association was found for both males (RR: 1.26 (95% CI: 1.12- 1.41)) and females (RR: 1.36 (95% CI: 1.22- 1.52)). Dong et al. performed a subgroup analysis by type of diabetes and found a 1.34-fold increased risk of thyroid cancer in the population with type 2 diabetes (95% CI: 1.17- 1.53). This systematic review and meta-analysis including 20 cohort studies and more than 300,000 individuals provides evidence that the risk of thyroid cancer was increased in approximately 30% of patients with diabetes.

As previously described, the incidence of both thyroid cancer and Hashimoto's thyroiditis has increased steadily over past decades. Although these increases may be caused by access, use and quality of diagnostic tools, chronically unresolved inflammation, as present in Hashimoto's thyroiditis, has been associated with increased risk of malignant disease. In fact, chronic infection or chronic inflammatory states are thought to be a causal factor in approximately 20% of all human cancers. (11) Xu et al. performed a systematic literature review and meta-analysis to understand the impact of Hashimoto's thyroiditis on the progression of papillary thyroid cancer. The meta-analysis included 39 original research articles and showed that Hashimoto's thyroiditis is a risk factor for thyroid cancer (pooled odds ratio (OR): 1.71 (95% CI: 1.57- 1.80)). On the contrary, prevalence of extrathyroidal extension (pooled OR: 0.79 (95% CI: 0.72- 0.86)), central lymph node metastasis (pooled OR: 0.80 (95% CI: 0.74- 0.87)), distant metastasis (pooled OR: 0.52 (95%: 0.31- 0.87)), *BRAF V600E* mutations (pooled OR: 0.47 (95% CI: 0.43- 0.52)), and recurrence (pooled OR: 0.32 (95% CI: 0.18- 0.58)) were significantly lower in patients with both Hashimoto's thyroiditis and thyroid cancer, suggesting that Hashimoto's thyroiditis is a protective factor against PTC progression. Xu et al. thus demonstrated that Hashimoto's thyroiditis seems to be a "double-edged sword" in thyroid cancer.

Iodine deficiency can have major thyroid-related health consequences including goiter, hypothyroidism, impaired mental function, and delayed physical development. Iodine deficiency before or just after birth has been associated with fetal and infant mortality, congenital anomalies and endemic cretinism. (12) Cretinism is caused by severe iodine deficiency *in utero* and is characterized by gross intellectual disability and varying degrees of short stature, deaf-mutism, and spasticity. (12) Li et al. performed a cross-sectional study including 31 neurological cretins and 85 controls to reassess thyroid status following iodine supplementation after birth. A significantly higher prevalence of subclinical hypothyroidism (P= 0.029) and thyroid nodules (P< 0.0001) was found in the cretin group compared to the control group, which highlights the irreversible impact of iodine deficiency *in utero* on the thyroid gland.

Endocrine disrupting chemicals

Exposure to endocrine disrupting chemicals (EDCs) as a potential modifiable risk factor for thyroid dysfunction and thyroid cancer is of growing interest to researchers. Although exposure to endocrine disrupting chemicals (EDCs) has been associated with changes in thyroid function, the potential carcinogenic effect of EDCs on the thyroid gland remains to be evaluated, as study results are inconsistent. (13)

Iodide uptake mediated by the sodium-iodide symporter (NIS) is identified as the first limiting step involved in the production of thyroid hormones. Perchlorate, nitrate, and thiocyanate competitively inhibit the NIS-mediated iodine uptake, thus modifying iodide uptake and affecting thyroid hormone synthesis. The review by Serrano-Nascimento et al. focusses on these anions, concluding that the impact of exposure to NIS-inhibitors is still inconclusive and controversial and further evaluation is needed.

Ayhan et al. examined the association between *in utero* exposure to chlordecone, an organochlorine insecticide with endocrine disruptive properties, and thyroid function in 124 boys and 161 girls at the age of 7 years. While they found that prenatal exposure to this EDC was associated with elevated levels of thyroid stimulating hormone (TSH) in the third quartile of cord-blood chlordecone concentrations for girls compared to the lowest quartile ($\beta_{adjusted}$: 0.22 (95% CI: 0.01; 0.44)), it was not associated with significant changes in free triiodothyronine (fT3) and free thyroxine (fT4) in girls and boys.

Tang et al. investigated the effect of early life exposure to triclosan, an antimicrobial chemical with potential endocrine disruptive properties, on thyroid hormone levels and histopathological changes of thyroid follicles. Zebrafish were exposed to triclosan at 0 (control), 3, 30, 100, 300, and 900 ng/mL. Increasing triclosan from 3 to 300 ng/mL reduced total triiodothyronine (TT3), fT3 and fT4 levels and induced histopathological changes within thyroid tissues.

To examine the toxic and endocrine disruptive effects of glyphosate-based herbicides in experimental models, Dal' Bó et al. investigated the effects of Roundup®, a glyphosate containing herbicide, on normal and papillary thyroid carcinoma cell lines. Exposure to Roundup® at acceptable occupational exposure levels (160µg/L) caused the death of 43% to 50% of the human thyroid-derived cell lines in 24 hours and 33% in 48 hours. Dal' Bó et al. concluded that Roundup® exposure presents a non-monotonic dual dose-response curve with significant cell death after low dose exposure with important proliferative effects.

Sousa-Vidal et al. assessed the effects of intrauterine exposure to di(2-ethylhexyl) phthalate (DHEP), a widely used EDC in the production of malleable plastics, on the hypothalamus-pituitary-thyroid (HPT) axis in offspring rats in adulthood. A decrease in serum TSH and T4 levels was found in female rats exposed to DHEP *in utero*, while an increase in serum TSH levels was found in

exposed male rats. These results confirm the impact of intrauterine DHEP exposure on the HPT axis and the potential increased susceptibility to develop thyroid dysfunction.

In conclusion, this research topic provides a broad overview of the complex and extensive interplay of endogenous and exogenous exposures potentially involved in thyroid dysfunction and thyroid cancer. It summarizes the recent achievements in disentangling the potential roles of these exposures, thereby providing a basis for future studies to better explore and understand reasons for the increasing trend in the incidence of thyroid cancer and thyroid disease. In turn, this may allow for the development of preventative strategies, personalized management, and exposure health policies to improve thyroid health.

Author contributions

MG, JC and CS contributed to conception and design of the study. MG wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Conflict of interest

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

Publisher's note

References

1. Lim H, Devesa SS, Sosa JA, Check D, Kitahara CM. Trends in thyroid cancer incidence and mortality in the united states 1974-2013. *JAMA* (2017) 317(13):1338–48. doi: 10.1001/jama.2017.2719

2. Powers AE, Marcadis AR, Lee M, Morris LGT, Marti JL. Changes in trends in thyroid cancer incidence in the united state To 2016. *JAMA* (2019) 322(24):2440–1. doi: 10.1001/jama.2019.18528

3. Miranda-Filho A, Lortet-Tieulent J, Bray F, Cao B, Franceschi S, Vaccarella S, et al. Thyroid cancer incidence trends by histology in 25 countries: A population-based study. *Lancet Diabetes Endocrinol* (2021) 9(4):225–34. doi: 10.1016/S2213-8587(21)00027-9

4. Furszyfer J, Kurland LT, McConahey WM, Woolner LB. And elveback, l Epidemiologic aspects of hashimoto's thyroiditis and graves' disease in Rochester, minnesot–1967), with special reference to temporal trends. *R.Metabolism* (1972) 21(3):197–204. doi: 10.1016/0026-0495(72)90041-8

5. Benvenga S, Trimarchi F. Changed presentation of hashimoto's thyroiditis in north-Eastern Sicily and calabria (Southern Italy) based on a 31-year experience. *Thyroid* (2008) 18(4):429–41. doi: 10.1089/thy.2007.0234

6. Rizzo M, Rossi RT, Bonaffini O, Scisca C, Altavilla G, Calbo L, et al. Increased annual frequency of hashimoto's thyroiditis between years 1988 and 2007 at a cytological unit of Sicily. *Ann Endocrinol* (2010) 71(6):525–34. doi: 10.1016/j.ando.2010.06.006

7. Ferrari SM, Fallahi P, Antonelli A, and Benvenga S. Environmental issues in thyroid diseases. *Front Endocrinol (Lausanne)* (2017) 8:50. doi: 10.3389/fendo.2017.00050

8. van Gerwen M, Alsen M, Genden E. It may not all be overdiagnosis: The potential role of environmental exposures in the thyroid cancer incidence increase. *Epidemiology* (2022) 33(5):607–10. doi: 10.1097/EDE.0000000000001519

9. Golden SH, Robinson KA, Saldanha I, Anton B, Ladenson PW. Clinical review: Prevalence and incidence of endocrine and metabolic disorders in the united states: a comprehensive review. *J Clin Endocrinol Metab* (2009) 94(6):1853–78. doi: 10.1210/jc.2008-2291

10. Kitahara CM, Platz EA, Beane Freeman LE, Black A, Hsing AW, Linet MS, et al. Physical activity, diabetes, and thyroid cancer risk: A pooled analysis of five prospective studies. *Cancer Causes Control* (2012) 23(3):463–71. doi: 10.1007/s10552-012-9896-y

11. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* (2010) 140(6):883–99. doi: 10.1016/j.cell.2010.01.025

12. Zimmermann MB, Jooste PL, Pandav CS. Iodine-deficiency disorders. *Lancet.* (2008) 372(9645):1251–62. doi: 10.1016/S0140-6736(08)61005-3

13. Alsen M, Sinclair C, Cooke P, Ziadkhanpour K, Genden E, van Gerwen M. Endocrine disrupting chemicals and thyroid cancer: An overview. *Toxics* (2021) 9(1):14. doi: 10.3390/toxics9010014

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 22 November 2021
doi: 10.3389/fendo.2021.771641

In Utero Chlordecone Exposure and Thyroid, Metabolic, and Sex-Steroid Hormones at the Age of Seven Years: A Study From the TIMOUN Mother-Child Cohort in Guadeloupe

Gülen Ayhan[1], Florence Rouget[2], Frank Giton[3], Nathalie Costet[4], Léah Michineau[4], Christine Monfort[4], Jean-Pierre Thomé[5], Philippe Kadhel[1], Sylvaine Cordier[4], Alejandro Oliva[6] and Luc Multigner[4]*

[1] Centre Hospitalier Universitaire (CHU) de Guadeloupe, Univ Antilles, Inserm, École des Hautes Études en Santé Publique (EHESP), Irset (Institut de recherche en santé, environnement et travail) -UMR_S 1085, Rennes, France, [2] Centre Hospitalier Universitaire (CHU) de Rennes, Univ Rennes, Inserm, École des Hautes Études en Santé Publique (EHESP), Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, Rennes, France, [3] Assistance Publique - Hôpitaux de Paris (AP-HP) Hôpital Henri Mondor, Pôle Biologie-Pathologie, IMRB U955, Créteil, France, [4] Univ Rennes, Inserm, École des Hautes Études en Santé Publique (EHESP), Irset (Institut de recherche en santé, environnement et travail) -UMR_S 1085, Pointe à Pitre, France, [5] LEAE-CART (Laboratoire d'Ecologie Animale et d'Ecotoxicologie-Centre de Recherche Analytique et Technologique), Université de Liège, Liège, Belgium, [6] Center for Interdisciplinary Studies, National University of Rosario, Rosario, Argentina

OPEN ACCESS

Edited by:
Maaike Van Gerwen, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Yi Wang, China Medical University, China
Corina Lesseur, Icahn School of Medicine at Mount Sinai, United States

****Correspondence:***
Gülen Ayhan
gulen.ayhan@inserm.fr

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *01 October 2021*
Accepted: *05 November 2021*
Published: *22 November 2021*

Citation:
Ayhan G, Rouget F, Giton F, Costet N, Michineau L, Monfort C, Thomé J-P, Kadhel P, Cordier S, Oliva A and Multigner L (2021) In Utero Chlordecone Exposure and Thyroid, Metabolic, and Sex-Steroid Hormones at the Age of Seven Years: A Study From the TIMOUN Mother-Child Cohort in Guadeloupe. Front. Endocrinol. 12:771641. doi: 10.3389/fendo.2021.771641

Background: Chlordecone is an endocrine-disrupting chemical with well recognized estrogenic and progestagenic properties. This organochlorine insecticide was extensively used in the French West Indies from 1973 to 1993 to control the banana root borer. Due to its poor degradation in the environment, permanently polluted soil is responsible for the current contamination of the food chain and human beings. We aimed to examine the relationship of *in utero* exposure to chlordecone and thyroid (thyroid stimulating hormone [TSH], free tri-iodothyronine [FT3], free thyroxine [FT4]), metabolic (insulin growth-factor 1, leptin, adiponectin), and sex-steroid (dehydroepiandrosterone [DHEA], total testosterone [TT], dihydrotestosterone [DHT], estradiol [E2]) hormone levels in children at the age of seven years who participated in TIMOUN, an ongoing birth cohort in Guadeloupe.

Methods: Chlordecone concentrations were measured in cord-blood at delivery. Thyroid, metabolic, and sex-steroid hormone levels were determined in the blood of children at seven years of age. Associations between *in utero* chlordecone exposure and hormone levels at seven years of age were assessed by multiple linear or logistic regression, controlling for confounding factors.

Results: Among the study population (210 boys and 228 girls), chlordecone and hormone measurements were available for 124 boys and 161 girls. We found the third quartile of *in utero* chlordecone exposure relative to the lowest quartile to be associated with elevated TSH levels in girls and elevated DHEA, TT, and DHT levels in both sexes. Complementary non-linear analysis (spline regression) confirmed a significant non-linear trend for TSH in girls and DHEA and DHT in boys.

Conclusion: *In utero* chlordecone exposure was associated with elevated levels of selected thyroid (TSH) and sex-steroid (DHEA, TT, and DHT) hormones at seven years in a non-monotonic dose response (inverted U) relationship. The implications for future health and reproductive function in puberty and adulthood should be determined.

Keywords: chlordecone, endocrine disruptors, *in utero* exposure, hormones, children

INTRODUCTION

Chlordecone is a persistent organochlorine insecticide that was extensively used in Guadeloupe and Martinique (French West Indies, FWI) from 1973 to 1993 to fight against the banana root borer (1). This pesticide undergoes no significant biotic or abiotic degradation in the environment (2). Although chlordecone has not been used since 1993, it persists in the soil of current and former banana fields where it has been spread. Simulation studies have shown that it would take up to 7 centuries for soils to be cleaned up through leaching (3). Chlordecone in soil is slowly drained by rainfall towards superficial water, ground water, and marine coastal waters and contaminates the terrestrial and aquatic ecosystems, including crops, livestock, and fishing products (4, 5). Most local animal and vegetable production and consumption is not influenced by the limited seasons that occur in the FWI, as in many tropical Caribbean areas. Therefore, human exposure to chlordecone in the FWI arises from the continuous consumption of contaminated foodstuffs. The analysis of blood samples has shown that a large proportion of the French West Indies population, both infants and adults, are contaminated by chlordecone (1, 6–8). Chlordecone crosses the human placental barrier, thus exposing the fetus during its development (9, 10).

Epidemiological studies conducted in the FWI have reported that chlordecone exposure is associated with an increased risk of prostate cancer in adult men, preterm birth in pregnant women, and altered growth, cognitive, motor or visual development in toddlers and children (7, 9–14).

Several biological mechanisms have been suggested to explain the unwanted effects of chlordecone on health. Chlordecone has the capacity to inhibit brain ATPases, as well as that to interact with multiple neurotransmitters (noradrenaline, dopamine, GABA, etc.) (15). Moreover, estrogenic and progestagenic-like properties have been clearly established both *in vivo* and *in vitro* (16–21). Chlordecone binds to estrogen receptors α (ERα) and β (ERβ), acting as an agonist of ERα and an antagonist of ERβ (22, 23). In addition to its interaction with nuclear ERs, chlordecone may activate alternative estrogen signaling pathways or other enzymes and receptors involved in steroid homeostasis (24–27). These data all support chlordecone an endocrine disruptor.

Endocrine-disrupting chemicals (EDCs) are exogenous agents that interfere with the synthesis, secretion, transport, metabolism, binding action, and/or elimination of natural blood hormones (28). Hormonal homeostasis is essential for a large variety of physiological processes, including growth, development, reproduction, energy balance, metabolism, and the regulation of body weight, among others (29). By interfering with hormonal homeostasis, EDCs may affect such physiological processes and lead to deleterious effects on many endocrine systems, with negative outcomes (30). Among the key characteristics of EDCs are those of being able to alter hormone synthesis, transport, metabolism, and clearance (31). Such alterations can result in changes in circulating hormone levels, which, in turn, can lead to negative health outcomes.

Few studies have investigated the relationship between chlordecone exposure and circulating hormone levels. No association was found among healthy adult Guadeloupian men between chlordecone exposure and circulating levels of dehydroepiandrosterone (DHEA), DHEA-sulphate, androstenedione, androstenediol, total testosterone (TT), free and bioavailable testosterone, dihydrotestosterone (DHT), estrone, E1-sulphate, or estradiol (E2) (32). In the TIMOUN Mother-Child Cohort Study in Guadeloupe, *in utero* exposure to chlordecone has been shown to be associated with increased thyroid stimulating hormone (TSH) levels at three months of age, but only for boys, without modification of free tri-iodothyronine (FT3) or free thyroxine (FT4) levels (33).

The *in utero* period of development is a recognized temporal window of vulnerability during which EDCs may exert their effects, resulting in a large spectrum of disorders, some of which are sexually dimorphic, later in life (30). Here, we investigated the relationship between prenatal (*in utero*) exposure to chlordecone and the circulating levels of thyroidal (TSH, FT3, FT4), metabolic (Insulin growth-factor 1 [IGF-1], adiponectin, leptin), and sex-steroid (DHEA, TT, E2) hormones in children at seven years of age who participated in the follow-up of the TIMOUN Mother-Child cohort study.

MATERIALS AND METHODS

Participants

This study was conducted in Guadeloupe (French West Indies), a Caribbean archipelago. The TIMOUN Mother-Child Cohort was established to investigate the consequences of prenatal (maternal or *in utero*) exposure to chlordecone on pregnancy and child development. From November 2004 to December 2007, 1,068 pregnant women from the general population were enrolled during their third-trimester prenatal visit at public health centers (University Hospital of Pointe-à-Pitre, General Hospital of Basse-Terre, and antenatal care units) (6). Questionnaires were administered at inclusion to assess their social, demographic, occupational, medical, and family characteristics, as well as lifestyle habits. At delivery, data concerning maternal diseases during pregnancy, delivery, and the health status and

anthropometric characteristics of the newborn at birth were collected by the medical staff and maternal and cord blood samples were obtained. Follow-up visits of the children were conducted afterwards at various ages (3, 7, and 18 months) to evaluate the development of the children (9, 10, 33). When the children were seven years of age (May 2011 to October 2015), 1,033 mothers initially included in the cohort were contacted for a follow-up interview and a medical examination of their child. Among them, 589 (57% of the initial cohort) were interviewed and had their child examined at the University Hospital of Guadeloupe. During the medical examination of the children, face-to-face interviews were conducted with their mothers to collect information about the socio-economic context in which the child was growing and the health of the child, including medication intake. A blood sample was also collected from the children at the end of the examination. We excluded five children from the present study because of major congenital anomalies or severe diseases that could affect hormone levels. We then retained those for whom at least one blood hormone concentration was successfully measured at seven years of age, leaving 438 children (210 boys and 228 girls). Among them, a cord-blood chlordecone measurement was available for 285 (124 boys and 161 girls). No children were treated with hormonal or anti-hormonal medications.

The study was approved by the relevant ethical committee for studies involving human subjects (Comité de Protection des Personnes Sud-Ouest et Outremer III (n° 2011-AOOSSI-40). Each parent provided written informed consent.

Blood Collection

Blood samples at birth (umbilical cord blood) and at seven years of age were collected in EDTA tubes and, after centrifugation, plasma samples were conserved in polypropylene Nunc® tubes at -30°C. They were transferred on dry ice by airmail to the Center for Analytical Research and Technology (CART) at Liège University in Belgium for organochlorine analysis and to the steroid mass spectrometry platform of the Mondor Institute for Biomedical Research, Créteil, France, for hormone analysis.

Organochlorine Analyses

Blood samples were analyzed for chlordecone, p,p′−dichlorodiphenyldichloroethylene (DDE, the major and most persistent metabolite of dichlorodiphenyltrichloroethane, DDT), and the non-dioxin-like polychlorinated biphenyl congener 153 (PCB-153) by high-resolution gas chromatography with Ni63 electron capture detection. Detailed information about the sampling, analysis, and quality assurance and control have been provided elsewhere (11, 34). Among PCBs, we selected PCB-153 because it correlates very well with the total PCB concentration in plasma (35). The analytical limit of detection (LD) was 0.06 µg/L for chlordecone in cord blood, 0.05 µg/L for DDE and PCB-153 in cord blood, and 0.02 µg/L for chlordecone in the children's blood.

Hormone Analyses

TSH, FT3, and FT4 were measured by immuno-radiometric assay (IM 3712 TSH Irma Kit, IM 1579 FT3, IM 1363 FT4, Beckman Coulter). Results were expressed as concentrations (milli-international units per liter for TSH and picomoles per milliliter for FT3 and FT4). The intra- and inter-assay coefficients of variation (CVs) were ≤3.7 and ≤8.6 for TSH, ≤6.4 and ≤5.5 for FT3, and ≤10.29 and ≤7.58% for FT4, respectively.

Insulin growth-factor 1 (IGF-1) was measured by radio-immunoassay assay (IGF1-RIACT, Cisbio Bioassays, Codolet, France) and concentrations are expressed in ng per milliliter. The intra-assay CV was ≤3.8 and the inter-assay CV ≤8.2%. Leptin was measured by enzyme-linked immuno-sorbent assay (BioVendor Human Leptin ELISA, Brno, Czech Republic) and concentrations are expressed in ng per milliliter. The intra-assay CV was ≤7.6 and the inter-assay CV ≤6.7%. Total adiponectin was measured by enzyme-linked immunosorbent assay (ALPCO, Salem, NH, USA) and concentrations are expressed in µg per milliliter. The intra-assay CV was ≤5.7 and the inter-assay CV ≤6.4%.

DHEA, TT, DHT, and E2 were assayed simultaneously by gas chromatography-mass spectrometry, as previously described (36). Briefly, deuterated steroid internal standards (CDN Isotopes, Inc., Point- Claire, Quebec, Canada) were added to all plasma samples, which were then extracted with 1-chlorobutane. The organic extracts were purified on conditioned high-purity silica LC-Si SPE columns (Varian, Les Ulis, France). All steroids were derivatized with pentafluorobenzoyl chloride (77253-1ml, Sigma-Aldrich, Steinheim, Germany). The final extracts were reconstituted in isooctane and then transferred to conical vials for injection into the GC system (GC-2010 Plus, Shimadzu, Japan), equipped with a 50% phenylmethylpolysiloxane VF-17MS capillary column (20 m x0.15 mm, internal diameter, 0.15 mm film thickness; Agilent Technologies, Les Ulis, France). A TQ8050 (Shimadzu, Japan) triple quadrupole mass spectrometer equipped with a chemical ionization source and operating in Q3 single-ion monitoring mode was used for detection. Concentrations were reported for DHEA in nmol per liter and for DHT, TT, and E2 in pmol per liter. The intra- and inter-assay CVs were ≤3.5 and ≤4.7 for DHEA, ≤2.2 and ≤2.1 for TT, ≤3.0 and ≤3.1 for DHT, and ≤3.5 and ≤4.1% for E2, respectively.

Lipid Analysis

Total cord plasma cholesterol and triglyceride concentrations were determined enzymatically (DiaSys Diagnostic Systems GmbH; Holzheim, Germany) and the total lipid concentration calculated as previously described (37).

Data and Statistical Analysis

All analyses were stratified by sex because of gender differences in hormone production and potential sexual dimorphism related to the effect of chlordecone. Continuous variables are described as means, medians, inter-quartile ranges, and percentiles. Mean ranks between unpaired groups were compared using the Mann Whitney test in descriptive bivariate analyses.

Cord-blood chlordecone concentrations were considered to be categorical (quartiles, based on their distribution in the

population study) or continuous variables after $\log_{10}$ transformation. Chlordecone values below the LOD were imputed by a maximum likelihood estimation method (38).

Associations between *in utero* chlordecone exposure and hormones with ≥90% detectable values (TSH, FT3, FT4, DHEA, IGF-1, and adiponectin for both sexes, and leptin for girls) were analyzed by multiple linear regression, allowing calculation of the β regression coefficient and its 95% confidence interval (95%CI). Hormones were included in the model after the imputation of values < LOD (38) and a $\log_{10}$ transformation, as they were log-normally distributed. Hormones with 14.3 to 72.6% detectable values (DHT, TT, and E2 for both sexes, and leptin for boys; see **Table 3**) were dichotomized according to their LOD (< LOD *vs* ≥ LOD) and potential associations with *in utero* chlordecone exposure analyzed using multiple logistic regression models, allowing estimation of the odds ratio (OR) and its 95%CI.

The following maternal covariates were considered to be potential confounding factors: age at delivery (years), geographic origin (Caribbean *vs* European), body mass index (BMI, kg/m^2), weight gain during pregnancy (insufficient, normal, excessive, or very excessive, according to the guidelines of the Institute of Medicine (39), education (< 12 years schooling *vs* ≥ 12 years schooling), smoking during pregnancy (never *vs* ever), and alcohol consumption during pregnancy (never *vs* ever). We also considered the following child covariates: exact age at examination (years), z-score height, z-score BMI, preterm birth (yes, no), small for gestational age (tenth percentile (40); yes, no), breastfeeding (yes, no), time of day when blood was drawn, and total cord-plasma lipid concentration (g/L). The characteristics of the samples were taken into account, as hemolysis was present in some (ranked by visual inspection as none or light, moderate, or strong) and could have affected the hormonal analysis. These covariates, including hemolysis, were included in all statistical models and then selected by applying a backward stepwise elimination procedure at $P < 0.2$. Missing values for each covariate were coded as an indicator variable to indicate missing. The Hosmer-Lemeshow goodness-of-fit test was used for the final logistic models.

We also implemented general additive models (GAM), including restricted cubic splines, to fit a potential non-linear association between *in utero* chlordecone exposure and hormone levels (41). Analyses were carried out using Stata 15.1 (College Station, Texas, USA) for linear and logistic regression modeling and SAS (SAS Institute Inc., North Carolina, USA) for GAM modeling. All tests were two-tailed, and P values <0.05 were considered statistically significant.

RESULTS

The mothers' and children's characteristics according to the sex of the child are presented in **Table 1**. Children included in the study had an average age of 7.7 years for boys and 7.6 years for girls. The geographical origin of the family was predominantly the Caribbean islands (~97%). The mothers' mean age at delivery was 32 years and very few reported alcohol consumption (~3%) or smoking (~2%) during pregnancy. Thirteen percent of the children were born preterm.

TABLE 1 | General characteristics of the study population according to sex.

VARIABLE	BOYS (N = 210)	GIRLS (N = 228)
Mothers' characteristics		
Age at delivery (mean, min, max)	32.0 (16.2, 45.1)	32.0 (15.1, 44.8)
Geographic origin		
Caribbean Islands	204 (97.1)	219 (96.0)
Europe	6 (2.9)	9 (4.0)
BMI in early pregnancy (mean, min, max)	24.8 (17.1, 40.7)	25.7 (15.6, 50.0)
Weight gain during pregnancy (n, %)		
Insufficient	66 (32.2)	58 (25.8)
Normal	28 (13.7)	26 (11.5)
Excessive	53 (25.8)	78 (34.7)
Very excessive	58 (28.3)	63 (28.0)
Education level (n, %)		
< 12 years schooling	112 (53.3)	117 (51.3)
≥ 12 years schooling	98 (46.7)	111 (48.7)
Smoking during pregnancy (n, %)	6 (2.9)	7 (3.1)
Alcohol during pregnancy (n, %)	4 (2.0)	5 (2.3)
Childs' characteristics		
Age (mean, min, max)	7.7 (7.1, 8.1)	7.6 (7.1, 8.2)
Z-score height (mean, min, max)	0.9 (-1.9, 3.8)	1.0 (-1.4, 3.6)
Z-score BMI (mean, min, max)	0.2 (-3.8, 3.7)	0.3 (-3.1, 2.9)
Prematurity (n, %)	29 (13.8)	28 (12.3)
Small for gestational age (n, %)	24 (11.4)	17 (7.5)
Breastfeeding (n, %)	183 (87.1)	192 (84.2)
Total cord blood lipids (mean, interquartile range) (g/L)	2.24 (1.82, 2.41)	2.29 (1.75, 2.55)

The distributions of organochlorine contaminants in cord blood and the blood of the children at seven years of age are presented in **Table 2**. The detection frequency and median concentration of chlordecone in cord blood was 79% and 0.25 µg/L for boys, and 76.4% and 0.21 µg/L for girls. At seven years of age, chlordecone was detected in 73.8% (median concentration = 0.06 µg/L) of the blood samples of boys and 71.1% (0.05 µg/L) of those of girls. DDE and PCB-153 were also detected in 79.5% (median concentration = 0.22 µg/L) and 56.7 (0.07 µg/L) of the cord blood samples of boys and 84.9% (0.31 µg/L) and 52.2% (0.06 µg/L) of those of girls, respectively. We observed no differences according to sex, regardless of the contaminants considered (**Table 2**). Spearman's rank correlation values (rho) between cord chlordecone and DDE or PCB-153 levels were 0.16 ($P = 0.002$) and -0.01 ($P = 0.83$), respectively. Cord-blood and child chlordecone concentrations were poorly correlated (rho = 0.09, $P = 0.14$).

The distribution of hormone concentrations in the blood of the children at seven years of age is presented in **Table 3**. The detection frequency was 100% for TSH, FT4, FT3, IGF-1, and adiponectin for both sexes. The detection frequency for DHEA was 94.1% for boys and 99.1% for girls, whereas it was 91.7% for leptin for girls. The detection frequency was between 14.3 and 72.6% for the other hormones (DHT, TT, and E2 for both sexes,

TABLE 2 | Detection and concentrations (µg/l) of organochlorine contaminants in cord and child blood samples.

Organochlorine	N	Detection frequency (%)	Minimum	p5	p25	p50	p75	p95	Maximum	P^a
Cord blood										
Chlordecone										
Boys	124	79.0	< DL	< DL	0.08	0.25	0.41	1.46	12.5	0.46
Girls	161	76.4	< DL	< DL	0.07	0.21	0.37	1.44	29.8	
DDE										
Boys	127	79.5	< DL	< DL	0.09	0.22	0.64	2.71	7.78	0.26
Girls	159	84.9	< DL	< DL	0.10	0.31	0.74	3.22	12.5	
PCB-153										
Boys	127	56.7	< DL	< DL	< DL	0.07	0.15	0.46	1.31	0.69
Girls	159	52.2	< DL	< DL	< DL	0.06	0.14	0.57	1.75	
Child blood										
Chlordecone										
Boys	210	73.8	< DL	< DL	< DL	0.06	0.11	0.37	7.01	0.37
Girls	225	71.1	< DL	< DL	< DL	0.05	0.11	0.30	2.15	

DL, Detection limits. Cord chlordecone: 0.06 µg/L, cord DDE: 0.05 µg/L, cord PCB-153: 0.05 µg/L, child chlordecone: 0.02 µg/L.
[a]*Mann-Whitney U test.*

and leptin for boys). Girls showed significantly higher plasma levels of FT3, DHEA, TT, DHT, E2, IGF-1, and leptin than boys, whereas TSH, FT4, and adiponectin did not differ according to sex (**Table 3**). TSH, FT3 and FT4 levels were within normal range expected at the age of seven years (42). For the other hormones, available information is scarce and there are no well-established reference values in large populations of children at the age of seven years.

The results of crude and adjusted linear or logistic regression analyses for *in utero* chlordecone exposure are presented in

TABLE 3 | Detection and concentrations of hormones in child blood samples according to sex.

Hormones	N	Detection frequency (%)	Minimum	p5	p25	p50	p75	p95	Maximum	P^a
TSH (mUI/L)										
Boys	210	100	0.59	0.99	1.49	2.10	2.88	4.19	7.15	0.22
Girls	226	100	0.35	0.85	1.44	2.00	2.63	4.28	6.63	
FT3 (pmol/L)										
Boys	210	100	2.90	4.90	5.50	6.20	6.90	8.10	12.7	0.05
Girls	228	100	3.70	4.70	5.60	6.40	7.10	9.40	12.8	
FT4 (pmol/L)										
Boys	210	100	13.2	14.9	15.9	16.9	18	20	21.6	0.70
Girls	228	100	13.3	14.6	15.6	16.85	18.2	20.3	26.9	
DHEA (nmol/L)										
Boys	203	94.1	< DL	< DL	1.63	2.93	5.03	8.94	19.9	0.01
Girls	219	99.1	< DL	1.00	2.23	3.44	5.69	10.1	29.0	
DHT (pmol/L)										
Boys	203	61.1	< DL	< DL	< DL	38.9	76.9	176.5	959.0	0.002
Girls	219	72.6	< DL	< DL	< DL	55.2	108.1	270.3	1112.3	
TT (pmol/L)										
Boys	203	36.0	< DL	< DL	< DL	< DL	112.2	261.7	438.9	0.02
Girls	219	46.1	< DL	< DL	< DL	< DL	140.1	264.4	712.2	
E2 (pmol/L)										
Boys	203	14.3	< DL	< DL	< DL	< DL	< DL	7.06	13.7	<0.0001
Girls	219	43.4	< DL	< DL	< DL	< DL	7.50	15.4	34.8	
IGF-1 (ng/mL)										
Boys	210	100	97	144	174	218.5	254	318	440	<0.0001
Girls	228	100	113	151	206.5	253.5	303.5	415	529	
Leptin (ng/mL)										
Boys	210	70.0	< DL	< DL	< DL	1.70	3.41	14.3	36.0	<0.0001
Girls	228	91.7	< DL	< DL	1.96	3.68	8.48	23.2	45.6	
Adiponectin (µg/mL)										
Boys	210	100	2.05	2.65	3.90	4.73	5.75	8.00	11.3	0.13
Girls	228	100	2.05	3.15	4.05	5.00	6.08	8.15	12.4	

DL, Detection limits. DHEA: 0.3 nmol/L, DHT: 28.3 pmol/L, testosterone 81.3 pmol/L, estradiol: 5.1 pmol/L, leptin: 1.0 ng/mL.
[a]*Mann-Whitney U test.*

Tables 4 to 6 for thyroid, metabolic, and sex-steroid hormones, respectively.

Multiple linear regression analysis showed TSH levels to be significantly higher in the third quartile of cord-blood chlordecone concentrations for girls than those in the lowest quartile (adjusted model, β = 0.22, 95%CI = 0.01-0.44; **Table 4**). Non-linear modelling using restricted cubic splines of the associations between cord-blood chlordecone concentrations (as continuous variables) and TSH levels in girls showed a significant non-linear trend (adjusted model, P = 0.04) (**Figure 1**). We obtained comparable results following additional adjustments for cord blood DDE or child blood chlordecone concentrations in the multivariable models (β = 0.18, 95%CI = -0.03-0.39, and β = 0.23, 95%CI = 0.01-0.45 for the third quartile relative to the lowest for cord-blood DDE and child blood chlordecone, respectively) (**Supplemental Table S1**). By contrast, we observed no association between *in utero* chlordecone exposure (as continuous values or categorized by quartiles) and FT4 or FT3 levels for either sex, regardless of the adjustment model (**Table 4** and **Supplemental Table S1**).

There were no significant associations between cord-blood chlordecone concentration (as continuous or categorized variable) and any metabolic hormones, whatever the adjustment model (**Table 5** and **Supplemental Table S2**).

In terms of sex-steroid hormones, we found significantly higher levels in the third quartile of cord blood chlordecone concentration than in the lowest quartile for DHEA (adjusted model, β = 0.54, 95%CI = 0.08-1.01, for boys; β = 0.36, 95%CI = 0.02-0.71, for girls), TT (adjusted model, OR = 3.22, 95%CI = 1.08-9.6, for boys; OR = 3.28, 95%CI = 1.32-8.17, for girls), and DHT (adjusted model, OR = 3.70, 95%CI = 1.29-10.6, for boys; OR = 3.20, 95%CI = 1.01-10.2, for girls) (**Table 6**). Supplementary adjustments for cord blood DDE or child blood chlordecone concentrations had a minimal impact on the estimates (**Supplemental Table S3**). By contrast, we observed no associations concerning E2, regardless of the adjustment model (**Table 6** and **Supplemental Table S3**). Non-linear modelling of the associations between cord blood chlordecone exposure as a continuous variable and sex-steroid hormone levels showed a significant non-linear trend for DHEA (P = 0.004) and DHT (P = 0.003) and a non-significant non-linear

TABLE 4 | Associations between *in utero* (cord blood) chlordecone exposure and thyroid hormone concentrations at seven years of age in children of the TIMOUN cohort.

Hormone	Sex (N)	Chlordecone (µg/L)	Unadjusted			Adjusted[a]		
			ß[b]	95% CI	*P*	ß[b]	95% CI	*P*
TSH [b] (mIU/L) (log_{10})	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	0.12	-0.12; 0.36	0.32	0.20	-0.04; 0.44	0.10
		0.20-0.40	0.01	-0.21; 0.23	0.92	0.05	-0.17; 0.26	0.68
		>0.40	0.02	-0.20; 0.24	0.85	0.10	-0.13; 0.33	0.38
		Log10	-0.01	-0.07; 0.04	0.71	-0.01	-0.05; 0.06	0.82
	Girls (159)	<0.07	Ref.			Ref.		
		0.07-0.19	0.10	-0.11; 0.32	0.34	0.10	-0.11; 0.32	0.51
		0.20-0.40	0.19	-0.02; 0.40	0.08	0.22	0.01; 0.44	0.04
		>0.40	0.05	-0.16; 0.27	0.63	0.08	-0.15; 0.30	0.50
		Log10	0.02	-0.04; 0.07	0.53	0.02	-0.03; 0.08	0.39
FT3 [b] (pmol/mL) (log_{10})	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	-0.32	-0.93; 0.28	0.29	-0.22	-0.76; 0.32	0.43
		0.20-0.40	0.14	-0.42; 0.70	0.62	0.03	-0.46; 0.52	0.92
		>0.40	0.24	-0.33; 0.81	0.41	-0.06	-0.57; 0.44	0.80
		Log10	0.11	-0.03; 0.26	0.11	0.05	-0.08; 0.17	0.45
	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	0.30	-0.23; 0.83	0.27	0.32	-0.21; 0.85	0.24
		0.20-0.40	0.37	-0.15; 0.89	0.16	0.42	-0.11; 0.94	0.12
		>0.40	0.18	-0.36; 0.71	0.52	0.18	-0.36; 0.73	0.51
		Log10	0.07	-0.07; 0.20	0.33	0.08	-0.06; 0.22	0.27
FT4 [b] (pmol/mL) (log_{10})	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	-0.32	-1.15; 0.50	0.44	-0.22	-1.08; 0.63	0.61
		0.20-0.40	-0.18	-0.95; 0.58	0.63	-0.25	-1.00; 0.51	0.52
		>0.40	-0.28	-1.06; 0.50	0.48	-0.35	-1.12; 0.41	0.36
		Log10	0.04	-0.15; 0.23	0.69	0.02	-0.17; 0.21	0.83
	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	-0.22	-1.06; 0.62	0.60	-0.13	-0.95; 0.69	0.76
		0.20-0.40	0.08	-0.75; 0.90	0.86	0.21	-0.61; 1.02	0.61
		>0.40	0.35	-0.50; 1.20	0.42	0.52	-0.32; 1.35	0.23
		Log10	0.11	-0.10; 0.32	0.30	0.17	-0.04; 0.38	0.12

[a]*The covariates for which we adjusted: For TSH boys: mothers' BMI in early pregnancy, alcohol during pregnancy; For TSH girls: alcohol during pregnancy, breastfeeding; For FT3 boys: geographic origin, mothers' age at delivery, breastfeeding, alcohol during pregnancy; For FT3 girls: geographic origin, mothers age at delivery, alcohol during pregnancy; For FT4 boys: mothers' age at delivery, breastfeeding, z-BMI; For FT4 girls: z-BMI.*

[b]*Beta coefficient of regression.*

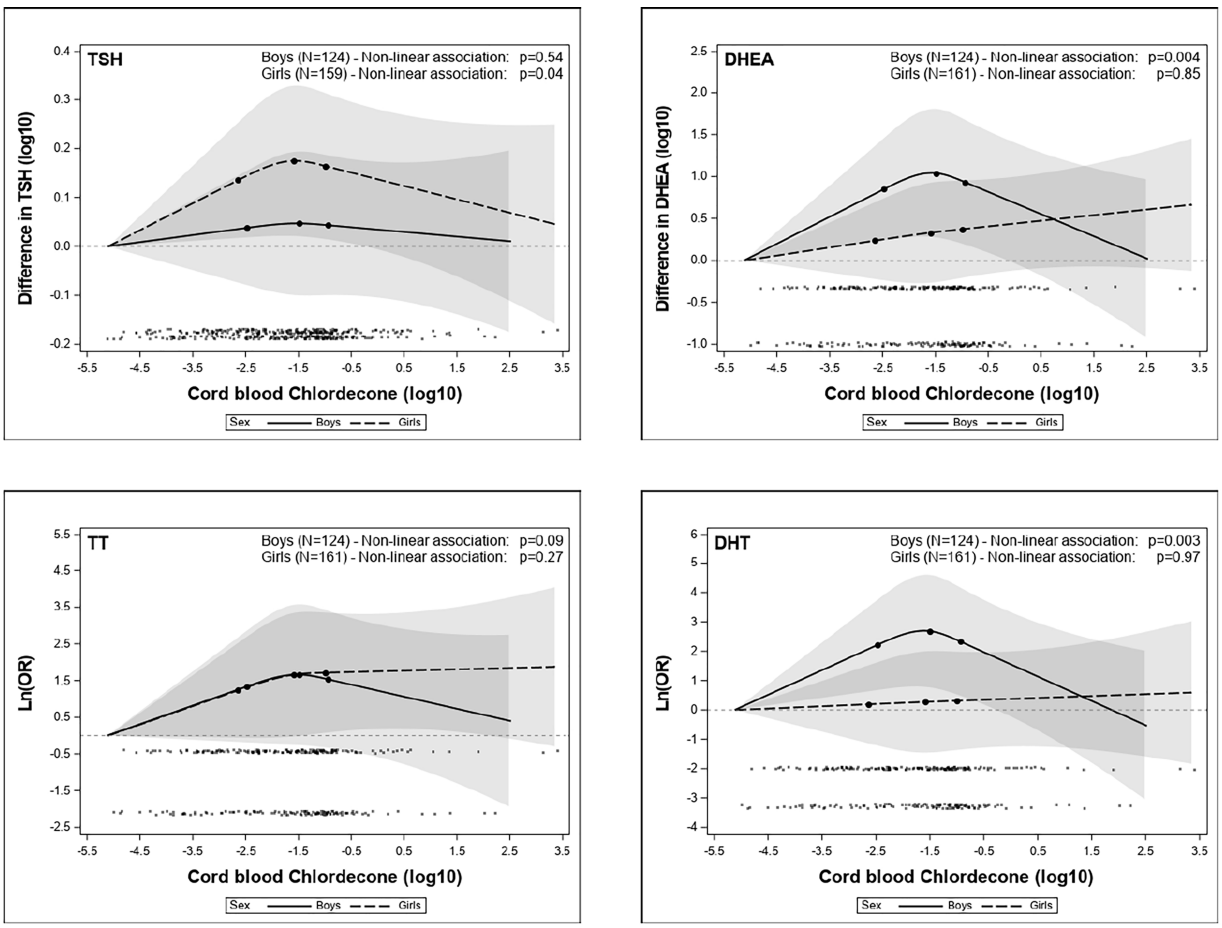


FIGURE 1 | Exposure-response associations (adjusted generalized additive models, restricted cubic splines). Horizontal axis: $\log_{10}$ (cord blood chlordecone concentration, µg/L). Vertical axis: difference in $\log_{10}$ TSH (mIU/L), $\log_{10}$ DHEA (nmol/L), Ln (OR) TT (above *vs* below the detection limit), and Ln (OR) DHT (above *vs* below the detection limit) estimated for various levels of exposure compared to the minimal exposure observed. Black points at the bottom of the graph represent the observed values of chlordecone concentrations.

trend for TT (P = 0.09) for boys only in adjusted models (**Figure 1**).

DISCUSSION

In the TIMOUN Mother-Child Cohort Study, we examined the association between prenatal (*in utero*) chlordecone exposure and thyroid, metabolic, and sex-steroid hormone levels in children at seven years of age. This age is a critical period during the process of child development, as it occurs at the end of the period of adiposity rebound (between five and seven years) (43) and before the onset of puberty, corresponding to the pre-pubertal surge from the adrenal gland (adrenarche) (44).

Blood chlordecone levels were lower in children at seven years of age than those observed at birth. Cord blood levels resulted from the *in-utero* transfer from mothers to their babies during the period of inclusion of pregnant women (2004–2007). Chlordecone levels in children at seven years of age (2011–2015) are the sum of what remains of the body burden at birth, in addition to continuous postnatal exposure. Considering the half-life of chlordecone in blood (5 to 6 months) (2), most of the *in utero* contribution had disappeared at seven years of age. Since 2005, the health authorities have been implementing preventive measures to reduce chlordecone exposure of the population. This has resulted in a reduction in chlordecone exposure, as estimated by blood measurements in the general population (8).

We found a non-monotonic (inverted-U) association between *in utero* exposure to chlordecone, assessed by concentrations in cord blood, and TSH levels for girls and DHEA, TT, and DHT levels for both boys and girls. Only the third quartile of *in utero* exposure was associated with significantly increased hormone levels. Supplementary adjustments for *in utero* DDE or postnatal chlordecone exposure did not change the results. Complementary non-linear analysis (spline regression) confirmed a significant non-

TABLE 5 | Associations between *in utero* (cord blood) chlordecone exposure and metabolic hormone concentrations at seven years of age in children of the TIMOUN cohort.

Hormone	Sex (N)	Chlordecone (µg/L)	Unadjusted			Adjusted [a]		
			ß[b] or OR[c]	95% CI	*P*	ß[b] or OR[c]	95% CI	*P*
IGF-1 [b] (ng/mL) (log_{10})	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	4.79	-24.6; 34.2	0.75	-7.20	-35.41; 21.01	0.61
		0.20-0.40	7.92	-19.2; 35.1	0.57	-1.96	-27.14; 23.23	0.88
		>0.40	1.65	-26.1; 29.4	0.91	-3.34	-28.79; 22.11	0.80
		Log10	1.63	-5.26; 8.51	0.64	-0.22	-6.57; 6.13	0.95
	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	-16.1	-49.1; 16.9	0.34	-15.6	-47.0; 15.8	0.33
		0.20-0.40	-13.1	-45.6; 19.3	0.42	-16.3	-47.6; 14.9	0.30
		>0.40	-9.4	-42.8; 24.1	0.58	-16.8	-49.1; 15.4	0.31
		Log10	0.35	-8.03; 8.73	0.93	-3.51	-11.6; 4.54	0.39
Adiponectin [b] (µg/mL) (log_{10})	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	0.62	-0.29; 1.53	0.18	0.79	-0.14; 1.72	0.10
		0.20-0.40	0.52	-0.32; 1.36	0.23	0.67	-0.17; 1.51	0.12
		>0.40	0.62	-0.24; 1.48	0.16	0.68	-0.17; 1.53	0.12
		Log10	0.15	-0.06; 0.36	0.17	0.18	-0.04; 0.39	0.11
	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	-0.32	-1.05; 0.41	0.39	-0.36	-1.08; 0.37	0.34
		0.20-0.40	-0.35	-1.07; 0.36	0.33	-0.32	-1.04; 0.39	0.37
		>0.40	0.06	-0.68; 0.80	0.87	0.06	-0.68; 0.79	0.88
		Log10	0.03	-0.15; 0.22	0.72	0.04	-0.15; 0.22	0.68
Leptin [c] (<LOD *vs* ≥ LOD)	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	1.75	0.45; 6.84	0.42	0.82	0.16; 4.30	0.81
		0.20-0.40	0.72	0.24; 2.19	0.57	0.36	0.09; 1.45	0.15
		>0.40	0.73	0.24; 2.26	0.59	0.40	0.10; 1.69	0.21
		Log10	0.86	0.65; 1.15	0.32	0.74	0.50; 1.08	0.12
Leptin [b] (ng/mL) (log_{10})	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	0.29	-0.16; 0.75	0.20	0.10	-0.15; 0.36	0.42
		0.20-0.40	0.30	-0.14; 0.75	0.18	0.02	-0.24; 0.27	0.91
		>0.40	0.29	-0.17; 0.75	0.22	0.004	-0.26; 0.26	0.98
		Log10	0.10	-0.02; 0.21	0.10	-0.003	-0.07; 0.06	0.94

[a]*The covariates for which we adjusted: For IGF-1 boys: geographical origin, mothers' age at delivery, z-BMI; For IGF-1 girls: geographical origin, mothers' age at delivery, mothers' BMI in early pregnancy, child z-score BMI; For Adiponectin boys: geographical origin, child z-score BMI; For Adiponectin girls: breastfeeding; For Leptin boys: mothers age at delivery, mothers education level, child z-score BMI; For Leptin girls: geographical origin, child z-score BMI.*

[b]*Beta coefficient of regression;*

[c]*Odds ratio.*

linear trend for TSH levels in girls and for DHEA and DHT levels in boys, as well as a statistically non-significant trend for TT levels in boys.

Thyroid Hormones

In the TIMOUN Mother-Child Cohort Study, we previously reported that cord-blood chlordecone concentrations were monotonously associated with increased TSH levels at three months of age in boys only, without modification of FT4 or FT3 levels (33). At seven years, we no longer observed such a profile in boys. However, we observed this profile in girls but with a non-monotonic dose-response pattern. In our population study, 7 (4.4%) of the 159 girls showed a slight increase in TSH levels to between 4.5 and 6.6 mIU/L, with strictly normal free thyroid hormone levels. The clinical importance of such a slight increase in plasma TSH levels (below 10 mIU/L) and the precise upper limit of the normal range for plasma TSH levels is still debated (45, 46). Although we cannot predict the clinical significance of our observations, the natural history of slight TSH elevations in healthy children who were not being drug treated show spontaneous normalization of TSH values (47). The biological mechanisms by which chlordecone could affect the thyroid axis are still unknown. A series of *in vivo* studies reported thyroid disruption in embryo and adult rare minnows exposed to chlordecone (48). However, complementary *in vitro* and *in silico* experiments showed only weak potency for the interaction of chlordecone with thyroid-related proteins (including thyroid receptors α and β) and suggested that thyroid alterations could be attributed to its interactions with ERs (48). Thus, the observed thyroid alterations in fish may have resulted from the well-recognized estrogenic activity of chlordecone. Whether a similar biological mechanism by which chlordecone could affect the thyroid axis in humans, and possibly differentially according to sex, is yet to be established.

Metabolic Hormones

We did not observe any association between *in utero* chlordecone exposure and metabolic hormone levels for either sex in our study population. To date, no experimental or toxicological studies have addressed the question of possible relationships between

TABLE 6 | Associations between *in utero* (cord blood) chlordecone exposure and steroid hormone concentrations at seven years of age in children of the TIMOUN cohort.

Hormone	Sex(N)	Chlordecone (µg/L)	Unadjusted			Adjusted[a]		
			ß[b] or OR[c]	95% CI	*P*	ß[b] or OR[c]	95% CI	*P*
DHEA [b] (nmol/L) (log_{10})	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	0.20	-0.31; 0.72	0.44	0.26	-0.24; 0.75	0.30
		0.20-0.40	0.54	0.06; 1.03	0.03	0.54	0.08; 1.01	0.02
		>0.40	0.37	-0.12; 0.86	0.13	0.39	-0.08; 0.86	0.11
		Log10	0.05	-0.08; 0.17	0.47	0.04	-0.08; 0.16	0.51
	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	0.20	-0.16; 0.55	0.27	0.18	-0.17; 0.52	0.32
		0.20-0.40	0.41	0.06; 0.75	0.02	0.36	0.02; 0.71	0.04
		>0.40	0.29	-0.07; 0.64	0.11	0.22	-0.13; 0.57	0.21
		Log10	0.09	0.004; 0.18	0.04	0.08	-0.01; 0.16	0.09
DHT [c](<LOD *vs* ≥ LOD)	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	1.97	0.67; 5.78	0.22	1.81	0.61; 5.39	0.29
		0.20-0.40	3.69	1.29; 10.56	0.02	3.70	1.29; 10.6	0.02
		>0.40	1.16	0.43; 3.15	0.77	1.17	0.43; 3.19	0.76
		Log10	0.99	0.77; 1.28	0.96	0.99	0.77; 1.28	0.96
	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	0.80	0.31; 2.03	0.63	0.81	0.31; 2.17	0.68
		0.20-0.40	2.64	0.88; 7.90	0.08	3.20	1.01; 10.2	0.05
		>0.40	1.05	0.40; 2.79	0.92	1.13	0.41; 3.15	0.81
		Log10	1.07	0.83; 1.37	0.63	1.08	0.83; 1.41	0.57
Testosterone [c](<LOD *vs* ≥ LOD)	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	1.02	0.34; 3.04	0.97	1.07	0.32; 3.58	0.91
		0.20-0.40	2.29	0.84; 6.23	0.11	3.22	1.08; 9.58	0.04
		>0.40	0.94	0.33; 2.63	0.90	1.33	0.43; 4.07	0.62
		Log10	1.03	0.80; 1.33	0.82	1.12	0.85; 1.47	0.43
	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	1.67	0.68; 4.06	0.26	1.89	0.76; 4.72	0.17
		0.20-0.40	3.11	1.27; 7.62	0.01	3.28	1.32; 8.17	0.01
		>0.40	2.09	0.83; 5.09	0.12	1.96	0.78; 4.88	0.15
		Log10	1.27	1.01; 1.61	0.04	1.25	0.98; 1.59	0.07
Estradiol [c](<LOD *vs* ≥ LOD)	Boys (124)	<0.07	Ref.			Ref.		
		0.07-0.19	0.70	0.17; 2.81	0.61	0.65	0.16; 2.65	0.55
		0.20-0.40	1.10	0.33; 3.61	0.88	1.14	0.34; 3.81	0.83
		>0.40	1.03	0.30; 3.52	0.96	1.05	0.30; 3.61	0.94
		Log10	0.99	0.73; 1.36	0.96	1.00	0.73; 1.37	0.99
	Girls (161)	<0.07	Ref.			Ref.		
		0.07-0.19	0.67	0.27; 1.62	0.37	0.72	0.29; 1.77	0.47
		0.20-0.40	1.26	0.53; 3.00	0.60	1.36	0.56; 3.26	0.50
		>0.40	0.90	0.37; 2.19	0.82	0.88	0.36; 2.16	0.78
		Log10	1.05	0.84; 1.31	0.70	1.03	0.82; 1.30	0.78

[a]*The covariates for which we adjusted: For DHEA boys: child z-score BMI; For DHEA girls: geographical origin; For DHT boys: breastfeeding; For DHT girls: mothers' age at delivery, smoking during pregnancy, child z-score BMI; For Testosterone boys: geographical origin, alcohol during pregnancy, breastfeeding; For testosterone girls: geographical origin, mothers' age at delivery; For Estradiol boys: mothers' BMI in early pregnancy; For estradiol girls: mothers' age at delivery.*

[b]*Beta coefficient of regression;*

[c]*Odds ratio.*

chlordecone exposure or its effects and metabolic hormones. During the follow-up of the TIMOUN Mother-Child Cohort at the age of seven years, we found no clear evidence supporting an adipogenic effect of *in utero* chlordecone exposure. Despite significantly higher adiposity in the third quartile of *in utero* chlordecone exposure (particularly in boys), we were unable to formally establish a significant non-linear trend (Costet N, Lafontaine A, Rouget F, Michineau L, Monfort C, Thomé JP, Kadhel P, Multigner L, Cordier S; unpublished data). Given the present results concerning metabolic hormones, it appears unlikely that these hormones mediate changes in adiposity, if any, in response to chlordecone exposure.

Sex Steroid Hormones

We observed increased levels of DHEA, TT, and DHT in children at seven years of age, but not E2, for the same third quartile of cord-blood chlordecone exposure relative to the lowest quartile. These hormones are located in the successive classical pathways of sex steroid production that include androstenedione and androstenediol, two alternative intermediate steps between DHEA and TT that we did not measure in the present study (29). Such relationships suggest that the increase in TT and DHT levels may result from an initial increase in the level of the substrate DHEA, consistent with the law of mass action, although increased enzyme activity in these

pathways (3-β-hydroxy steroid dehydrogenase, 17-β- hydroxy steroid dehydrogenase, 5-α reductase) cannot be excluded. However, E2 levels were not modified, regardless of the level of *in utero* chlordecone exposure. Chlordecone is a recognized inhibitor of aromatase, the enzyme that converts TT to E2 (26). Thus, we cannot exclude the possibility that chlordecone-mediated inhibition of aromatase prevents increased E2 levels, despite an excess of TT as substrate. Finally, the origin of the increased levels of DHEA may be from any step upstream of cholesterol involving liver cytochrome P450 enzymes. Interestingly, experimental studies in rodents have shown that chlordecone induces cytochromes P450 enzymes (49) and may impair cholesterol homeostasis and tissue distribution (27, 50).

Non-Monotonic Dose-Response

The non-monotonic dose-response (NMDR) we observed between *in utero* chlordecone exposure and certain hormones (inverted U-shaped curve) is characterized by associations at intermediate exposure concentrations and no association at lower and higher exposure concentrations. Such a relationship is not entirely unexpected, as it is recognized that certain EDCs may exhibit such patterns in experimental studies (51, 52). NMDRs can arise from numerous molecular mechanisms, such as opposing effects induced by multiple receptors differing in their affinity, receptor desensitization, negative feedback with increasing dose, or dose-dependent modulation of metabolism (53). In the TIMOUN Mother-Child Cohort Study, we previously observed a similar NMDR between *in utero* chlordecone exposure and birth weight in overweight and obese mothers (13).

Strengths and Limits

The main strengths of this study lie in its prospective design, its being a population-based cohort study, and the exposure and outcome measurements. Prenatal (*in utero*) chlordecone exposure was determined using cord-blood samples, providing a representative measure of fetal exposure during the entire pregnancy, because the half-life of chlordecone in blood is approximately six months (54) and mothers were continuously exposed *via* the dietary intake of contaminated foods (6). Co-exposure to other EDCs was also considered, such as that to DDE and PCB-153, as well as childhood chlordecone exposure. We simultaneously measured a large number of hormones using standardized methods and gas chromatography–mass spectrometry for sex-steroid hormones, a method considered to be the gold standard for steroid hormone assays (55). Nevertheless, associations we observed in the present study, including the U-shaped relationships, should be interpreted with caution and we cannot exclude that it may result from a chance finding resulting from residual confounding or multiple comparisons.

CONCLUSION

This study shows that prenatal (*in utero*) exposure to chlordecone is associated with increased levels of TSH in girls and increased levels of DHEA, TT, and DHT in boys and girls in a non-monotonic dose-response relationship at seven years of age. Additional studies are necessary to explore the biological mechanisms involved in these associations and, in parallel, to identify whether such changes are predictive of a subsequent occurrence of disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because supporting data cannot be made openly available due to ethical concerns. The TIMOUN team can provide the data on request, subject to appropriate approvals. Requests to access the datasets should be directed to Gülen Ayhan, gulen.ayhan@inserm.fr.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Protection des Personnes Sud-Ouest et Outremer III (n° 2011-AOOSSI-40). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LM, PK, SC, and AO contributed to the conception and design of the study. FR and LMi contributed to the acquisition of data. LMi and CM organized the database and GA, NC, and LMu performed the statistical analyses. J-PT performed the chemical analysis and FG the hormonal analysis and both wrote the corresponding sections of the manuscript. GA and LMu wrote the first draft of the manuscript. All authors contributed to the revision of the manuscript, read, and approved the submitted version.

FUNDING

This work was supported by grants from the General Health Directorate (DGS RMC11129NNA & R17142NN), and the Fondation de France (N° 69263).

ACKNOWLEDGMENTS

We are grateful to all the families of the Timoun cohort, the staff of the follow-up at seven years (Marie-Fred Noyon, Nathalie Surville Barland, Annie-Claude Coriolan, Chantale Emeville, Colette Danquin, Sabrina Mimifir, Tania Plumain, Indira Oujagir, Agnès Desiré, Katia Galbas, Stéphanie Reine), Catherine Adam, at the LEAE-CART for her valuable help in the organochlorine analysis, and Regine Hierso for her essential help in blood sample processing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.771641/full#supplementary-material

REFERENCES

1. Multigner L, Kadhel P, Rouget F, Blanchet P, Cordier S. Chlordecone Exposure and Adverse Effects in French West Indies Populations. *Environ Sci Pollut Res* (2016) 23:3–8. doi: 10.1007/s11356-015-4621-5
2. Agency for Toxic Substances and Diseases Registry (ATSDR). *Toxicological Profile for Mirex and Chlordecone*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service (2020). Available at: https://www.atsdr.cdc.gov/toxprofiles/tp66.pdf (Accessed November 1, 2021).
3. Cabidoche YM, Achard R, Cattan P, Clermont-Dauphin C, Massat F, Sansoulet J. Long-Term Pollution by Chlordecone of Tropical Volcanic Soils in the French West Indies: A Simple Leaching Model Accounts for Current Residue. *Environ Pollut* (2009) 157:1697–705. doi: 10.1016/j.envpol.2008.12.015
4. Bocquené G, Franco A. Pesticide Contamination of the Coastline of Martinique. *Mar Pollut Bull* (2005) 51:612–9. doi: 10.1016/j.marpolbul.2005.06.026
5. Dubuisson C, Héraud F, Leblanc JC, Gallotti S, Flamand C, Blateau A, et al. Impact of Subsistence Production on the Management Options to Reduce the Food Exposure of the Martinican Population to Chlordecone. *Regul Toxicol Pharmacol* (2007) 49:5–16. doi: 10.1016/j.yrtph.2007.04.008
6. Guldner L, Multigner L, Heraud F. Pesticide Exposure of Pregnant Women in Guadeloupe: Ability of a Food Frequency Questionnaire to Estimate Blood Concentration of Chlordecone. *Environ Res* (2010) 110:146–51. doi: 10.1016/j.envres.2009.10.015
7. Kadhel P, Monfort C, Costet N,J. Chlordecone Exposure, Length of Gestation, and Risk of Preterm Birth. *Am* (2014) 179:536–44. doi: 10.1093/aje/kwt313
8. Dereumeaux C, Saoudi A, Guldner L, Pecheux M, Chesneau J, Thomé JP, et al. Chlordecone and Organochlorine Compound Levels in the French West Indies Population in 2013–2014. *Environ Sci Pollut Res* (2020) 27:41033–45. doi: 10.1007/s11356-019-07181-9
9. Dallaire R, Muckle G, Rouget F, Kadhel P, Bataille H, Guldner L, et al. Cognitive, Visual, and Motor Development of 7-Month-Old Guadeloupean Infants Exposed to Chlordecone. *Environ Res* (2012) 118:79–85. doi: 10.1016/j.envres.2012.07.006
10. Boucher O, Simard MN, Muckle G, Rouget F, Kadhel P, Bataille H, et al. Exposure to an Organochlorine Pesticide (Chlordecone) and Development of 18-Month-Old Infants. *Neurotoxicology* (2013) 35:162–8. doi: 10.1016/j.neuro.2013.01.007
11. Multigner L, Ndong JR, Giusti A, Romana M, Delacroix-Maillard H, Cordier S, et al. Chlordecone Exposure and Risk of Prostate Cancer. *J Clin Oncol* (2010) 28:3457–62. doi: 10.1200/JCO.2009.27.2153
12. Costet N, Pelé F, Comets E, Rouget F, Monfort C, Bodeau-Livinec F, et al. Perinatal Exposure to Chlordecone and Infant Growth. *Environ Res* (2015) 142:123–34. doi: 10.1016/j.envres.2015.06.023
13. Hervé D, Costet N, Kadhel P, Rouget F, Monfort C, Thomé JP, et al. Prenatal Exposure to Chlordecone, Gestational Weight Gain, and Birth Weight in a Guadeloupean Birth Cohort. *Environ Res* (2016) 151:436–44. doi: 10.1016/j.envres.2016.08.004
14. Saint-Amour D, Muckle G, Gagnon-Chauvin A, Rouget F, Monfort C, Michineau L, et al. Visual Contrast Sensitivity in School-Age Guadeloupean Children Exposed to Chlordecone. *Neurotoxicology* (2020) 78:195–201. doi: 10.1016/j.neuro.2020.02.012
15. Hammond B, Katzenellenbogen BS, Krauthammer N, McConnell J. Estrogenic Activity of the Insecticide Chlordecone (Kepone) and Interaction With Uterine Estrogen Receptors. *Proc Natl Acad Sci USA* (1979) 76:6641–5. doi: 10.1073/pnas.76.12.6641
16. Palmiter RD, Mulvihill ER. Estrogenic Activity of the Insecticide Kepone on the Chicken Oviduct. *Science* (1978) 201(4353):356–8. doi: 10.1126/science.78523
17. Eroschenko VP. Estrogenic Activity of the Insecticide Chlordecone in the Reproductive Tract of Birds and Mammals. *J Toxicol Environ Health* (1981) 8:731–42. doi: 10.1080/15287398109530109
18. Das SK, Tan J, Johnson DC, Dey SK. Differential Spatiotemporal Regulation of Lactoferrin and Progesterone Receptor Genes in the Mouse Uterus by Primary Estrogen, Catechol Estrogen, and Xenoestrogen. *Endocrinology* (1998) 139:2905–15. doi: 10.1210/endo.139.6.6051
19. Das S, Thomas P. Pesticides Interfere With the Nongenomic Action of a Progestogen on Meiotic Maturation by Binding to its Plasma Membrane Receptor on Fish Oocytes. *Endocrinology* (1999) 140:1953–6. doi: 10.1210/endo.140.4.6781
20. Vonier PM, Crain DA, McLachlan JA, Guillette LJ, Arnold SF. Interaction of Environmental Chemicals With the Estrogen and Progesterone Receptors From the Oviduct of the American Alligator. *Environ Health Perspect* (1996) 104:1318–22. doi: 10.1289/ehp.961041318
21. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. Interaction of Estrogenic Chemicals and Phytoestrogens With Estrogen Receptor β. *Endocrinology* (1998) 139:4252–63. doi: 10.1210/endo.139.10.6216
22. Lemaire G, Mnif W, Mauvais P, Balaguer P, Rahmani R. Activation of α- and β-Estrogen Receptors by Persistent Pesticides in Reporter Cell Lines. *Life Sci* (2006) 79:1160–9. doi: 10.1016/j.lfs.2006.03.023
23. Thomas P, Dong J. Binding and Activation of the Seven-Transmembrane Estrogen Receptor GPR30 by Environmental Estrogens: A Potential Novel Mechanism of Endocrine Disruption. *J Steroid Biochem Mol Biol* (2006) 102:175–9. doi: 10.1016/j.jsbmb.2006.09.017
24. Ray S, Xu F, Li P, Sanchez NS, Wang H, Das SK. Increased Level of Cellular Bip Critically Determines Estrogenic Potency for a Xenoestrogen Kepone in the Mouse Uterus. *Endocrinology* (2007) 148:4774–85. doi: 10.1210/en.2007-0537
25. Scippo ML, Argiris C, Van De Weerdt C, Muller M, Willemsen P, Martial J, et al. Recombinant Human Estrogen, Androgen and Progesterone Receptors for Detection of Potential Endocrine Disruptors. *Anal Bioanal Chem* (2004) 378:664–9. doi: 10.1007/s00216-003-2251-0
26. Benachour N, Moslemi S, Sipahutar H, Seralini GE. Cytotoxic Effects and Aromatase Inhibition by Xenobiotic Endocrine Disrupters Alone and in Combination. *Toxicol Appl Pharmacol* (2007) 222:129–40. doi: 10.1016/j.taap.2007.03.033
27. Lee J, Scheri RC, Zhang Y, Curtis LR. Chlordecone, a Mixed Pregnane X Receptor (PXR) and Estrogen Receptor Alpha (Erα) Agonist, Alters Cholesterol Homeostasis and Lipoprotein Metabolism in C57BL/6 Mice. *Toxicol Appl Pharmacol* (2008) 233:193–202. doi: 10.1016/j.taap.2008.08.011
28. Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, et al. Research Needs for the Risk Assessment of Health and Environmental Effects of Endocrine Disrupters: A Report of the U.S. EPA-Sponsored Workshop. *Environ Health Perspect* (1996) 104:715–40. doi: 10.1289/ehp.96104s4715
29. Jameson L, De Groot L. *Endocrinology: Adult and Pediatric, 2-Volume Set - 7th Edition*. Philadelphia: Elsevier (2015).
30. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. *Endocr Rev* (2009) 30:293–342. doi: 10.1210/er.2009-0002
31. La Merrill MA, Vandenberg LN, Smith MT, Goodson W, Browne P, Patisaul HB, et al. Consensus on the Key Characteristics of Endocrine-Disrupting Chemicals as a Basis for Hazard Identification. *Nat Rev Endocrinol* (2020) 16:45–57. doi: 10.1038/s41574-019-0273-8
32. Emeville E, Giton F, Giusti A, Oliva A, Fiet J, Thomé JP, et al. Persistent Organochlorine Pollutants With Endocrine Activity and Blood Steroid Hormone Levels in Middle-Aged Men. *PloS One* (2013) 8(6):e66460. doi: 10.1371/journal.pone.0066460
33. Cordier S, Bouquet E, Warembourg C, Massart C, Rouget F, Kadhel P, et al. Perinatal Exposure to Chlordecone, Thyroid Hormone Status and Neurodevelopment in Infants: The Timoun Cohort Study in Guadeloupe (French West Indies). *Environ Res* (2015) 138:271–8. doi: 10.1016/j.envres.2015.02.021
34. Debier C, Pomeroy P, Dupont C. Quantitative Dynamics of PCB Transfer From Mother to Pup During Lactation in UK Grey Seals Halichoerus Grypus. *Mar Ecol Prog Ser* (2003) 247:237–48. doi: 10.3354/meps247237
35. Glynn AW, Wolk A, Aune M, Atuma S, Zettermark S, Mahle-Schmid M, et al. Serum Concentrations of Organochlorines in Men: A Search for Markers of Exposure. *Sci Total Environ* (2000) 263:197–208. doi: 10.1016/S0048-9697(00)00703-8
36. Giton F, Fiet J, Cornu JN, Cussenot O, Bélanger A, Urien S, et al. Serum Sex Steroids Measured in Middle-Aged European and African-Caribbean Men by Gas Chromatography-Mass Spectrometry. *Eur J Endocrinol* (2011) 165:917–24. doi: 10.1530/EJE-11-0551
37. Bernert JT, Turner WE, Patterson DG, Needham LL. Calculation of Serum "Total Lipid" Concentrations for the Adjustment of Persistent Organohalogen

Toxicant Measurements in Human Samples. *Chemosphere* (2007) 68:824–31. doi: 10.1016/j.chemosphere.2007.02.043
38. Jin Y, Hein M, Hines CJDJA. Analysis of Lognormally Distributed Exposure Data With Repeated Measures and Values Below the Limit of Detection Using SAS. *Ann Occup Hyg* (2011) 55:97–112. doi: 10.1093/annhyg/meq061
39. Rasmussen KM, Yaktine AL. Institute of Medicine and National Research Council. Weight Gain During Pregnancy: Reexamining the Guidelines. *Washingt Natl Acad Press Natl Acad Sci* (2009) 1:2. doi: 10.17226/12584
40. AUDIPOG. *French Population-Based Fetal Growth Charts* (2008). Available at: https://www.audipog.net/Courbes-morpho/ (Accessed September 4, 2021).
41. Desquilbet L, Mariotti F. Dose-Response Analyses Using Restricted Cubic Spline Functions in Public Health Research. *Stat Med* (2010) 29:1037–57. doi: 10.1002/sim.3841
42. Kapelari K, Kirchlechner C, Högler W, Schweitzer K, Virgolini I, Moncayo R. Pediatric Reference Intervals for Thyroid Hormone Levels From Birth to Adulthood: A Retrospective Study. *BMC Endocr Disord* (2008) 8:15. doi: 10.1186/1472-6823-8-15
43. Rolland-Cachera MF, Deheeger M, Guilloud-Bataille M, Avons P, Patois E, Sempé M. Tracking the Development of Adiposity From One Month of Age to Adulthood. *Ann Hum Biol* (1987) 14:219–29. doi: 10.1080/03014468700008991
44. Rosenfield RL. Normal and Premature Adrenarche. *Endocr Rev* (2021) bnab009:1–32. doi: 10.1210/endrev/bnab009
45. Fatourechi V. Subclinical Hypothyroidism: An Update for Primary Care Physicians. *Mayo Clin Proc* (2009) 84:65–71. doi: 10.4065/84.1.65
46. Salerno M, Capalbo D, Cerbone M, De Luca F. Subclinical Hypothyroidism in Childhood-Current Knowledge and Open Issues. *Nat Rev Endocrinol* (2016) 12:734–46. doi: 10.1038/nrendo.2016.100
47. Lazar L, Frumkin RBD, Battat E, Lebenthal Y, Phillip M, Meyerovitch J. Natural History of Thyroid Function Tests Over 5 Years in a Large Pediatric Cohort. *J Clin Endocrinol Metab* (2009) 94:1678–82. doi: 10.1210/jc.2008-2615
48. Yang L, Zha J, Guo Y, Zhou B. Evaluation and Mechanistic Study of Chlordecone-Induced Thyroid Disruption: Based on *In Vivo*, *In Vitro* and in Silico Assays. *Sci Total Environ* (2020) 716. doi: 10.1016/j.scitotenv.2020.136987
49. Kaminsky LS, Piper LJ, McMartin DN, Fasco MJ. Induction of Hepatic Microsomal Cytochrome P-450 by Mirex and Kepone. *Toxicol Appl Pharmacol* (1978) 43(2):327–38. doi: 10.1016/0041-008x(78)90012-1
50. Carpenter HM, Curtis LR. Low Dose Chlordecone Pretreatment Altered Cholesterol Disposition Without Induction of Cytochrome P-450. *Drug Metab Dispos* (1991) 19:673–8.
51. Fagin D. Toxicology: The Learning Curve. *Nature* (2012) 490:462–5. doi: 10.1038/490462a
52. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, et al. Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. *Endocr Rev* (2012) 33:378–455. doi: 10.1210/er.2011-1050
53. Lagarde F, Beausoleil C, Belcher SM, Belzunces LP, Emond C, Guerbet M, et al. Non-Monotonic Dose-Response Relationships and Endocrine Disruptors: A Qualitative Method of Assessment. *Environ Health* (2015) 14:13. doi: 10.1186/1476-069X-14-13
54. Cohn WJ, Boylan JJ, Blanke RV, Fariss MW, Howell JR, Guzelian PS. Treatment of Chlordecone (Kepone) Toxicity With Cholestyramine. *N Engl J Med* (1978) 298:243–8. doi: 10.1056/NEJM197802022980504
55. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Position Statement: Utility, Limitations, and Pitfalls in Measuring Testosterone: An Endocrine Society Position Statement. *J Clin Endocrinol Metab* (2007) 92:405–13. doi: 10.1210/jc.2006-1864

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 11 February 2022
doi: 10.3389/fendo.2022.816970

Establishment of Reference Intervals for Thyroid-Associated Hormones Using refineR Algorithm in Chinese Population at High-Altitude Areas

Chaochao Ma[1†], Jian Zhong[1†], Yutong Zou[1†], Zhijuan Liu[2], Honglei Li[1], Jinrong Pang[2], Xiaoxing Liu[3], Zejipuchi[4], Liping Tian[5], Li'an Hou[1], Danchen Wang[1], Xinqi Cheng[1] and Ling Qiu[1,6]*

[1] Department of Laboratory Medicine, Peking Union Medical College Hospital, Peking Union Medical College & Chinese Academy of Medical Science, Beijing, China, [2] Department of Clinical Laboratory, People's Hospital of Tibet Autonomous Region, Lhasa, China, [3] Department of Clinical Laboratory, Ali District People's Hospital, Ali, China, [4] Department of Clinical Laboratory, Sang Zhu Zi District People's Hospital, Shigatse, China, [5] Department of Clinical Laboratory, Maternal and Child Health Hospital, Nyingchi, China, [6] State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China

Objectives: Diagnosis of thyroid disease among individuals dwelling at high altitude remains a challenge. Reference intervals (RIs) for thyroid-associated hormones among Tibetans living at various high altitudes were established to improve diagnosis.

Methods: One thousand two hundred eighty-one subjects were randomly recruited from Nyingchi, Shigatse/Lhasa, and Ali of Tibet. Thyroid-stimulating hormone (TSH), free triiodothyronine (FT3), and free thyroxine (FT4) were measured by the Cobas e601 electrochemiluminescence analyzer. We used multiple linear regression and variance component analysis to assess the effect of sex, age, and altitude on hormones. RIs were established by refineR algorithm and compared with those provided by the manufacturer.

Results: Serum TSH was significantly lower in males than in females, while FT3 and FT4 were higher in males. Both FT3 and FT4 decreased with increasing age. FT3 increased with altitude, while TSH and FT4 were less influenced by altitude. The RI for TSH was 0.764–5.784 μIU/ml, while for FT4, the RIs were 12.36–19.38 pmol/L in females and 14.84–20.18 pmol/L in males. The RIs for FT3 at Nyingchi, Shigatse/Lhasa, and Ali in females were 4.09–4.98, 4.31–5.45, and 4.82–5.58 pmol/L, while in males, the values were 4.82–5.41, 4.88–5.95, and 5.26–6.06 pmol/L, respectively. The obtained RIs for TSH and FT4 were generally higher, while that for FT3 was narrower than the RIs provided by Cobas.

Conclusions: Specific RIs were established for thyroid-associated hormones among Tibetans, which were significantly different from those provided by the manufacturer.

Keywords: high altitude, reference interval, refineR algorithm, thyrotropin, thyroid hormones

OPEN ACCESS

Edited by:
Maaike Van Gerwen, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Paul Horn, Cincinnati Children's Hospital Medical Center, United States
Nalini Selveindran, Hospital Putrajaya, Malaysia

***Correspondence:**
Ling Qiu
lingqiubj@163.com

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *17 November 2021*
Accepted: *04 January 2022*
Published: *11 February 2022*

Citation:
Ma C, Zhong J, Zou Y, Liu Z, Li H, Pang J, Liu X, Zejipuchi, Tian L, Hou L'a, Wang D, Cheng X and Qiu L (2022) Establishment of Reference Intervals for Thyroid-Associated Hormones Using refineR Algorithm in Chinese Population at High-Altitude Areas. Front. Endocrinol. 13:816970. doi: 10.3389/fendo.2022.816970

INTRODUCTION

Thyroid disease is a prevalent health problem which may lead to potentially devastating healthy consequences (1). In China, the weighted prevalence of overt hyperthyroidism, overt hypothyroidism, subclinical hyperthyroidism, and subclinical hypothyroidism are known to be 0.78%, 1.02%, 0.44%, and 12.93%, respectively (2). Delayed diagnosis of thyroid disease can lead to adverse effects such as heart failure, atrial fibrillation, and mortality from other cardiovascular diseases, and redundant treatment due to misdiagnosis may also increase disease burden of patients or trigger adverse drug reactions (1, 3, 4). It was estimated that the rate of overdiagnosis of subclinical hypothyroidism was up to 16% due to physiological elevation of thyroid-stimulating hormone (TSH) (5, 6). Therefore, accurate and timely diagnosis is essential. The diagnosis of thyroid disease is predominantly based on laboratory measurements, of which the accuracy is mainly related to the applicability of reference interval (RIs). For some special groups such as the elderly (7), pregnant women (8), minority, and so on, general RIs may be not applicable. Similarity, habitants in the Tibet Plateau with an average altitude of more than 4,000 m may have their hypothalamic–pituitary–thyroid (HPT) axis altered for adaptation to severe cold, hypobaric hypoxia, sleep disorder, and other unfavorable conditions. This long-term adaptation may lead to thyroid-related hormone levels in Tibetans different from those in the plain (9–11). Even if the effects of sex, age (12–14), region, sampling time (15), and pregnancy status (16) on thyroid-related hormones are well discussed, however, only few articles (10, 11, 17) have assessed the influence of altitude on thyroid-associated hormones, let alone establishing altitude-specific RIs.

Currently, direct and indirect methods are the two sampling techniques applied to establish RIs. The direct method is the accepted standard method using *a priori* or posteriori sampling approach to provide a high internal validity and a minimal bias. However, the process is always costly and time-consuming and has poor feasibility (18). Since it is very difficult to define the apparently healthy individuals for specific groups such as the elderly, pregnant women, and people living in a special environment, the application of the direct method is restricted (18). Conversely, the indirect method with the simple and low-cost performance in a real-world environment utilizes the data mining technique to estimate the healthy distribution from the mixed distribution (19, 20). Thus, it can be used for the establishment of RIs when healthy individuals cannot be defined and obtained using direct sampling. The refineR algorithm has been previously proposed for the problem of "data mining" RIs (21). An open-source R code for the establishment of RIs using refineR algorithm has been developed by Ammer (21) and its effectiveness has been also proved. The core of the algorithm is still parameter combination and optimal search. Compared with the forward approach proposed by other indirect algorithms, the refineR algorithm adopts an inverse modeling approach to separate the healthy distribution of observed test results and identifies the optimal model for RI establishment. In short, using indirect methods such as refineR algorithm to establish RIs might be more preferable in Tibetan population, as the chronic exposure to high altitude may induce changes of thyroid-associated hormones for environmental adaptation (10, 11).

To help make more accurate clinical decisions on thyroid-related diseases, we used the variance component model to explore the effects of age, sex, and altitude on thyroid-associated hormones in Tibetan population. Furthermore, we established sex- and altitude-specific RIs for thyroid-related hormones in Tibetans based on the refineR algorithm and evaluated the application.

METHOD AND MATERIAL

Subjects

From September 2016 to August 2018, we used a standard questionnaire to recruit participants at Ali (altitude I: 4,298–4,352 m), Shigatse/Lhasa (altitude II: 3,670–3,835 m), and Nyingchi (altitude III: about 2,900 m) of Tibet Autonomous Region in China. One thousand two hundred eighty-one indigenous Tibetan subjects were randomly enrolled in our study by the following criteria:

i. Subjects self-report that they are currently in good health and have no major organ system disease,
ii. age ≥19 years,
iii. the subjects were Tibetan,
iv. subjects with no hospitalization in the past 6 months and no illness in the past 4 weeks, and
v. subjects were required to have lived in Tibet for >1 year.

Ethical Approval

This study has been approved by the Ethics Committee of the People's Hospital of Tibet Autonomous Region (Approval No.ME-TBHP-2017-021) and the Ethics Committee of Peking Union Medical College Hospital (Approval No. S-K530). All the subjects had signed the informed consent form.

Sample Collection

All subjects were requested to maintain on a normal diet and to avert night shifts or strenuous exercise 24 h before tests. After sitting for 10 to 15 min, fasting venous blood samples of subjects were drawn into red-capped procoagulant-containing Vacuette 5-mL tubes with gel (Greiner Bio-One, Kremsm €unster, Austria) by well-trained nurses and centrifuged at 3,000rpm for 10 min.

Analytical Performance of Analytes

The levels of thyroid-related hormones including TSH, FT3, and FT4 were measured using the Cobas e601 electrochemiluminescence analyzer (Roche, Basel, Switzerland) with corresponding reagents, calibrators, and quality controls supplied by the manufacturer.

Quality Control

In this study, sample quality was strictly controlled, and all sample collection, processing, and testing personnel were uniformly trained. Serum samples were centrifuged, packaged and frozen within 30 min after sampling, and transported to the Department of Laboratory Medicine of Peking Union Medical College Hospital for unified measurement through a cold chain transportation system with strict temperature control. For all test items, two levels of quality control were implemented before and after each batch test. Samples can be tested only after the quality control is qualified. In addition, the Department of Clinical Laboratory of Peking Union Medical College Hospital is accredited by both ISO15189 and CAP. All the above test items were evaluated by the National Health and Health Commission, and the results were all qualified.

Data Cleaning and Statistical Analysis

Data cleaning and statistical analysis were performed using R programming language (V.4.0.5) and MedCalc Statistical software 18.116.6 (Mariakerke, Belgium). Shapiro–Wilk tests and frequency distribution histograms were used to describe the distributions of the items. If the variables satisfy a normal distribution, data were described as mean ± standard deviations and the Tukey method was used to identify outliers, whereas data rejecting the normality hypothesis were described as medians with quartiles. The Box–Cox method was applied to improve the normality of data before using the Tukey method to identify the outliers. Furthermore, when establishing reference intervals, the Box–Cox was performed again. The standardized regression coefficients of sex, altitude, and age were calculated by multiple linear regression. The variance component model was used to calculate the variation of thyroid-related hormones in gender, age, and altitude, that is, standard deviation or coefficient of variation. The residual standard deviation in the variance component model represents individual variation of thyroid-related hormones. Furthermore, standard deviation ratio (SDR) was expressed as (SDsex, SDaltitude, SDage)/SDresidual and was employed to judge whether the RI of thyroid-related hormones needs to be divided into several partitioning by sex, age, or altitude, and 0.4 is often used as a judgment threshold for thyroid-related hormones.

The refineR algorithm (21) was implemented using refineR package (version 1.0.0) for the aim of establishing RIs for thyroid-associated hormones. Two-sided P <0.05 was considered statistically significant.

RESULTS

Baseline Information of Participants Enrolled in the Study

In total, 1,281 participants were enrolled in our study (Nyingchi: n = 363; Shigatse/Lhasa: n = 473; Ali: n = 445). Females accounted for 65.8%, 55.0%, and 51.9% of the recruits at altitudes III, II, and I, respectively. The median age of the Tibetan population at altitudes III, II, and I was 42, 42, and 34 years, respectively. The results of TPO-Ab and TG-Ab measured by Cobas are shown in **Table 1**.

Effect of Sex, Altitude, and Age on Thyroid-Associated Hormones

Source variations of each thyroid-related hormone were analyzed as shown in **Tables 2**, **3**. Sex has apparently effects on TSH, FT4, and FT3, showing that females have higher TSH levels but lower FT4 and FT3 levels than males. As shown in **Figure 1**, both FT3

TABLE 1 | Baseline information of individuals enrolled in this study.

Index	Altitude III	Altitude II	Altitude I
n	363	473	445
Age (years)	42 (32, 51)	42 (32, 52)	34 (28, 43)
Sex (female%)	65.8%	55.0%	51.9%
TPO-Ab (IU/L)	13.25 (11.15, 16.21)	12.52 (10.02, 18.46)	14.51 (11.45, 9.28)
TG-Ab (IU/L)	10.00 (10.00, 10.54)	10.45 (10.00, 14.28)	14.00 (11.23, 18.97)

Altitude Ⅲ, Nyingchi (altitude: ~2,900 m); altitude Ⅱ, Shigatse/Lhasa (altitude: 3,670–3,835 m); altitude I, Ali (altitude: 4,298–4,352 m); TG-Ab, thyroglobulin antibody; TPO-Ab, thyroid peroxidase antibody.

TABLE 2 | Effect of sex, altitude, and age on thyroid-associated hormones.

Index	Sex		Altitude				Age					
	β	*P*	AI 1		AI 2		A1		A2		A3	
			β	*P*	*B*	*P*	*β*	*P*	*β*	*P*	*B*	*P*
TSH	−0.060	0.038	0.077	0.026	−0.027	0.446	0.041	0.253	0.093	0.009	0.151	<0.001
FT3	0.425	<0.001	0.164	<0.001	0.309	<0.001	−0.185	<0.001	−0.257	<0.001	−0.250	<0.001
FT4	0.300	<0.001	0.000	0.991	0.076	0.020	−0.060	0.075	−0.216	<0.001	−0.235	<0.001

β, standardized regression coefficient; female is the reference for sex groups. AI 1 and AI 2 are the dummy variable of altitude, Nyingchi (altitude: ~2,900 m) is the reference level, AI 1 stands for Shigatse/Lhasa relative to Nyingchi and AI 2 stands for Ali relative to Nyingchi; A1, A2, and A3 are the dummy variable of age, 19~29 years is the reference level, A1 stands for 30~39 years relative to 19~29 years, A2 stands for 40~49 years relative to 19~29 years, A3 stands for age 50 or older relative to 19~29 years.

TABLE 3 | Results of variance component model for thyroid-associated hormones.

Index	SDRresi	Sex		Altitude		Age	
		SD	SDR	SD	SDR	SD	SDR
TSH	2.18	0.33	0.2	0.00	0.0	0.34	0.2
FT3	0.50	0.38	0.8	0.21	0.4	0.17	0.3
FT4	2.14	0.96	0.4	0.23	0.1	0.71	0.3

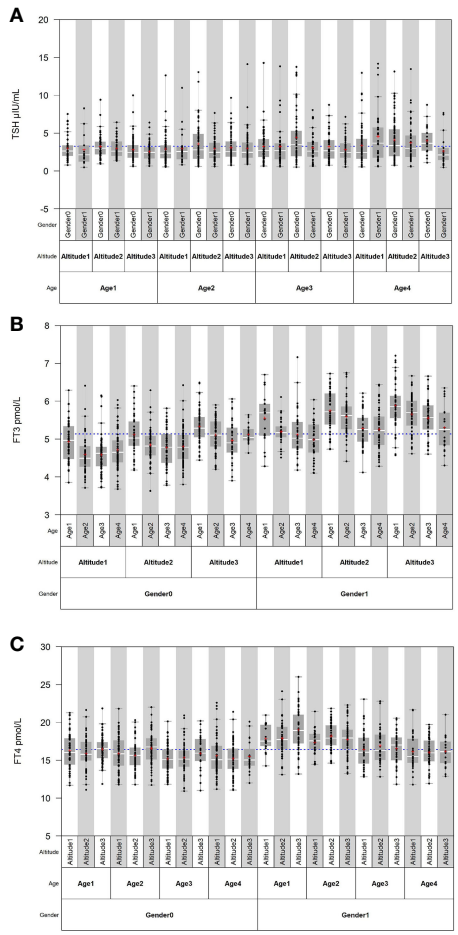


FIGURE 1 | Distribution of thyroid-related hormones in Tibetan population by sex, age, and altitude. **(A–C)** Distribution of TSH, FT3, and FT4 in Tibetan population by sex, age, and altitude.

and FT4 decreased with increasing age. Tibetans aged over 50 years have significant higher levels of TSH than those aged between 19 and 29 years ($P < 0.001$). FT3 increased with altitude ($P < 0.001$), while TSH and FT4 were less influenced by altitude. The results of multiple linear regression in **Table 2** and the variance component analysis in **Table 3** indicated that sex- and altitude-specific RIs for FT3 should be considered when the SDR ≥0.4. The RIs for FT4 should be partitioned by sex with SDRsex of 0.4.

RIs of Thyroid-Associated Hormones for the Population at High Altitude

The optimal parametrical models for RI establishment calculated by the refineR algorithm are shown in **Figure 2** and **Appendix 1**. The specific results are shown in **Table 4**. RIs for FT3 were divided by sex and altitude, while RIs for FT4 were divided by sex. The RIs for FT3 and FT4 were overall higher in males than in females. RIs for FT3 elevated with increasing altitude as the RIs at altitudes III, II, and I were 4.09–4.98, 4.31–5.45, and 4.82–5.58 pmol/L in females and 4.82–5.41, 4.88–5.95, and 5.26–6.06 pmol/L in males. The total RIs for TSH, FT3, and FT4 were 0.764–5.784 μIU/ml, 4.01–6.23 pmol/L, and 12.19–20.70 pmol/L, respectively. Compared with the RIs provided by the manufacturer, the lower and upper limits of the obtained RIs for TSH and FT4 were higher, and the RI for FT3 was narrower.

DISCUSSION

The current study utilized the refineR algorithm to formulate sex- and/or altitude-specific TSH, FT4 and FT3 RIs for indigenous Tibetans living at high altitude. Sex, age, and altitude had no effect on TSH levels in Tibetans and the RI for TSH was 0.764–5.784 μIU/ml. The level of FT3 was influenced by both sex and altitude, with the upper and lower limits of the RIs being higher for males than females and both increasing with altitude. The limits of TSH as well as FT4 were found to be significantly higher in our study as compared with those provided by the manufacturer. A narrower RI range for FT3 was also found. Therefore, to avoid misdiagnosis or underdiagnosis of thyroid disease, it is recommended to apply the appropriate specific RI for the Tibetan population.

Thyroid-related hormones are closely related to the metabolism of the body, short-term stress, or long-term adaptation. Due to the particularity of the living environment, Tibetan people may have hormone levels inconsistent with those in the plain areas. The present study found little effects of altitude on serum TSH among Tibetans, which is consistent with the results of an observation study (11). Relatively short half-life of TSH (17 to 93 min) (15), pulsatile secretion manners (15, 22) and feedback regulation of the HPT axis may roughly explain the phenomenon, although the exact mechanism is unclear. In euthyroid people, synthetic TSH is released in a manner combining a basal (non-pulsatile) and a pulsatile form and can be promptly cleared after action on the thyroid gland. The frequency, mass, and duration of a pulsatile can be rapidly adjusted to maintain the dynamic stability of TSH under the control of the HPT axis in response to environmental changes like hypobaric hypoxia, cold, and other harsh conditions on the plateau (10, 23). Meanwhile, the levels of thyroid-associated hormones were apparently elevated at higher altitude, with FT3 rising more obviously. Our results roughly concurred with previous studies which reported significant increases in thyroid-associated hormones for people following short-term or prolonged exposure to high-altitude environments (11, 17, 24, 25). Elevated thyroid-associated hormones in high-altitude

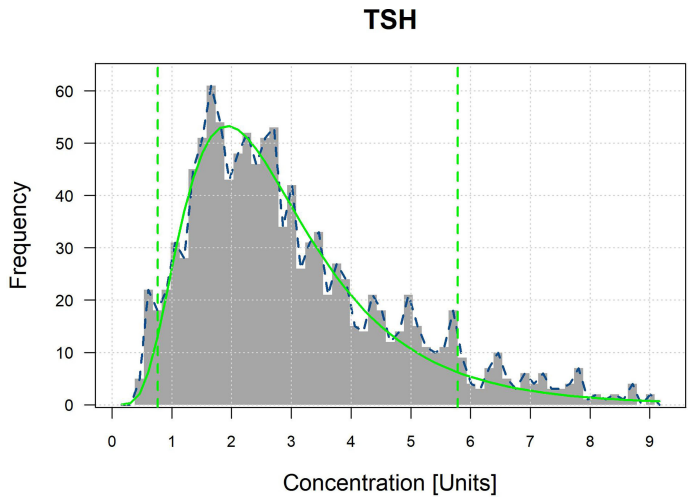


FIGURE 2 | Optimal parametrical models calculated by refineR algorithm for TSH.

TABLE 4 | Reference interval of thyroid-associated hormones.

Index	Units	Group	refineR				Manufacturer	
			Lower limits of RI	90% CI	Upper limits of RI	90% CI	Lower limits of RI	Upper limits of RI
TSH	μIU/ml	Total	0.764	0.2276–0.9228	5.784	4.0818–8.2030	0.270	4.220
FT3	pmol/L	Total	4.01	3.909–4.240	6.23	5.701–6.554	3.10	6.80
		Female	3.98	3.803–4.298	5.79	5.370–6.077		
		Altitude III	4.09	3.777–4.424	4.98	4.769–5.324		
		Altitude II	4.31	4.051–4.628	5.45	5.119–5.762		
		Altitude I	4.82	4.411–5.048	5.58	5.379–5.848		
		Male	4.58	4.141–5.088	6.53	5.955–6.711		
		Altitude III	4.82	4.633–4.995	5.41	5.260–5.590		
		Altitude II	4.88	4.480–5.244	5.95	5.575–6.271		
		Altitude I	5.26	5.019–5.592	6.06	5.852–6.420		
FT4	pmol/L	Total	12.19	11.417–13.599	20.70	18.854–21.721	12.00	20.00
		Female	12.36	11.422–14.316	19.38	17.207–20.776		
		Male	14.84	12.407–16.318	20.18	18.890–21.825		

Altitude Ⅲ, Nyingchi (altitude: ~2,900 m); altitude Ⅱ, Shigatse/Lhasa (altitude: 3,670–3,835 m); altitude I, Ali (altitude: 4,298–4,352 m).

environments facilitate the resistance of the body to the harsh environment of high altitude and seem to be independent on pituitary gland secretion of TSH (24, 26). Furthermore, altitude-dependent FT3 rise may also be relevant to physiological changes caused by the relative lack of iodine, as studies have shown that Tibetan regions have lower urinary iodine levels and a higher risk of iodine deficiency compared with mainland China (27, 28). Mouse models also showed that iodine deficiency could induce changes in deiodinase activity in the thyroid or peripheral tissues to affect the conversion of T4 to T3 (29), and make monocarboxylate transporter 8, one of the most important T3 relevant transporters, upregulated in the thyroid gland to transport more T3 into blood circulation (30).

Gender and age are important factors to be considered in the establishment of RIs for thyroid-related hormones. However, there is still a great controversy about whether the RIs should be partitioned by age (7, 12, 14, 31). In our study, the impact of age on RIs for TSH, FT3, and FT4 in Tibetans was modest. Although it was clear from the results that TSH levels were significant higher in the group aged over 50 years than in the group aged between 19 and 29 years, the RI could not be partitioned by age in our study and the RI for TSH was 0.764–5.784 μIU/ml. Zhai et al., using the same instrument as we did, reported that the RIs for TSH in Chinese plain population <65 and ≥65 years were 0.76–6.57 and 0.75–8.86 mIU/L, respectively (14). Difference in the upper limits may be explained by the age composition of the population living at different altitudes in China, as in the case of the three regions of Tibet, where the proportion of people aged >65 years was only approximately 5% (32). Obvious discrepancies were observed for TSH, FT3, and FT4 levels between males and females, although the effect of sex on TSH level was not as remarkable as those on FT3 or FT4, which was

consistent with previous studies (14, 27, 33, 34). Thus, sex-specific RIs for FT4 and FT3, but not for TSH, were proposed in our study and results showed that both the upper and lower limits of RIs for FT3 and FT4 were higher in males than in females, which can be interpreted by the potential effect of sex hormones on the HPT axis (10, 35).

At the end of this study, we compared the RIs calculated by the refineR algorithm with those proposed by the manufacturer. Higher upper and lower reference limits were found in Tibetans for TSH and FT4 (TSH: 0.764–5.784 *vs.* 0.270–4.220 μIU/ml, FT4: 12.19–20.70 *vs.* 12.00–20.00 pmol/L), as well as a narrower range for FT3 (4.01–6.23 *vs.* 3.10–6.80 pmol/L). These discrepancies suggested that the direct use of the RIs of the manufacturer may lead to underdiagnosis of hyperthyroidism or overdiagnosis of subclinical hypothyroidism. The study highlights the importance of establishing appropriated RIs in high-altitude laboratories by an indirect method like the refineR algorithm. However, it is important that 90% CI of RIs show that the widths of CI of FT3 and FT4 are suitable. However, the 90% CI of the upper limit of reference interval for TSH is relatively wide, possibly due to the large variation of the TSH for populations at high altitude.

Our research has both advantages and disadvantages. Firstly, it is the first multicenter cross-sectional study to set up RIs for thyroid-related hormones in Tibetan population dwelling at the plateau, and the refineR algorithm was creatively used under this situation. Secondly, a detailed questionnaire was used to assist in screening subjects from Nyingchi, Shigatse, Lhasa, and Ali. Unequivocally, there are limitations in the current study, such as the lack of thyroid ultrasonography results and the utilization of only a single testing system. However, the refineR algorithm can help identify healthy individuals from a distribution mixed with a small proportion of pathological individuals and then obtain RIs according to the optimal parameter models. Considering that conventional definitions of apparent health, such as normal blood pressure and BMIs in the normal range, may not be applicable to high-altitude dwellers, the method in our study may be a preference to establish RIs. In addition, the RIs established for thyroid-related hormones may be only applicable to the Roche platform, as non-negligible discrepancy among assay methodologies has been revealed (13). Overall, we have currently established RIs for Tibetans living at high altitude for generations, and further studies should use real-world data to compare the prevalence of thyroid disease in populations dwelling at different altitudes for generations.

CONCLUSION

Altitude- and/or sex-specific RIs for thyroid-related hormones were established in Chinese people living at high-altitude areas based on the refineR algorithm. Significant differences have been found while comparing the obtained RIs with the RIs provided by the manufacturer. Therefore, establishment of specific RIs according to regional characteristics of Tibet should be recommended to avoid underdiagnosis or misdiagnosis of thyroid disease.

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 the Ethics Committee of the People's Hospital of Tibet Autonomous Region (Approval No.ME-TBHP-2017-021) and the Ethics Committee of Peking Union Medical College Hospital (Approval No. S-K530). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LQ designed the study. CM analyzed the data. CM, JZ, and YZ wrote this manuscript. LH, DW, and CM performed detection of the thyroid-related hormones. XC, LQ, and DW made suggestions for the revision of the manuscript. HL, JP, ZL, XL, ZP, and LT helped in participant recruitment and sample collection. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The work was supported by the Special Foundation Project for Human Resources Development of the Tibet Autonomous Region (2016000), Science and Technology Program of the Tibet Autonomous Region (2015XZ01G20), Beijing Key Clinical Specialty for Laboratory Medicine - Excellent Project (No. ZK201000) and Capital's Funds for Health Improvement and Research (CFH-2020-1-4014).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.816970/full#supplementary-material

REFERENCES

1. Taylor PN, Albrecht D, Scholz A, Gutierrez-Buey G, Lazarus JH, Dayan CM, et al. Global Epidemiology of Hyperthyroidism and Hypothyroidism. *Nat Rev Endocrinol* (2018) 14(5):301–16. doi: 10.1038/nrendo.2018.18
2. Li Y, Teng D, Ba J, Chen B, Du J, He L, et al. Efficacy and Safety of Long-Term Universal Salt Iodization on Thyroid Disorders: Epidemiological Evidence From 31 Provinces of Mainland China. *Thyroid* (2020) 30(4):568–79. doi: 10.1089/thy.2019.0067
3. Chaker L, Bianco AC, Jonklaas J, Peeters RP. Hypothyroidism. *Lancet* (2017) 390(10101):1550–62. doi: 10.1016/s0140-6736(17)30703-1
4. De Leo S, Lee SY, Braverman LE. Hyperthyroidism. *Lancet* (2016) 388 (10047):906–18. doi: 10.1016/s0140-6736(16)00278-6
5. Shan Z, Chen L, Lian X, Liu C, Shi B, Shi L, et al. Iodine Status and Prevalence of Thyroid Disorders After Introduction of Mandatory Universal Salt Iodization for 16 Years in China: A Cross-Sectional Study in 10 Cities. *Thyroid* (2016) 26(8):1125–30. doi: 10.1089/thy.2015.0613
6. Biondi B, Cappola AR, Cooper DS. Subclinical Hypothyroidism: A Review. *JAMA* (2019) 322(2):153–60. doi: 10.1001/jama.2019.9052
7. Mooijaart SP, Du Puy RS, Stott DJ, Kearney PM, Rodondi N, Westendorp RGJ, et al. Association Between Levothyroxine Treatment and Thyroid-Related Symptoms Among Adults Aged 80 Years and Older With Subclinical Hypothyroidism. *JAMA* (2019) 322(20):1977–86. doi: 10.1001/jama.2019.17274
8. Dickens LT, Cifu AS, Cohen RN. Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum Period. *Jama* (2019) 321 (19):1928–9. doi: 10.1001/jama.2019.5321
9. Feldt-Rasmussen U, Effraimidis G, Klose M. The Hypothalamus-Pituitary-Thyroid (HPT)-Axis and its Role in Physiology and Pathophysiology of Other Hypothalamus-Pituitary Functions. *Mol Cell Endocrinol* (2021) 525:111173. doi: 10.1016/j.mce.2021.111173
10. Keenan DM, Pichler Hefti J, Veldhuis JD, Von Wolff M. Regulation and Adaptation of Endocrine Axes at High Altitude. *Am J Physiol Endocrinol Metab* (2020) 318(2):E297–309. doi: 10.1152/ajpendo.00243.2019
11. von Wolff M, Nakas CT, Tobler M, Merz TM, Hilty MP, Veldhuis JD, et al. Adrenal, Thyroid and Gonadal Axes are Affected at High Altitude. *Endocr Connect* (2018) 7(10):1081–9. doi: 10.1530/EC-18-0242
12. Cappola AR. The Thyrotropin Reference Range Should Be Changed in Older Patients. *JAMA* (2019) 322(20):1961–2. doi: 10.1001/jama.2019.14728
13. Kahapola-Arachchige KM, Hadlow N, Wardrop R, Lim EM, Walsh JP. Age-Specific TSH Reference Ranges Have Minimal Impact on the Diagnosis of Thyroid Dysfunction. *Clin Endocrinol (Oxf)* (2012) 77(5):773–9. doi: 10.1111/j.1365-2265.2012.04463.x
14. Zhai X, Zhang L, Chen L, Lian X, Liu C, Shi B, et al. An Age-Specific Serum Thyrotropin Reference Range for the Diagnosis of Thyroid Diseases in Older Adults: A Cross-Sectional Survey in China. *Thyroid* (2018) 28(12):1571–9. doi: 10.1089/thy.2017.0715
15. van der Spoel E, Roelfsema F, van Heemst D. Within-Person Variation in Serum Thyrotropin Concentrations: Main Sources, Potential Underlying Biological Mechanisms, and Clinical Implications. *Front Endocrinol (Lausanne)* (2021) 12:619568. doi: 10.3389/fendo.2021.619568
16. Zhang J, Li W, Chen QB, Liu LY, Zhang W, Liu MY, et al. Establishment of Trimester-Specific Thyroid Stimulating Hormone and Free Thyroxine Reference Interval in Pregnant Chinese Women Using the Beckman Coulter UniCel™ DxI 600. *Clin Chem Lab Med* (2015) 53(9):1409–14. doi: 10.1515/cclm-2014-0615
17. Liu D, Li M, Wang Y, Jiang S, You L, Li F, et al. Effects of Moderate Altitude on Fasting Blood Glucose, Lipid Profile and Thyroid Hormones in Healthy Volunteers. *Chin J Pathophysiol* (2019) 35(09):1683–8. doi: 10.3969/j.issn.1000-4718. 2019.09.023
18. Ma C, Wang X, Wu J, Cheng X, Xia L, Xue F, et al. Real-World Big-Data Studies in Laboratory Medicine: Current Status, Application, and Future Considerations. *Clin Biochem* (2020) 84:21–30. doi: 10.1016/j.clinbiochem.2020.06.014
19. Jones GRD, Haeckel R, Loh TP, Sikaris K, Streichert T, Katayev A, et al. Indirect Methods for Reference Interval Determination - Review and Recommendations. *Clin Chem Lab Med* (2018) 57(1):20–9. doi: 10.1515/cclm-2018-0073
20. Ma C, Cheng X, Xue F, Li X, Yin Y, Wu J, et al. Validation of an Approach Using Only Patient Big Data From Clinical Laboratories to Establish Reference Intervals for Thyroid Hormones Based on Data Mining. *Clin Biochem* (2020) 80:25–30. doi: 10.1016/j.clinbiochem.2020.03.012
21. Ammer T, Schützenmeister A, Prokosch HU, Rauh M, Rank CM, Zierk J. Refiner: A Novel Algorithm for Reference Interval Estimation From Real-World Data. *Sci Rep* (2021) 11(1):16023. doi: 10.1038/s41598-021-95301-2
22. Roelfsema F, Boelen A, Kalsbeek A, Fliers E. Regulatory Aspects of the Human Hypothalamus-Pituitary-Thyroid Axis. *Best Pract Res Clin Endocrinol Metab* (2017) 31(5):487–503. doi: 10.1016/j.beem.2017.09.004
23. Mendoza A, Hollenberg AN. New Insights Into Thyroid Hormone Action. *Pharmacol Ther* (2017) 173:135–45. doi: 10.1016/j.pharmthera.2017.02.012
24. Basu M, Pal K, Malhotra AS, Prasad R, Sawhney RC. Free and Total Thyroid Hormones in Humans at Extreme Altitude. *Int J Biometeorol* (1995) 39(1):17–21. doi: 10.1007/bf01320888
25. Richalet JP, Letournel M, Souberbielle JC. Effects of High-Altitude Hypoxia on the Hormonal Response to Hypothalamic Factors. *Am J Physiol Regul Integr Comp Physiol* (2010) 299(6):R1685–92. doi: 10.1152/ajpregu.00484.2010
26. Nepal O, Pokhrel BR, Khanal K, Gyawali P, Malik SL, Koju R, et al. Thyroid Hormone Levels in Highlanders- a Comparison Between Residents of Two Altitudes in Nepal. *Kathmandu Univ Med J (KUMJ)* (2013) 11(41):18–21. doi: 10.3126/kumj.v11i1.11017
27. Ning P, Ren Q, Teng D, Zhang Z, Lv X, Meng S, et al. Current Iodine Nutrition Status and Prevalence of Thyroid Disorders in Tibetan Adults in an Oxygen-Deficient Plateau, Tibet, China: A Population-Based Study. *Thyroid* (2020) 30(5):759–66. doi: 10.1089/thy.2019.0669
28. Zou Y, Li H, Pang J, Liu X, Zejipuchi, Tian L, et al. An Evaluation of Urine and Serum Iodine Status in the Population of Tibet, China: No Longer an Iodine-Deficient Region. *Nutrition* (2021) 82:111033. doi: 10.1016/j.nut.2020.111033
29. Lavado-Autric R, Calvo RM, de Mena RM, de Escobar GM, Obregon MJ. Deiodinase Activities in Thyroids and Tissues of Iodine-Deficient Female Rats. *Endocrinology* (2013) 154(1):529–36. doi: 10.1210/en.2012-1727
30. Hu Z, Zhuo X, Shi Y, Liu X, Yuan J, Li L, et al. Iodine Deficiency Up-Regulates Monocarboxylate Transporter 8 Expression of Mouse Thyroid Gland. *Chin Med J (Engl)* (2014) 127(23):4071–6. doi: 10.3760/cma.j.issn.0366-6999.20141314
31. Carle A, Pedersen IB, Knudsen N, Perrild H, Ovesen L, Andersen S, et al. Hypothyroid Symptoms Fail to Predict Thyroid Insufficiency in Old People: A Population-Based Case-Control Study. *Am J Med* (2016) 129(10):1082–92. doi: 10.1016/j.amjmed.2016.06.013
32. Liao G, Liu Z. A Study on Situation and Characteristics of the Age Structure of Tibet Population : Based on the Analysis of the Sixth Census Data. *Northwest Population J* (2014) 35(02):13–6. doi: 10.15884/j.cnki.issn.1007-0672.2014.02.008
33. Li ZZ, Yu BZ, Wang JL, Yang Q, Ming J, Tang YR. Reference Intervals for Thyroid-Stimulating Hormone and Thyroid Hormones Using the Access TSH 3rd IS Method in China. *J Clin Lab Anal* (2020) 34(5):e23197. doi: 10.1002/jcla.23197
34. Zou Y, Wang D, Cheng X, Ma C, Lin S, Hu Y, et al. Reference Intervals for Thyroid-Associated Hormones and the Prevalence of Thyroid Diseases in the Chinese Population. *Ann Lab Med* (2021) 41(1):77–85. doi: 10.3343/alm.2021.41.1.77
35. Hadlow NC, Rothacker KM, Wardrop R, Brown SJ, Lim EM, Walsh JP. The Relationship Between TSH and Free T_4 in a Large Population is Complex and Nonlinear and Differs by Age and Sex. *J Clin Endocrinol Metab* (2013) 98 (7):2936–43. doi: 10.1210/jc.2012-4223

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.

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 24 February 2022
doi: 10.3389/fendo.2022.801925

Hashimoto's Thyroiditis: A "Double-Edged Sword" in Thyroid Carcinoma

Jiangyue Xu[1†], Ke Ding[2†], Lan Mu[1], Jiangsheng Huang[1], Fei Ye[1], Yu Peng[1], Can Guo[1] and Chutong Ren[1]*

[1] Department of General Surgery Thyroid Specialty, The Second Xiangya Hospital of Central South University, Changsha, China, [2] Department of Urology, Xiangya Hospital, Central South University, Changsha, China

Background: The prevalence of thyroid carcinoma (TC) and Hashimoto's thyroiditis (HT) has been increasing dramatically over the past decades. We investigated the relationship between HT and TC.

Methods: We followed the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines for carrying out and reporting this meta-analysis. The literature from January 1, 2010 to December 31, 2020, regardless of region and publication type, was searched comprehensively in PubMed, Embase, Web of Science, and Cochrane Library databases. After careful selection and data extraction, the pooled odds ratio of various clinical characteristics in 39 studies were calculated. Publication bias was analyzed using funnel plots.

Results: Meta-analysis of 39 original research articles showed HT to be a risk factor of TC (pooled odds ratio = 1.71; 95% confidence interval, 1.57–1.80; $p < 0.00001$) and papillary thyroid carcinoma (1.67, 1.51–1.85, <0.00001). Patients with papillary thyroid carcinoma (PTC) combined with HT were more likely to have multifocal carcinomas. The prevalence of an extrathyroidal extension, metastasis, $BRAF^{V600E}$ mutation, and recurrence was significantly lower in patients with PTC combined with HT.

Conclusions: HT is a "double-edged sword" in TC patients. HT increases the risk of TC and PTC but is a protective factor against PTC progression.

Keywords: Hashimoto's thyroiditis (HT), thyroid carcinoma (TC), meta-analysis, papillary thyroid carcinoma, $BRAF^{V600E}$ mutation

OPEN ACCESS

Edited by:
Catherine Fiona Sinclair, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Laura Sterian Ward, State University of Campinas, Brazil
Christoph Reiners, University Hospital Würzburg, Germany
Zhihong Wang, China Medical University, China

***Correspondence:**
Chutong Ren
2204120602@csu.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *26 October 2021*
Accepted: *18 January 2022*
Published: *24 February 2022*

Citation:
Xu J, Ding K, Mu L, Huang J, Ye F, Peng Y, Guo C and Ren C (2022) Hashimoto's Thyroiditis: A "Double-Edged Sword" in Thyroid Carcinoma. Front. Endocrinol. 13:801925. doi: 10.3389/fendo.2022.801925

1 INTRODUCTION

Thyroid carcinoma (TC) is the most common malignant disease of the endocrine system (1, 2). The overall survival from TC is high, and the prognosis is good, especially for papillary thyroid carcinoma (PTC). However, in recent decades, TC incidence has been increasing dramatically (3). The incidence of autoimmune thyroid diseases, especially Hashimoto's thyroiditis (HT), has also increased in recent decades (4). About one-third of PTC patients also have HT, and the number of people with PTC combined with HT is increasing (5, 6). The underlying reason for this increased

Abbreviations: HT, Hashimoto's thyroiditis; TC, thyroid carcinoma; PTC, papillary thyroid carcinoma; TSH, thyroid-stimulating hormone

incidence may be improved diagnostic tools (e.g., fine-needle aspiration, high-resolution ultrasound, and detection of thyroid gland-specific antibodies). About 20%–30% of HT patients will eventually experience hypothyroidism, and an increased serum level of thyroid-stimulating hormone (TSH) may promote TC occurrence (5, 6). In addition, exposure to adverse environmental factors can make people more vulnerable to thyroiditis (7, 8).

In 1893, Rudolf Virchow was the first to propose a link between chronic inflammation and cancer development. In the next century, his hypothesis was confirmed in several human diseases (9, 10). The most convincing evidence for his hypothesis was (i) the link between chronic inflammatory diseases of the intestine (Crohn's disease and ulcerative proctocolitis) and colon adenocarcinoma, (ii) chronic infection with the hepatitis B virus or hepatitis C virus and liver cancer, and (iii) chronic gastritis and gastric cancer caused by *Helicobacter pylori* infection. Similarly, it has been postulated that having HT (as the most common type of thyroiditis) also carries an increased risk of TC. Many scholars have investigated this hypothesis, but most studies have been influenced by selection biases and imprecise indicators (11). Hence, studies have reached controversial (or even contrary) conclusions. Some studies have shown that HT may be a tumor-promoting factor (12). Other studies have reported HT to not have a relationship with the higher incidence of TC (13). HT has different influences on PTC in several aspects: certain clinical manifestations, sex, lymph-node metastasis, $BRAF^{V600E}$ mutation, and recurrence (14–18). These factors affect the aggressiveness, treatment effect, and prognosis of PTC directly. Analyses of original studies and meta-analysis that had controversial conclusions revealed that biases usually arose because the (i) diagnostic criteria for HT were not uniform (B-ultrasound, antibodies, or pathology), (ii) the number of original research was limited, and (iii) the definition of the indicators used was not clear, such as lymph-node metastasis (metastasis of central lymph nodes or metastasis of lateral lymph nodes, or both).

Taking into account the limitations of such studies, an updated systematic review and meta-analysis is necessary. To better understand the impact of HT on PTC progression, we undertook a meta-analysis through a comprehensive search of the literature.

2 METHODS

2.1 Registration

We registered (CRD42021265538) a systematic review entitled "The relationship between Hashimoto's thyroiditis and thyroid carcinoma: a meta-analysis" in PROSPERO. We have been updating information in that systematic review.

2.2 Search Strategy

We followed the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines for carrying out and reporting this meta-analysis (19). The MOOSE checklist is shown in **Supplementary Table S1**. The literature from January 1, 2010 to December 31, 2020, regardless of region and publication type, was searched comprehensively in PubMed, Embase, Web of Science, and Cochrane Library databases. We used the following medical subject headings (MeSH) terms: "Thyroid neoplasms" and "Hashimoto disease". We combined the MeSH terms and entry words when constructing searches. In addition, we also use the related article function to expand the search scope. If multiple original studies involving the same population were published, we selected the latest and most comprehensive research. Exported citations were managed, and duplicate data were deleted, using EndNote™ (https://endnote.com/).

2.3 Inclusion and Exclusion Criteria

The inclusion criteria were the following: (i) the study type must be prospective, retrospective, randomized controlled trial, or case–control; (ii) the research focus was HT and thyroid nodules, TC, or PTC; (iii) patients must be adults (≥18 years); and (iv) HT, TC, or PTC was diagnosed by pathology.

The exclusion criteria were the following: (i) the study design was not a prospective, retrospective, or randomized controlled trial; (ii) those that were editorials, letters, comments, case reports, or laboratory-animal research; and (iii) data were incomplete, and extracting the data needed in the meta-analysis was not possible.

According to these criteria, two researchers (JX and LM) conducted a preliminary screening by reading the title and abstract of the article. For the articles remaining after the preliminary screening, JX and LM read the full text and followed the inclusion and exclusion criteria strictly for final screening of the article. This screening process was carried out independently by JX and LM. Disputes in the screening process were resolved through negotiation or help of a third author (KD).

2.4 Extraction and Quality Assessment of Data

JX and LM used identical standardized data-extraction tables. The latter included the characteristics of the original research (author, publication year, region, and research type) and clinical characteristics of thyroid nodules or TC related to HT (main results were the malignancy prevalence of thyroid nodules and lymph-node metastasis in PTC; secondary results were multifocality, extrathyroidal expansion, $BRAF^{V600E}$ mutation, recurrence, and distant metastasis). Disputes between JX and LM were resolved by KD. If the target data of the original study could not be obtained, then the corresponding author of the original study was contacted.

We used the Newcastle–Ottawa Scale (NOS) score to assess the quality of included studies (20). The NOS focuses on the choice of study subjects (four items), comparability between groups (two items) and measurement of results (three items, applicable to cohort studies), or exposure degree (three items, applicable to case–control studies). The NOS score of each study varied from 0 to 9 points (1 point for each item, 9 points in total). An original study with a NOS score ≥6 points was considered to be of "high quality."

2.5 Statistical Analyses

The meta-analysis of all data was carried out using Revman 5.4 with Cochrane Training (https://training.cochrane.org/). The odds ratio (OR) and 95% confidence interval (95%CI) of each study were calculated. The χ^2 test and I^2 test were employed to evaluate heterogeneity. For the χ^2 test, $p < 0.10$ was considered to denote significant heterogeneity. For the I^2 test, heterogeneity types were divided into "none" (0%–25%), "mild" (25%–50%), "moderate" (50%–75%), and "severe" (75%–100%). If $I^2 > 50\%$, the random-effects model was chosen for the meta-analysis; otherwise, the fixed-effects model (FEM) was chosen. We drew funnel plots and observed their symmetry to identify publication bias, but this was mainly for meta-analysis containing ≥10 studies. If the number of included studies was too small, evaluation of the symmetry of the funnel chart was not possible. For sensitivity analysis, we observed changes in the overall results by eliminating individual studies one-by-one and utilizing different effect models.

3 RESULTS

3.1 Literature Search

We identified 2,644 records in the last decade by searching PubMed, Embase, Web of Science, and Cochrane Library databases. A total of 527 studies were excluded because they contained duplicate data. A total of 1,948 studies were removed after screening of the title and abstract. JX and LM screened the full text of the remaining 169 studies independently. A total of 101 studies were excluded because they did not conform to the inclusion criteria, and an additional 29 studies were excluded because data could not be extracted. Finally, 39 studies were included for further analyses (14–16, 21–56). **Figure 1** is a flowchart of how studies were chosen.

3.2 Characteristics and Methodological Quality of Included Studies

The characteristics and methodological quality of included studies are described in detail in **Table 1**. The included studies were retrospective except for the studies by Cortes and colleagues (38) and Carvalho and collaborators (14). According to the NOS score, studies were of high quality except for the study by Kim and colleagues (26), which received a NOS score of 5 points.

In the included studies, most scholars focused on HT and its relationship to PTC. However, a few studies simultaneously analyzed multiple pathological types of TC (PTC, follicular, medullary, and undifferentiated).

3.3 HT as a Risk Factor of TC and PTC

We wished to control for a confounding bias. Hence, we assessed if HT plays a part in multiple pathological types of TC (PTC, follicular, medullary, and undifferentiated) and whether HT is a risk factor in PTC patients (**Figure 2**).

In 2,497 HT patients, 1,109 were finally diagnosed with TC regardless of the pathological type (**Figure 2A**). In 17,847 patients without HT, 5,742 were diagnosed with TC. The pooled OR was 1.71 (95%CI, 1.57–1.80; $p < 0.00001$, $I^2 = 40\%$, FEM), which provided evidence that HT is a risk factor for TC.

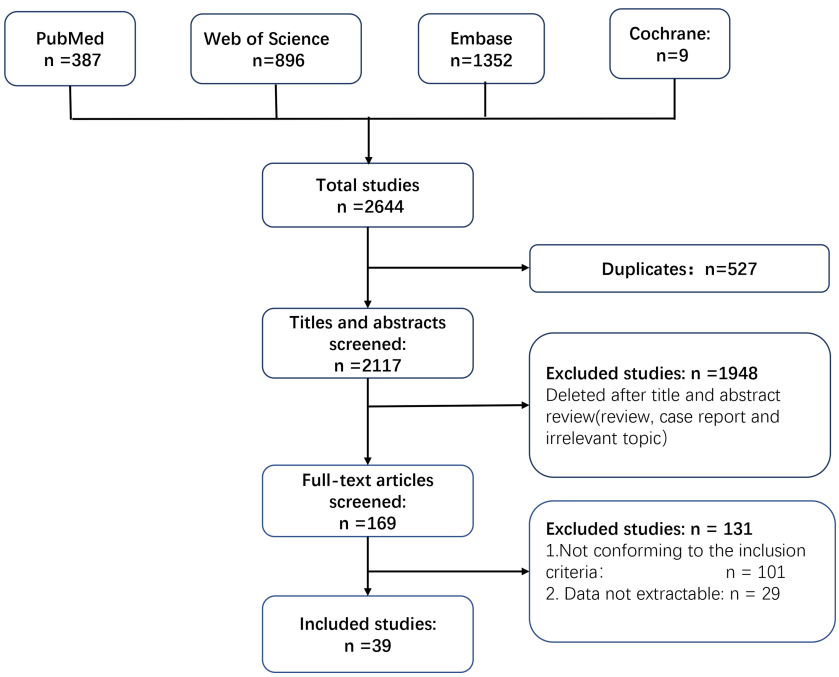


FIGURE 1 | Flow diagram of systemic review procedure.

TABLE 1 | The clinical characteristics of included studies.

Author	Type of Study	Nation	Year of Publication	With HT/without HT	Pathological Type	NOS Quality Assessment Scale	Reference Number
Kim SS	Retrospective	Korea	2011	146/254	PTC	7	(21)
Kim HS	Retrospective	Korea	2010	105/218	PTMC(>5mm)	6	(22)
Huang BY	Retrospective	China	2011	85/1,703	PTC	7	(23)
Ahn D	Retrospective	Korea	2011	58/211	PTC	8	(24)
Jeong JS	Retrospective	Korea	2012	359/998	PTC	7	(25)
Kim YS	Retrospective	Korea	2013	316/931	PTC	5	(26)
Cordioli MI	Retrospective	Brazil	2013	35/59	PTC	8	(27)
Jara SM	Retrospective	America	2013	226/269	PTC	6	(28)
Marotta V	Retrospective	Italy	2013	54/92	PTC	6	(16)
Lim JY	Retrospective	Korea	2013	964/1,983	PTC	6	(29)
Lang BH	Retrospective	Korea	2014	331/514	PTC (<2cm)	6	(30)
Kwak HY	Retrospective	Korea	2014	40/306	PTC	7	(31)
Konturek A	Retrospective	Poland	2014	130/643	PTC	7	(32)
Kim SK	Retrospective	Korea	2015	1006/2,326	PTC	7	(33)
Kim SJ	Retrospective	Korea	2016	204/1,576	PTC	6	(34)
Carvalho MS	Prospective	Brazil	2016	191/442	PTC	7	(14)
Dobrinja C	Retrospective	Italy	2016	70/90	PTC	7	(35)
Zhang Y	Retrospective	China	2014	247/1,488	PTC	6	(36)
Girardi FM	Retrospective	Brazil	2015	148/269	PTC	6	(37)
Cortes MCS	Prospective	Brazil	2018	45/68	PTC	8	(38)
Lee I	Retrospective	Korea	2020	1,174/1,754	PTC	7	(15)
Yang Y	Retrospective	China	2014	92/199	PTMC	7	(39)
Zeng R-C	Retrospective	China	2016	222/397	PTC	8	(40)
Yoon YH	Retrospective	Korea	2012	56/139	PTC	7	(41)
Paulson LM	Retrospective	America	2012	61/78	PTC	8	(42)
Park JY	Retrospective	Korea	2014	169/484	PTC	7	(43)
Ding J	Retrospective	China	2020	233/1,106	PTC	8	(44)
Zhu F	Retrospective	China	2016	129/105	PTC	7	(45)
Song E	Retrospective	Korea	2018	305/1,064	PTC	8	(46)
Nam HY	Retrospective	Korea	2016	22/15	PTC	6	(47)
Giagourta I	Retrospective	Greece	2013	441/939	PTC	7	(48)
Consorti F	Retrospective	Italy	2010	24/76	PTC	6	(49)
Jackson D	Retrospective	America	2019	52/307	TC	6	(50)
Kim ES	Retrospective	Korea	2010	41/201	PTC	6	(51)
Lun Y	Retrospective	China	2013	256/2,222	PTC	6	(55)
Vasileiadis I	Retrospective	Greece	2014	246/590	PTC	6	(53)
Zhang L	Retrospective	China	2012	653/5,456	PTC	6	(54)
Paparodis R	Retrospective	America	2013	567/2,151	DTC	7	(55)
Zeng, Rong	Retrospective	China	2016	30/1,168	PTC	8	(56)

HT, Hashimoto's thyroiditis; PTC, papillary thyroid carcinoma; PTMC, papillary thyroid microcarcinoma; TC, thyroid carcinoma; DTC, differentiated thyroid carcinoma.

Six studies focused on the relationship between HT and PTC (**Figure 2B**). In 1,889 HT patients, 822 were diagnosed with PTC. In 15,491 patients without HT, 4,935 were diagnosed with PTC. The pooled OR was 1.67 (95%CI 1.51–1.85; $p < 0.00001$, $I^2 = 18\%$, FEM).

3.4 HT and Multiple Clinical Characteristics in PTC Patients

3.4.1 Multifocality and Extrathyroidal Extension

Nineteen studies reported the multifocality of PTC patients with or without HT. In PTC patients with HT, 2,018 of 5,793 patients had multifocal carcinomas. In PTC patients without HT, 4,443 of 14,880 patients had multifocal carcinomas (**Figure 3A**). The pooled OR was 1.17 (95%CI, 1.09–1.25; $p < 0.00001$, $I^2 = 0\%$, FEM). The funnel plot did not show an obvious publication bias (**Figure 3B**).

Ten studies reported the extrathyroidal extension of PTC patients with or without HT. In PTC patients with HT, 1,618 of 3,497 patients had an extrathyroidal extension. In PTC patients without HT, 4,495 of 9,433 patients had an extrathyroidal extension (**Figure 3C**). The pooled OR was 0.79 (95%CI, 0.72–0.86; $p < 0.00001$, $I^2 = 38\%$, FEM). The funnel plot did not show an obvious publication bias (**Figure 3D**).

3.4.2 Lymph-Node Metastasis and Distant Metastasis

Eleven studies reported metastasis of the central lymph nodes of PTC patients with or without HT. In PTC patients with HT, 1,784 of 3,847 patients had metastasis of the central lymph nodes. In PTC patients without HT, 4,835 of 9,685 patients had metastasis of the central lymph nodes (**Figure 4A**). The pooled OR was 0.80 (95%CI, 0.74–0.87; $p < 0.00001$, $I^2 = 37\%$, FEM). The funnel plot did not show an obvious publication bias (**Figure 4B**).

Five studies reported metastasis of lateral lymph nodes of PTC patients with or without HT. In PTC patients with HT, 116 of 898 patients had metastasis of lateral lymph nodes. In PTC

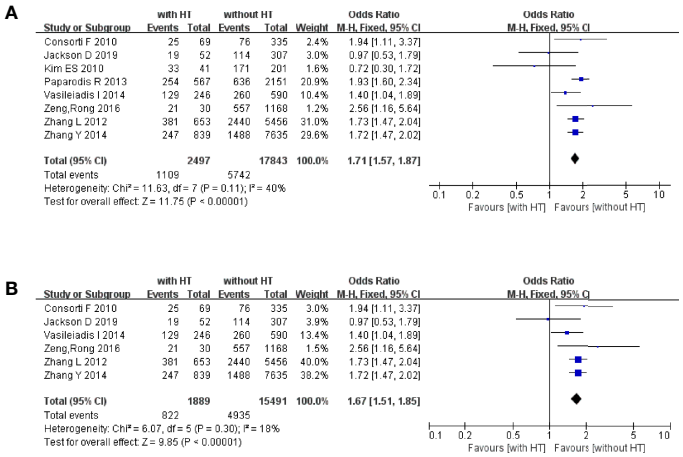


FIGURE 2 | HT as a risk factor of TC and PTC: **(A)** forest plot of HT and TC and **(B)** forest plot of HT and PTC.

patients without HT, 412 of 3,189 patients had metastasis of lateral lymph nodes (**Figure 4C**). The pooled OR was 0.77 (95% CI, 0.59–0.99; p = 0.04, I^2 = 26%, FEM).

Five studies reported distant metastasis in PTC patients with or without HT. In PTC patients with HT, 19 of 778 patients had distant metastasis. In PTC patients without HT, 70 of 1,555 patients had distant metastasis (**Figure 4D**). The pooled OR was 0.52 (95%CI, 0.31–0.87; p = 0.01, I^2 = 0%, FEM).

3.4.3 $BRAF^{V600E}$ Mutation and Recurrence

Eight studies reported the $BRAF^{V600E}$ mutation in PTC patients with or without HT. In PTC patients with HT, 2,327 of 3,348 patients had the $BRAF^{V600E}$ mutation. In PTC patients without HT, 6,839 of 8,309 patients had the $BRAF^{V600E}$ (**Figure 5A**). The pooled OR was 0.47 (95%CI, 0.43–0.52; p < 0.00001, I^2 = 0%, FEM).

Five studies reported the recurrence of PTC in patients with or without HT. In PTC patients with HT, 13 of 724 patients had tumor recurrence. In PTC patients without HT, 304 of 3,337 patients suffered tumor recurrence (**Figure 5B**). The pooled OR was 0.32 (95%CI, 0.18–0.58; p = 0.0002, I^2 = 0%, FEM).

3.5 Sensitivity Analysis

Removal of the data of each study did not influence the pooled OR of the whole analysis. Transformation of the FEM or random-effects model did not influence the results. Hence, our analysis was reliable.

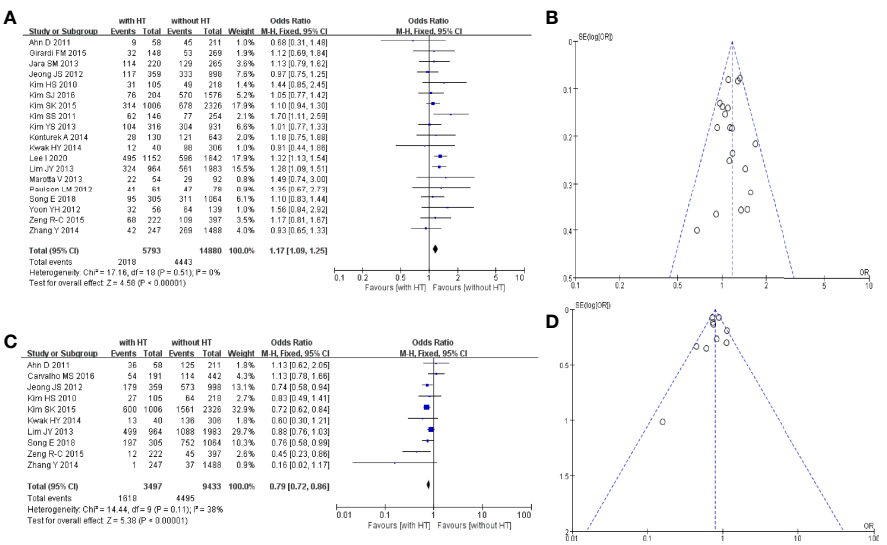


FIGURE 3 | HT and multifocality of PTC patients: **(A)** forest plot and **(B)** funnel plot. HT and extrathyroidal extension of PTC patients: **(C)** forest plot and **(D)** funnel plot.

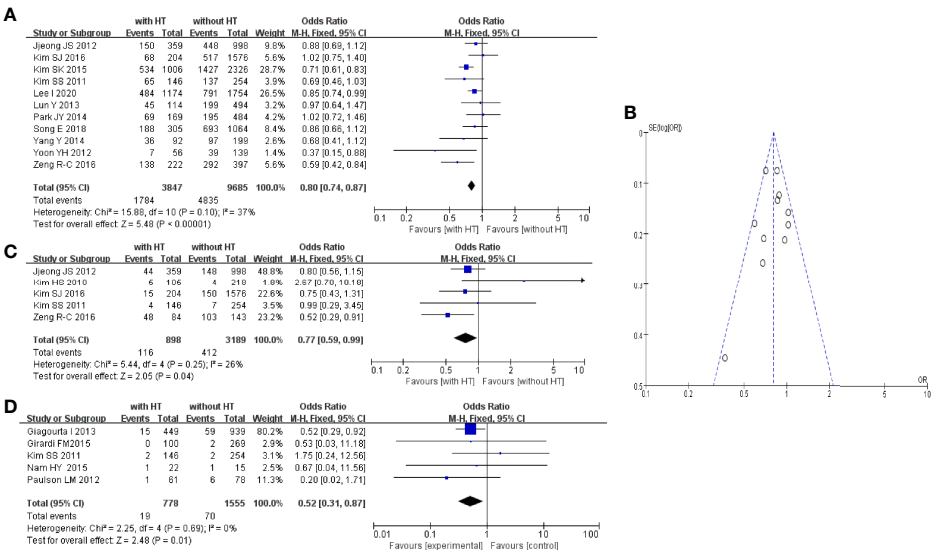


FIGURE 4 | HT and central LN metastasis: **(A)** forest plot, **(B)** funnel plot; **(C)** Forest plot of HT and lateral LN metastasis; **(D)** forest plot of HT and distant metastasis.

4 DISCUSSION

The relationship between HT and TC was discussed first by Dailey in 1955 (57). Whether HT plays a part in the development and progression of TC has been controversial since then. We revealed a complicated relationship between HT and TC (especially PTC). HT appears to be a "double-edged sword" in TC. It is a potential risk factor in TC (especially PTC) patients. However, PTC patients with HT usually carry a better prognosis because they have a lower risk of extrathyroidal extension, $BRAF^{V600E}$ mutation, metastasis, or recurrence.

The relationship between autoimmune thyroid disease and TC has been controversial for decades. Giagurta and colleagues compared the prevalence of autoimmune thyroiditis between people with PTC and individuals with benign thyroid nodules over the past 16 years. They found that patients with thyroid nodules and autoimmune thyroiditis were not more likely to have malignant thyroid nodules than individuals without autoimmune thyroiditis (48). Del Rio and colleagues conducted a prospective cohort study of 9,851 patients who underwent assessment of thyroid nodules between 1995 and 2017 (13). They showed that, for people with HT, the risk of malignant thyroid nodules was increased significantly. Our meta-analysis showed that the risk of TC or PTC in thyroid nodules for people with HT was increased. Therefore, we concluded that HT is a risk factor of TC or PTC.

Three possible pathogenic mechanisms can explain the results of our meta-analysis. First, the inflammatory response creates a favorable environment for malignant transformation. The damage wrought by cytokines and growth factors to stromal cells leads to changes in

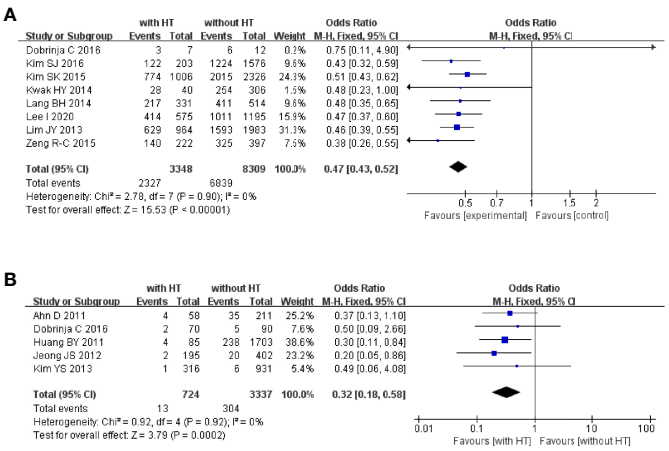


FIGURE 5 | **(A)** Forest plot of HT and BRAFV600E mutation; **(B)** forest plot of HT and recurrence.

stromal reactivity, which, in turn, can lead to the malignant transformation of epithelial cells (58). In addition, the infiltration of immune cells to the thyroid gland may promote abnormal repair of DNA, thereby inducing PTC (59). Second, TSH is not only an endogenous stimulator of thyroid-hormone production, it is also a growth factor for thyroid cells (60). An increased level of TSH in most HT patients stimulates follicular epithelial hyperplasia, which promotes PTC. Third, expression of some oncogenes, such as RET/PTC gene rearrangement (61) and p63 mutation (62), may be involved in the transformation from HT to PTC. In contrast, the $BRAF^{V600E}$ mutation, which is usually mutually exclusive with RET/PTC gene rearrangement (63), is more common in PTC without HT, a finding that is consistent with our meta-analysis.

Usually, TC (especially PTC) is considered to be less aggressive and to carry a better prognosis than that of a malignant tumor. However, the complicated extrathyroidal extension and various types of metastasis can be fatal in some circumstances. PTC shows a high tendency to spread to regional lymph nodes. The central region is the main region of lymph-node involvement in 20%–90% of PTC patients (64). Lymph-node metastasis is the main risk factor for PTC recurrence and is highly correlated with progression-free survival and overall survival in PTC patients (65, 66). Our meta-analysis showed PTC patients with HT to have a lower prevalence of lymph-node metastasis, distant metastasis, and recurrence. This conclusion is not consistent with the conclusions reached by Mao et al. (67) or Sun and colleagues (68). One reason for the different results could be a confounding bias (e.g., they did not distinguish lymph nodes from different areas).

According to our meta-analysis, PTC patients with HT had more favorable clinical characteristics and a better prognosis than PTC patients not suffering from HT. The latter is more common in young women. Thus, patients with HT are prone to be anxious and to undergo ultrasound of the thyroid gland frequently, so discovery of a malignant thyroid nodule at an early stage is likely. Multifocal carcinomas are independent risk factors for the TC prognosis. HT patients are more likely to have multifocal carcinomas, which leads to more aggressive and radical surgery, so their recurrence risk is lower than that of PTC patients without HT. Marotta and colleagues showed lymphocyte infiltration in HT patients to be a protective factor against PTC progression (16). Zhang et al. suggested that the $BRAF^{V600E}$ mutation can help to predict the prognosis of PTC (69). The $BRAF^{V600E}$ mutation is a marker of more aggressive behavior of PTC. In addition, the $BRAF^{V600E}$ mutation is less common in PTC patients with HT than in PTC patients without HT. The results of our meta-analysis and the studies stated above suggest that HT is a protective factor against PTC progression, but the mechanism of action merits further study.

Our meta-analysis explored the relationship between PTC and HT from occurrence to progression, but had two main limitations. First, the included studies were mainly retrospective: more prospective studies and real-world studies are needed to draw more accurate conclusions. Second, the included studies involved mainly Asian patients. More prospective cohort studies involving multiple ethnicities are needed to further clarify the relationship between HT and PTC.

5 CONCLUSIONS

HT is a double-edged sword in TC patients. HT increases the risk of TC and PTC but is a protective factor against PTC progression.

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 the ethics committee of Second Xiangya Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JX and KD provided main effort in the procedure of meta-analysis and manuscript editing. LM, FY, and CG offered great help in the data extraction and data analysis. JH and CR gave many valuable suggestions to this article. CR also contributed to the manuscript revision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key R&D Program of China under Grant 2019YFE0190500.

ACKNOWLEDGMENTS

We would like to acknowledge the librarians at the Libraries of Central South University for their efforts in obtaining primary resources for this meta-analysis. Additionally, we would like to acknowledge professors, colleagues, friends, and family members who assisted and gave encouragements in the writing procedure of this article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.801925/full#supplementary-material

REFERENCES

1. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, et al. 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):1–133. doi: 10.1089/thy.2015.0020
2. Londero SC, Krogdahl A, Bastholt L, Overgaard J, Pedersen HB, Hahn CH, et al. Papillary Thyroid Carcinoma in Denmark, 1996-2008: Outcome and Evaluation of Established Prognostic Scoring Systems in a Prospective National Cohort. *Thyroid* (2015) 25(1):78–84. doi: 10.1089/thy.2014.0294
3. Blomberg M, Feldt-Rasmussen U, Andersen KK, Kjaer SK. Thyroid Cancer in Denmark 1943-2008, Before and After Iodine Supplementation. *Int J Cancer* (2012) 131(10):2360–6. doi: 10.1002/ijc.27497
4. Rizzo M, Rossi RT, Bonaffini O, Scisca C, Altavilla G, Calbo L, et al. Increased Annual Frequency of Hashimoto's Thyroiditis Between Years 1988 and 2007 at a Cytological Unit of Sicily. *Ann Endocrinol (Paris)* (2010) 71(6):525–34. doi: 10.1016/j.ando.2010.06.006
5. Antonelli A, Ferrari SM, Corrado A, Di Domenicantonio A, Fallahi P. Autoimmune Thyroid Disorders. *Autoimmun Rev* (2015) 14(2):174–80. doi: 10.1016/j.autrev.2014.10.016
6. 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
7. Latina A, Gullo D, Trimarchi F, Benvenga S. Hashimoto's Thyroiditis: Similar and Dissimilar Characteristics in Neighboring Areas. Possible Implications for the Epidemiology of Thyroid Cancer. *PloS One* (2013) 8(3):e55450. doi: 10.1371/journal.pone.0055450
8. Benvenga S, Guarneri F. Molecular Mimicry and Autoimmune Thyroid Disease. *Rev Endocr Metab Disord* (2016) 17(4):485–98. doi: 10.1007/s11154-016-9363-2
9. Fung J, Lai CL, Yuen MF. Hepatitis B and C Virus-Related Carcinogenesis. *Clin Microbiol Infect* (2009) 15(11):964–70. doi: 10.1111/j.1469-0691.2009.03035.x
10. Balkwill F, Mantovani A. Inflammation and Cancer: Back to Virchow? *Lancet* (2001) 357(9255):539–45. doi: 10.1016/S0140-6736(00)04046-0
11. Guan H, de Morais NS, Stuart J, Ahmadi S, Marqusee E, Kim MI, et al. Discordance of Serological and Sonographic Markers for Hashimoto's Thyroiditis With Gold Standard Histopathology. *Eur J Endocrinol* (2019) 181(5):539–44. doi: 10.1530/EJE-19-0424
12. Silva de Morais N, Stuart J, Guan H, Wang Z, Cibas ES, Frates MC, et al. The Impact of Hashimoto Thyroiditis on Thyroid Nodule Cytology and Risk of Thyroid Cancer. *J Endocr Soc* (2019) 3(4):791–800. doi: 10.1210/js.2018-00427
13. Del Rio P, Montana Montana C, Cozzani F, Rossini M, Loderer T, Dall'Aglio E, et al. Is There a Correlation Between Thyroiditis and Thyroid Cancer? *Endocrine* (2019) 66(3):538–41. doi: 10.1007/s12020-019-01935-8
14. Carvalho MS, Rosario PW, Mourao GF, Calsolari MR. Chronic Lymphocytic Thyroiditis Does Not Influence the Risk of Recurrence in Patients With Papillary Thyroid Carcinoma and Excellent Response to Initial Therapy. *Endocrine* (2017) 55(3):954–8. doi: 10.1007/s12020-016-1185-1
15. Lee I, Kim HK, Soh EY, Lee J. The Association Between Chronic Lymphocytic Thyroiditis and the Progress of Papillary Thyroid Cancer. *World J Surg* (2020) 44(5):1506–13. doi: 10.1007/s00268-019-05337-9
16. Marotta V, Guerra A, Zatelli MC, Uberti ED, Di Stasi V, Faggiano A, et al. BRAF Mutation Positive Papillary Thyroid Carcinoma Is Less Advanced When Hashimoto's Thyroiditis Lymphocytic Infiltration Is Present. *Clin Endocrinol (Oxf)* (2013) 79(5):733–8. doi: 10.1111/cen.12194
17. Kim WW, Ha TK, Bae SK. Clinical Implications of the BRAF Mutation in Papillary Thyroid Carcinoma and Chronic Lymphocytic Thyroiditis. *J Otolaryngol Head Neck Surg* (2018) 47(1):4. doi: 10.1186/s40463-017-0247-6
18. Sakiz D, Sencar ME, Calapkulu M, Ozturk Unsal I, Aktas L, Ucan B, et al. The Effects of Chronic Lymphocytic Thyroiditis on Clinicopathologic Factors in Papillary Thyroid Cancer. *Endocr Pract* (2021) 27(12):1199–204. doi: 10.1016/j.eprac.2021.07.011
19. Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, et al. Meta-Analysis of Observational Studies in Epidemiology: A Proposal for Reporting. Meta-Analysis Of Observational Studies in Epidemiology (MOOSE) Group. *JAMA* (2000) 283(15):2008–12. doi: 10.1001/jama.283.15.2008
20. Stang A. Critical Evaluation of the Newcastle-Ottawa Scale for the Assessment of the Quality of Nonrandomized Studies in Meta-Analyses. *Eur J Epidemiol* (2010) 25(9):603–5. doi: 10.1007/s10654-010-9491-z
21. Kim SS, Lee BJ, Lee JC, Kim SJ, Jeon YK, Kim MR, et al. Coexistence of Hashimoto's Thyroiditis With Papillary Thyroid Carcinoma: The Influence of Lymph Node Metastasis. *Head Neck* (2011) 33(9):1272–7. doi: 10.1002/hed.21594
22. Kim HS, Choi YJ, Yun JS. Features of Papillary Thyroid Microcarcinoma in the Presence and Absence of Lymphocytic Thyroiditis. *Endocr Pathol* (2010) 21(3):149–53. doi: 10.1007/s12022-010-9124-9
23. Huang BY, Hseuh C, Chao TC, Lin KJ, Lin JD. Well-Differentiated Thyroid Carcinoma With Concomitant Hashimoto's Thyroiditis Present With Less Aggressive Clinical Stage and Low Recurrence. *Endocr Pathol* (2011) 22(3):144–9. doi: 10.1007/s12022-011-9164-9
24. Ahn D, Heo SJ, Park JH, Kim JH, Sohn JH, Park JY, et al. Clinical Relationship Between Hashimoto's Thyroiditis and Papillary Thyroid Cancer. *Acta Oncol* (2011) 50(8):1228–34. doi: 10.3109/0284186X.2011.602109
25. Jeong JS, Kim HK, Lee CR, Park S, Park JH, Kang SW, et al. Coexistence of Chronic Lymphocytic Thyroiditis With Papillary Thyroid Carcinoma: Clinical Manifestation and Prognostic Outcome. *J Korean Med Sci* (2012) 27(8):883–9. doi: 10.3346/jkms.2012.27.8.883
26. Kim YS, Choi HJ, Kim ES. Papillary Thyroid Carcinoma With Thyroiditis: Lymph Node Metastasis, Complications. *J Korean Surg Soc* (2013) 85(1):20–4. doi: 10.4174/jkss.2013.85.1.20
27. Cordioli MI, Cury AN, Nascimento AO, Oliveira AK, Mello M, Saieg MA. Study of the Histological Profile of Papillary Thyroid Carcinomas Associated With Hashimoto's Thyroiditis. *Arq Bras Endocrinol Metabol* (2013) 57(6):445–9. doi: 10.1590/s0004-27302013000600006
28. Jara SM, Carson KA, Pai SI, Agrawal N, Richmon JD, Prescott JD, et al. The Relationship Between Chronic Lymphocytic Thyroiditis and Central Neck Lymph Node Metastasis in North American Patients With Papillary Thyroid Carcinoma. *Surgery* (2013) 154(6):1272–80; discussion 80-2. doi: 10.1016/j.surg.2013.07.021
29. Lim JY, Hong SW, Lee YS, Kim BW, Park CS, Chang HS, et al. Clinicopathologic Implications of the BRAF(V600E) Mutation in Papillary Thyroid Cancer: A Subgroup Analysis of 3130 Cases in a Single Center. *Thyroid* (2013) 23(11):1423–30. doi: 10.1089/thy.2013.0036
30. Lang BH, Chai YJ, Cowling BJ, Min HS, Lee KE, Youn YK. Is BRAFV600E Mutation a Marker for Central Nodal Metastasis in Small Papillary Thyroid Carcinoma? *Endocr Relat Cancer* (2014) 21(2):285–95. doi: 10.1530/ERC-13-0291
31. Kwak HY, Chae BJ, Eom YH, Hong YR, Seo JB, Lee SH, et al. Does Papillary Thyroid Carcinoma Have a Better Prognosis With or Without Hashimoto Thyroiditis? *Int J Clin Oncol* (2015) 20(3):463–73. doi: 10.1007/s10147-014-0754-7
32. Konturek A, Barczynski M, Nowak W, Wierzchowski W. Risk of Lymph Node Metastases in Multifocal Papillary Thyroid Cancer Associated With Hashimoto's Thyroiditis. *Langenbecks Arch Surg* (2014) 399(2):229–36. doi: 10.1007/s00423-013-1158-2
33. Kim SK, Woo JW, Lee JH, Park I, Choe JH, Kim JH, et al. Chronic Lymphocytic Thyroiditis and BRAF V600E in Papillary Thyroid Carcinoma. *Endocr Relat Cancer* (2016) 23(1):27–34. doi: 10.1530/ERC-15-0408
34. Kim SJ, Myong JP, Jee HG, Chai YJ, Choi JY, Min HS, et al. Combined Effect of Hashimoto's Thyroiditis and BRAF(V600E) Mutation Status on Aggressiveness in Papillary Thyroid Cancer. *Head Neck* (2016) 38(1):95–101. doi: 10.1002/hed.23854
35. Dobrinja C, Makovac P, Pastoricchio M, Cipolat Mis T, Bernardi S, Fabris B, et al. Coexistence of Chronic Lymphocytic Thyroiditis and Papillary Thyroid Carcinoma. Impact on Presentation, Management, and Outcome. *Int J Surg* (2016) 28(Suppl 1):S70–4. doi: 10.1016/j.ijsu.2015.12.059
36. Zhang Y, Dai J, Wu T, Yang N, Yin Z. The Study of the Coexistence of Hashimoto's Thyroiditis With Papillary Thyroid Carcinoma. *J Cancer Res Clin Oncol* (2014) 140(6):1021–6. doi: 10.1007/s00432-014-1629-z
37. Girardi FM, Barra MB, Zettler CG. Papillary Thyroid Carcinoma: Does the Association With Hashimoto's Thyroiditis Affect the Clinicopathological

Characteristics of the Disease? *Braz J Otorhinolaryngol* (2015) 81(3):283–7. doi: 10.1016/j.bjorl.2014.04.006
38. Cortes MCS, Rosario PW, Mourao GF, Calsolari MR. Influence of Chronic Lymphocytic Thyroiditis on the Risk of Persistent and Recurrent Disease in Patients With Papillary Thyroid Carcinoma and Elevated Antithyroglobulin Antibodies After Initial Therapy. *Braz J Otorhinolaryngol* (2018) 84(4):448–52. doi: 10.1016/j.bjorl.2017.05.005
39. Yang Y, Chen C, Chen Z, Jiang J, Chen Y, Jin L, et al. Prediction of Central Compartment Lymph Node Metastasis in Papillary Thyroid Microcarcinoma. *Clin Endocrinol (Oxf)* (2014) 81(2):282–8. doi: 10.1111/cen.12417
40. Zeng R-C, Jin L-P, Chen E-D, Dong S-Y, Cai Y-F, Huang G-L, et al. Potential Relationship Between Hashimoto's Thyroiditis and BRAF(V600E) Mutation Status in Papillary Thyroid Cancer. *Head Neck* (2016) 38(Suppl 1):E1019–E25. doi: 10.1002/hed.24149
41. Yoon YH, Kim HJ, Lee JW, Kim JM, Koo BS. The Clinicopathologic Differences in Papillary Thyroid Carcinoma With or Without Co-Existing Chronic Lymphocytic Thyroiditis. *Eur Arch Otorhinolaryngol* (2012) 269 (3):1013–7. doi: 10.1007/s00405-011-1732-6
42. Paulson LM, Shindo ML, Schuff KG. Role of Chronic Lymphocytic Thyroiditis in Central Node Metastasis of Papillary Thyroid Carcinoma. *Otolaryngol Head Neck Surg* (2012) 147(3):444–9. doi: 10.1177/0194599812445727
43. Park JY, Kim DW, Park HK, Ha TK, Jung SJ, Kim DH, et al. Comparison of T Stage, N Stage, Multifocality, and Bilaterality in Papillary Thyroid Carcinoma Patients According to the Presence of Coexisting Lymphocytic Thyroiditis. *Endocr Res* (2015) 40(3):151–5. doi: 10.3109/07435800.2014.977911
44. Ding J, Wu W, Fang J, Zhao J, Jiang L. Male Sex Is Associated With Aggressive Behaviour and Poor Prognosis in Chinese Papillary Thyroid Carcinoma. *Sci Rep* (2020) 10(1):4141. doi: 10.1038/s41598-020-60199-9
45. Zhu F, Shen YB, Li FQ, Fang Y, Hu L, Wu YJ. The Effects of Hashimoto Thyroiditis on Lymph Node Metastases in Unifocal and Multifocal Papillary Thyroid Carcinoma: A Retrospective Chinese Cohort Study. *Med (Baltimore)* (2016) 95(6):e2674. doi: 10.1097/MD.0000000000002674
46. Song E, Jeon MJ, Park S, Kim M, Oh HS, Song DE, et al. Influence of Coexistent Hashimoto's Thyroiditis on the Extent of Cervical Lymph Node Dissection and Prognosis in Papillary Thyroid Carcinoma. *Clin Endocrinol (Oxf)* (2018) 88(1):123–8. doi: 10.1111/cen.13475
47. Nam HY, Lee HY, Park GC. Impact of Co-Existent Thyroiditis on Clinical Outcome in Papillary Thyroid Carcinoma With High Preoperative Serum Antithyroglobulin Antibody: A Retrospective Cohort Study. *Clin Otolaryngol* (2016) 41(4):358–64. doi: 10.1111/coa.12520
48. Giagourta I, Evangelopoulou C, Papaioannou G, Kassi G, Zapanti E, Prokopiou M, et al. Autoimmune Thyroiditis in Benign and Malignant Thyroid Nodules: 16-Year Results. *Head Neck* (2014) 36(4):531–5. doi: 10.1002/hed.23331
49. Consorti F, Loponte M, Milazzo F, Potasso L, Antonaci A. Risk of Malignancy From Thyroid Nodular Disease as an Element of Clinical Management of Patients With Hashimoto's Thyroiditis. *Eur Surg Res* (2010) 45(3-4):333–7. doi: 10.1159/000320954
50. Jackson D, Handelsman RS, Farra JC, Lew JI. Increased Incidental Thyroid Cancer in Patients With Subclinical Chronic Lymphocytic Thyroiditis. *J Surg Res* (2020) 245:115–8. doi: 10.1016/j.jss.2019.07.025
51. Kim ES, Lim DJ, Baek KH, Lee JM, Kim MK, Kwon HS, et al. Thyroglobulin Antibody Is Associated With Increased Cancer Risk in Thyroid Nodules. *Thyroid* (2010) 20(8):885–91. doi: 10.1089/thy.2009.0384
52. Lun Y, Wu X, Xia Q, Han Y, Zhang X, Liu Z, et al. Hashimoto's Thyroiditis as a Risk Factor of Papillary Thyroid Cancer May Improve Cancer Prognosis. *Otolaryngol–Head Neck Surg Off J Am Acad Otolaryngol-Head Neck Surg* (2013) 148(3):396–402. doi: 10.1177/0194599812472426
53. Vasileiadis I, Boutzios G, Charitoudis G, Koukoulioti E, Karatzas T. Thyroglobulin Antibodies Could be a Potential Predictive Marker for Papillary Thyroid Carcinoma. *Ann Surg Oncol* (2014) 21(8):2725–32. doi: 10.1245/s10434-014-3593-x
54. Zhang L, Li H, Ji QH, Zhu YX, Wang ZY, Wang Y, et al. The Clinical Features of Papillary Thyroid Cancer in Hashimoto's Thyroiditis Patients From an Area With a High Prevalence of Hashimoto's Disease. *BMC Cancer* (2012) 12:610. doi: 10.1186/1471-2407-12-610
55. Paparodis R, Imam S, Todorova-Koteva K, Staii A, Jaume JC. Hashimoto's Thyroiditis Pathology and Risk for Thyroid Cancer. *Thyroid* (2014) 24 (7):1107–14. doi: 10.1089/thy.2013.0588
56. Zeng R, Shou T, Yang K-X, Shen T, Zhang J-P, Zuo R-X, et al. Papillary Thyroid Carcinoma Risk Factors in the Yunnan Plateau of Southwestern China. *Ther Clin Risk Manag* (2016) 12:1065–74. doi: 10.2147/TCRM.S105023
57. Dailey ME, Lindsay S, Skahen R. Relation of Thyroid Neoplasms to Hashimoto Disease of the Thyroid Gland. *AMA Arch Surg* (1955) 70 (2):291–7. doi: 10.1001/archsurg.1955.01270080137023
58. Buyukasik O, Hasdemir AO, Yalcin E, Celep B, Sengul S, Yandakci K, et al. The Association Between Thyroid Malignancy and Chronic Lymphocytic Thyroiditis: Should It Alter the Surgical Approach? *Endokrynol Pol* (2011) 62(4):303–8.
59. Jankovic B, Le KT, Hershman JM. Clinical Review: Hashimoto's Thyroiditis and Papillary Thyroid Carcinoma: Is There a Correlation? *J Clin Endocrinol Metab* (2013) 98(2):474–82. doi: 10.1210/jc.2012-2978
60. Nicolson NG, Brown TC, Korah R, Carling T. Immune Cell Infiltrate-Associated Dysregulation of DNA Repair Machinery may Predispose to Papillary Thyroid Carcinogenesis. *Surgery* (2020) 167(1):66–72. doi: 10.1016/j.surg.2019.02.024
61. McLeod DS, Watters KF, Carpenter AD, Ladenson PW, Cooper DS, Ding EL. Thyrotropin and Thyroid Cancer Diagnosis: A Systematic Review and Dose-Response Meta-Analysis. *J Clin Endocrinol Metab* (2012) 97(8):2682–92. doi: 10.1210/jc.2012-1083
62. Wirtschafter A, Schmidt R, Rosen D, Kundu N, Santoro M, Fusco A, et al. Expression of the RET/PTC Fusion Gene as a Marker for Papillary Carcinoma in Hashimoto's Thyroiditis. *Laryngoscope* (1997) 107(1):95–100. doi: 10.1097/00005537-199701000-00019
63. Unger P, Ewart M, Wang BY, Gan L, Kohtz DS, Burstein DE. Expression of P63 in Papillary Thyroid Carcinoma and in Hashimoto's Thyroiditis: A Pathobiologic Link? *Hum Pathol* (2003) 34(8):764–9. doi: 10.1016/s0046-8177(03)00239-9
64. Kim KH, Suh KS, Kang DW, Kang DY. Mutations of the BRAF Gene in Papillary Thyroid Carcinoma and in Hashimoto's Thyroiditis. *Pathol Int* (2005) 55(9):540–5. doi: 10.1111/j.1440-1827.2005.01866.x
65. Kim SY, Kim BW, Pyo JY, Hong SW, Chang HS, Park CS. Macrometastasis in Papillary Thyroid Cancer Patients Is Associated With Higher Recurrence in Lateral Neck Nodes. *World J Surg* (2018) 42(1):123–9. doi: 10.1007/s00268-017-4158-5
66. Pelizzo MR, Boschin IM, Toniato A, Piotto A, Pagetta C, Gross MD, et al. Papillary Thyroid Carcinoma: 35-Year Outcome and Prognostic Factors in 1858 Patients. *Clin Nucl Med* (2007) 32(6):440–4. doi: 10.1097/RLU.0b013e31805375ca
67. Mao J, Zhang Q, Zhang H, Zheng K, Wang R, Wang G. Risk Factors for Lymph Node Metastasis in Papillary Thyroid Carcinoma: A Systematic Review and Meta-Analysis. *Front Endocrinol (Lausanne)* (2020) 11:265. doi: 10.3389/fendo.2020.00265
68. Sun W, Lan X, Zhang H, Dong W, Wang Z, He L, et al. Risk Factors for Central Lymph Node Metastasis in CN0 Papillary Thyroid Carcinoma: A Systematic Review and Meta-Analysis. *PloS One* (2015) 10(10):e0139021. doi: 10.1371/journal.pone.0139021
69. Zhang Q, Liu SZ, Zhang Q, Guan YX, Chen QJ, Zhu QY. Meta-Analyses of Association Between BRAF(V600E) Mutation and Clinicopathological Features of Papillary Thyroid Carcinoma. *Cell Physiol Biochem* (2016) 38 (2):763–76. doi: 10.1159/000443032

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.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 24 May 2022
doi: 10.3389/fendo.2022.850235

Clinicopathologic Characteristics and Outcomes of Massive Multinodular Goiter: A Retrospective Cohort Study

*Qiang Chen, Anping Su, Xiuhe Zou, Feng Liu, Rixiang Gong, Jingqiang Zhu, Zhihui Li and Tao Wei**

Department of Thyroid Surgery, West China Hospital, Sichuan University, Chengdu, China

Background: Thyroidectomy for massive goiters is challenging because of the increased risk of tracheomalacia, combined sternotomy, postoperative morbidity, and mortality, whereas studies investigating the clinicopathologic characteristics, postoperative morbidities, and surgical outcomes of massive goiters are limited.

Methods: Patients with goiters undergoing thyroid surgery between 2009 and 2019 were retrospectively reviewed. A total of 227 patients were enrolled and divided into massive goiter group and large goiter group according to the weight of the goiter. Clinicopathologic characteristics, postoperative morbidities, and surgical outcomes were compared between the two groups.

Results: Seventy-four patients (32.6%) had a goiter weighing more than 250 g and 153 patients (67.4%) were categorized in the large goiter group. Compared to large goiter patients, massive goiter patients had higher rates of retrosternal extension (82.4% vs. 30.7%), combined sternotomy (12.2% vs. 1.3%), intensive care unit admission (25.7% vs. 7.2%), transient hypoparathyroidism (41.9% vs. 25.5%), and transient recurrent laryngeal nerve palsy (10.8% vs. 3.3%) as well as prolonged length of hospital stay ($P < 0.05$).

Conclusions: Massive goiter patients were at increased risk of combined sternotomy, intensive care unit admission, postoperative morbidities as well as prolonged length of hospital stay after thyroidectomy compared to large goiter patients, but most of them can be treated through a cervical approach with a favorable outcome.

Keywords: multinodular goiter, thyroidectomy, retrosternal extension, massive goiter, sternotomy

OPEN ACCESS

Edited by:
Catherine Fiona Sinclair, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Erivelto Martinho Volpi, Centro de referencia no ensino do diagnóstico por imagem (CETRUS), Brazil
Serena Ippolito, PO Ospedale del Mare, Italy

***Correspondence:**
Tao Wei
surgeonweitao@163.com

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *07 January 2022*
Accepted: *24 February 2022*
Published: *24 May 2022*

Citation:
Chen Q, Su A, Zou X, Liu F, Gong R, Zhu J, Li Z and Wei T (2022) Clinicopathologic Characteristics and Outcomes of Massive Multinodular Goiter: A Retrospective Cohort Study. Front. Endocrinol. 13:850235. doi: 10.3389/fendo.2022.850235

INTRODUCTION

Multinodular goiter (MNG) is a common thyroid disorder, which is estimated to affect about 1.5 billion people worldwide (1, 2). Iodine deficiency is considered to be the most common cause of goiter in iodine-deficient areas, and the incidence of nodular goiter has declined with the popularization and implementation of iodized salt intake in the last decades (3, 4). However, the prevalence of nodular goiter, in iodine repletion countries, is reported in a range from 13% to 45% (5). Most nodular goiters are generally asymptomatic and slow growing, while surgical intervention should be considered for patients with compressive symptoms, disfigurement, or suspected

malignancy. The severity of compressive symptoms depends on the size and location of the goiter, which may worsen owing to the rapid growth or hemorrhage. In a recent study (6), shortness of breath was noted in 40% of patients with large cervical goiter (>100 g) and 52% with substernal goiter.

Surgical intervention is the most effective treatment for massive goiters that offers a definitive effect on alleviation of compressive symptoms. However, patients with massive goiters pose airway and surgical challenges due to the airway deformity and distorted neck anatomy. In addition, retrosternal extension, with a reported incidence of 12%~46%, is another challenge to surgeons because of the risk of an extra-thoracic approach (7, 8). Therefore, it is important to conduct a detailed preoperative evaluation as well as peri-operative management in patients with massive goiters. Although there have been many studies focusing on large goiters (>100 g), there are few studies targeting massive goiters.

The aim of this study was to investigate the clinicopathologic characteristics, postoperative morbidities, and surgical outcomes of patients with massive goiters.

MATERIALS AND METHODS

Study Design

We conducted a retrospective study of patients who underwent thyroidectomy as treatment for goiter in West China Hospital, Sichuan University from September 2009 to December 2019. Clinical and pathological data were retrieved from electronic medical record system from the Computer Information Resource Center of West China Hospital. Demographic and clinicopathologic variables, including age, gender, compressive symptoms, retrosternal extension, surgical procedures, postoperative morbidities, length of hospital stay, concurrent thyroid malignancy, and surgical outcomes, were analyzed and compared between the large goiter group and the massive goiter group. Patients with incomplete record, in absence of preoperative CT record, or in absence of goiter weight record as well as an ectopic goiter were excluded. This study was designed according to the STROBE criteria and was approved by the Institutional Review Board of West China Hospital, Sichuan University.

Peri-Operative Management

All patients underwent a preoperative thyroid function test including thyroid stimulating hormone, free triiodothyronine, free thyroxine, thyroglobulin, and antibodies against thyroperoxidase and thyroglobulin as well as serum parathyroid hormone (PTH). Patients with hyperthyroidism in the study were treated with methimazole (10-30 mg/day; Merck KGaA, Darmstadt, Germany) to achieve a euthyroid state prior to surgery. Both ultrasonography (US) and cervicothoracic CT scan were performed in all patients to assess the size, location, and adjacent structures of the goiter. A fiberoptic laryngoscopy was performed preoperatively to assess vocal cord mobility. Post-operative laryngoscopy was selectively performed in patients with voice change. Fine-needle aspiration cytology (FNAC) was selectively performed in patients with suspected malignancy based on preoperative US findings. Moreover, an esophageal or tracheal endoscopy was performed in patients with suspected malignancy or significant symptoms of dysphagia and dyspnea.

Serum calcium and PTH levels were routinely assessed for all patients on the first day after surgery. Intravenous and oral calcium supplementations with 1,25-dihydroxyvitamin D3 (1.8~2.4 g/day; Caltrate, Wyeth, New Jersey, USA) were prescribed if the patient exhibited symptoms of hypocalcemia. The dosage of these medications was gradually tapered off with the normalization of serum calcium and PTH levels.

Definitions

Although there is no uniform definition of a large goiter, several studies defined a large goiter as the gross weight of more than 100 g (6, 7). Therefore, a large goiter was also defined as a goiter with a gross weight > 100 g, and a massive goiter was defined as a goiter with a gross weight >250 g in the present study. Similarly, a substernal goiter was defined as a goiter extending below the plane of the thoracic inlet on computed tomography (CT) scan in the supine position.

Hypoparathyroidism was defined as a serum PTH level lower than 1.6 pmol/L with a concurrent serum calcium level <2.0 mmol/L. Permanent hypoparathyroidism was defined as postoperative hypocalcemia for more than 6 months. Vocal cord palsy was diagnosed if vocal cord immobility was confirmed by laryngoscopy. Permanent recurrent laryngeal nerve (RLN) palsy was defined as persistent vocal cord palsy for more than 12 months postoperatively.

Statistical Analysis

Normally or nonnormally distributed continuous variables were described as mean ± standard deviation (SD) or median, respectively. Comparisons between two groups were conducted using Pearson's chi-square test or Fisher's exact test for categorical variables, whereas continuous variables were compared using Student's *t*-test or Mann-Whitney *U* test. Statistically significant was accepted when $P < 0.05$. All statistical analyses were performed using SPSS Statistics software version 21.0 (IBM Inc., Chicago, IL, USA).

RESULTS

Patient Characteristics

From September 2009 to December 2019, a total of 452 thyroidectomies were performed for nodular goiters in our department. Finally, a total of 227 patients who met the criteria were included (**Figure 1**). Patient demographics and characteristics are summarized in **Table 1**. There were 171 females and 56 males, with a median age of 58 years (range, 17-87 years). The most common compressive symptom was dyspnea (44.9%), followed by dysphagia (21.6%). Of these, 159 patients (70.0%) had a normal thyroid function, while 21 patients (9.3%) exhibited hyperthyroidism and 47 (20.7%) exhibited hypothyroidism. Total thyroidectomy or near total thyroidectomy was performed in 93 patients (41.0%), lobectomy in 62 patients (27.3%), Dunhill's

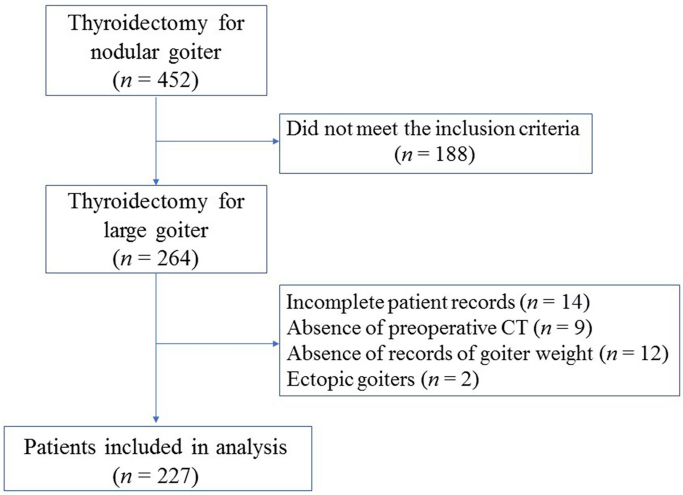


FIGURE 1 | Flow chart of patients' inclusion.

operation in 29 patients (12.8%), and 43 patients (18.9%) underwent completion thyroidectomy. In addition, concurrent central lymph node dissection (level VI) was performed in 21 patients (9.3%).

TABLE 1 | Demographic and clinical characteristics of patients.

	n = 227
Age at presentation (y), median (range)	58 (17-87)
Gross thyroid weight (g), mean ± SD	284.2 ± 54.6
Gender, *n* (%)	
Male	56 (24.7)
Female	171 (75.3)
Preoperative thyroid function, *n* (%)	
Euthyroidism	159 (70.0)
Hyperthyroidism	21 (9.3)
Hypothyroidism*	47 (20.7)
Hashimoto thyroiditis, *n* (%)	35 (15.4)
Preoperative symptoms, *n* (%)	
Dyspnea	102 (44.9)
Dysphagia	49 (21.6)
Hoarseness	13 (5.7)
Neck pain	35 (15.4)
Retrosternal extension, *n* (%)	108 (47.6)
Surgical procedure, *n* (%)	
Lobectomy	62 (27.3)
Dunhill's operation#	29 (12.8)
Total or near total thyroidectomy	93 (41.0)
Completion thyroidectomy	43 (18.9)
Central lymph node dissection	21 (9.3)
Sternotomy	11 (4.8)
Pathology, *n* (%)	
Benign	204 (89.9)
Malignant	23 (10.1)
Length of hospital stay (d), mean ± SD	8.7 ± 2.6

SD, standard deviation.
**including subclinical hypothyroidism.*
#Dunhill's operation: one side lobectomy and one side subtotal thyroidectomy.

Retrosternal extension was observed in 108 patients (47.6%). Based on careful review of CT images, the inferior limit of goiter extended over the aortic arch in 9 patients (4.0%) and to the tracheal bifurcation in 2 patients (0.8%). Malignancy was identified in 23 patients (10.1%) and the type of malignancies included papillary microcarcinoma (n = 13), papillary carcinoma (n = 8), and follicular papillary carcinoma (n = 2). Among them, 16 cases were diagnosed by FNAC, while other 7 cases with negative FNAC results were confirmed by intraoperative frozen section examination or by paraffin pathological diagnosis.

The mean ± SD weight of goiter was 284.2 ± 54.6 g. One hundred and fifty-three patients (67.4%) were categorized in the large goiter group and 74 patients (32.6%) had a goiter weight >250 g. As shown in **Table 2**, massive goiter patients were more frequently from rural areas ($P = 0.032$) with a longer duration course of goiter ($P < 0.001$) compared to large goiter patients. The rates of compressive symptoms ($P < 0.05$) and retrosternal extension ($P < 0.001$) were significantly higher in the massive goiter group. Moreover, patients with massive goiters more frequently had a previous thyroidectomy ($P < 0.001$) with significantly higher rates of hypothyroidism ($P = 0.002$), combined sternotomy ($P < 0.001$), and prolonged length of hospital stay ($P < 0.001$).

Primary Outcomes

Postoperative outcomes are shown in **Table 3**. One case of permanent hypoparathyroidism was recorded in the massive goiter group. There was a higher incidence of transient hypoparathyroidism in the massive goiter group compared to the large goiter group (41.9% vs. 25.5%, $P = 0.012$). Moreover, there was an increased risk of transient RLN palsy in the massive group ($P = 0.032$). No statistical differences were observed in permanent RLN palsy and permanent hypoparathyroidism between the two groups. Additionally, massive goiter patients had a significantly higher rate of intensive care unit (ICU) admission ($P < 0.001$). None of the patients in both groups

TABLE 2 | Age and gender demographics of the double Sinopharm Vaccinated cohort in this study.

	Large goiters (≤250 g) (*n* = 153)	Massive goiters (>250 g) (*n* = 74)	P
Age at presentation (y), median (range)	54 (17-72)	63 (26-87)	0.526
Gender, *n* (%)			0.119
Male	46 (30.1)	15 (20.3)	
Female	107 (69.9)	59 (79.7)	
BMI (kg/m^2)			0.408
<25	74(36)	40(4)	
≥25	43	30	
Duration of goiter (y), mean ± SD	4.3 ± 2.3	10.5 ± 5.2	<0.001
Resident region, *n* (%)			0.032
Urban	66 (43.1)	21 (28.4)	
Rural	87 (56.9)	53 (71.6)	
Hypothyroidism*, *n* (%)	23 (15.0)	24 (32.4)	0.002
History of thyroidectomy[§], *n* (%)	19 (12.4)	24 (32.4)	<0.001
Hashimoto thyroiditis, *n* (%)	23 (15.0)	9 (16.2)	0.817
Retrosternal extension, *n* (%)	47 (30.7)	61 (82.4)	<0.001
Dyspnea	41 (26.8)	61 (82.4)	<0.001
Dysphagia	24 (15.7)	25 (33.8)	0.002
Hoarseness	5 (3.3)	8 (10.8)	0.032[†]
Sternotomy, *n* (%)	2 (1.3)	9 (12.2)	<0.001[†]
Postoperative pathology, *n* (%)			0.002
Benign	144 (94.1)	60 (81.1)	
Malignant	9 (5.9)	14 (18.9)	
Length of hospital stay (d), mean ± SD	7.9 ± 2.4	9.8 ± 2.5	<0.001

SD, standard deviation.
**Including subclinical hypothyroidism.*
[§]Including lobectomy and subtotal thyroidectomy.
[†]Fisher's exact test.

TABLE 3 | Comparison of postoperative complications between large goiters and massive goiters.

	Large goiters (≤250 g) (*n* = 153)	Massive goiters (>250 g) (*n* =74)	*P*
Transient hypoparathyroidism, *n* (%)	39 (25.5)	31 (41.9)	0.012
Permanent hypoparathyroidism, *n* (%)	0	1 (1.4)	0.326*
Transient RLN palsy, *n* (%)	5 (3.3)	8 (10.8)	0.032*
Permanent RLN palsy, *n* (%)	1 (0.7)	3 (4.1)	0.103*
Surgical reintervention for bleeding, *n* (%)	2 (1.3)	4 (5.4)	0.090*
Wound infection, *n* (%)	5 (3.3)	3 (4.1)	0.718*
ICU admission, *n* (%)	11 (7.2)	19 (25.7)	<0.001

RLN, recurrent laryngeal nerve; ICU, intensive care unit.
**Fisher's exact test.*

required tracheostomy, while 5 massive goiter patients (6.7%) required prolonged intubation to prevent tracheomalacia, all of which were successfully extubated a few days later. Despite the higher rate of concurrent malignancy in the massive group, neither the patients in the large group nor in the massive group suffered recurrent disease with a mean follow-up time of 6.7 months (range, 3–18 months) and 14.2 months (range, 9–24 months), respectively.

DISCUSSION

Goiter is defined as an enlargement of the thyroid gland that usually presents as a swelling in the front of the neck, which can be further classified as endemic or non-endemic, diffuse or nodular, and toxic or nontoxic (9). Most goiters are asymptomatic and do not require surgical intervention, while surgery is recommended in patients with compressive symptoms, suspected malignancy, drug-resistant hyperthyroidism, or retrosternal extension (10–12). Patients with large goiters frequently present with shortness of breath, dysphagia, and voice change; however, the most common symptom is nonspecific. In our study, the most common symptom was dyspnea, and patients in the massive goiter group had a much higher rate of compressive symptoms (for example, dyspnea, dysphagia, and hoarseness). Compressive symptoms may be directly related to goiter size, while goiter size is correlated with growth time: the longer the growth time, the larger the size. Consistent with the data of Agarwal et al. (13), we found that the duration course of goiter in the massive goiter group was longer than that in the large goiter group. These results, in a word, justify that a massive goiter potentially results from an untreated goiter with further progression for a long time.

Goiter can affect the thyroid gland diffusely or only involve one lobe. Although performing total thyroidectomy or lobectomy remains controversial, lobectomy may be an appropriate procedure for goiters which are isolated to one lobe. However, in a prospective study with a mean follow-up time of 14.5 years, Beatriz et al. (14) found that about 15% of patients (394/2,675) with MNG required completion thyroidectomy for recurrence. In this study, 43 patients (18.9%) underwent completion thyroidectomy; however, we could not conclude that the patients had recurrence of MNG because it is difficult to evaluate whether residual goiter remained after the initial surgery. Moreover, whether nodular disease involves the contralateral lobe is difficult to assess. Interestingly, we found that a higher proportion of patients with massive goiter had a previous thyroidectomy. In general, the residual thyroid tissue after lobectomy can secrete sufficient thyroid hormone to maintain daily metabolism, while thyroxine will be insufficient if the remnant of thyroid tissue is small or involved in diseases, such as Hashimoto thyroiditis or nodular goiter. Low-level of thyroid hormone stimulates the synthesis and secretion of TSH; the latter one plays an important role in the development of nodular thyroid hyperplasia (15, 16). As shown in the present study, the patients with massive goiter were found to have a higher rate of hypothyroidism, which suggests that close surveillance of thyroid function and appropriate supplementation of thyroxine may play an important role in preventing goiter progression.

Retrosternal extension, namely substernal goiter, is frequently associated with a large goiter. Although numerous definitions have been proposed and compared, there is no consensus on the definition of substernal goiter. In the literature, the reported incidence of substernal goiter varies from 12% to 46% due to the use of different criteria (7, 8). In the present study, 108 patients (47.6%) were confirmed with retrosternal extension, and all of them located in the anterior mediastinum. Moreover, we identified that the rate of retrosternal extension in the massive group was higher than that in the large group, which is consistent with a previous report (12). Most substernal goiters can be managed through a cervical approach; sternotomy should be taken into consideration if the extension is beyond the level of the aortic arch, in combination with malignancy, or in the presence of a posterior mediastinal goiter, ectopic goiter, or recurrent goiter (17–19). Only 11 patients (4.8%) in the study required a combined sternotomy, and all the patients who underwent a combined sternotomy had a goiter extending below the level of the aortic arch. Similar to the report by Sancho et al. (20), in which a goiter weight of over 250 g is an independent predictor of an increased need for additional sternotomy, suggesting that a much higher rate of sternotomy would be performed in patients with massive goiters. Cervical and thoracic CT scans play a valuable role in evaluating the depth and extent of substernal goiter and its relationship with surrounding structures, which is helpful in planning the surgical strategy and anesthesia intubation.

Total thyroidectomy for massive goiter is challenging, which is associated with a significantly higher risk of RLN and parathyroid damage (21, 22). Our data showed that the rate of RLN injury was 11.2%, which was comparable between the two groups. Lin et al. (23) reported that the incidence of right-sided RLN injury was more common than that of left-sided RLN injury. However, no such difference was noted between right-sided and left-sided RLN injuries in this study. In our experience, adequate exposure is crucial in ensuring safe thyroidectomy and preventing the potential risk of RLN injury. Recently, continuous intraoperative nerve monitoring has been reported as a useful and effective modality in identifying and preserving the integrity of the nerve, which effectively lower the incidence of postoperative vocal cord palsy (24). Postoperative hypoparathyroidism is not uncommon in large goiters due to an increased risk of inadvertent dissection or devascularization of parathyroid glands. The incidence of transient hypoparathyroidism was significantly higher in the massive goiter group, whereas the rate of permanent hypoparathyroidism was similar between the two groups. This may have resulted from the reason that patients undergoing lobectomy, Dunhill's procedure, and completion thyroidectomy were also enrolled in the present study.

The incidence of malignancy in MNG has been reported to vary from 4% to 17% (25). In our study, the overall incidence of malignancy was 10.1% (23/227), and it was 18.9% (14/74) in the massive goiter group, which was significantly higher than that in the large goiter group. Whether there is an increased risk of malignancy in large goiter remains controversial; however, malignancy implies an increased risk of RLN and parathyroid injury due to the necessity of lymph node dissection. Although the relationship between goiter size and malignancy has not been clearly determined, it has been reported that malignancy was significantly higher in intrathoracic goiter (26, 27). On the basis of the study, a detailed preoperative US evaluation with FNAC is indispensable for massive goiters, even though the diagnostic accuracy of malignancy was relatively low in our study (69.6%). This can be attributed to the limitations of sonographic detection of surrounding structures, the deep location of the tumor, and/or tumor size <1.0 cm. In our experience, intraoperative frozen section examination provides a supplementary modality for the diagnosis of malignant nodules.

Some limitations should be noted in our study. First, retrospective analysis in general is subject to informational biases: data accuracy is closely dependent on the registration/codification process. Second, the present study was conducted in a single institution with a limited sample size. Finally, the rate of retrosternal extension was not accurately assessed and compared because there is no uniform definition of a retrosternal goiter. However, in our opinion, we prefer to define a substernal goiter that extending below the plane of the thoracic inlet on the basis of that researchers in different facilities and circumstances can easily and uniformly use this anatomic definition.

CONCLUSIONS

Massive goiter (>250 g) patients were at higher risk of combined sternotomy, ICU admission, postoperative morbidities as well as prolonged length of hospital stay after thyroidectomy compared to large goiter patients. Although massive goiters were associated with an increased rate of retrosternal extension, most of them can be removed through a cervical approach. The overall surgical

outcomes for massive goiters may potential less favorable than for large goiters, whereas most massive goiters can be safely excised with minimal morbidity with an experienced surgeon and a dedicated planning.

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 study was approved by the Institutional Review Board of West China Hospital, Sichuan University. Written informed consent to participate in this study was provided by the patients or their legal guardian.

AUTHOR CONTRIBUTIONS

QC, data collection, analysis, drafting of manuscript, interpretation of data, final approval, and accountability for all aspects of the work. AS, data collection, interpretation of data, and final approval. XZ, interpretation of data, analysis, and final approval. FL and RG, data collection, analysis, and final approval. JZ, interpretation of data, critical review, revising, and final approval. ZL, interpretation of data, critical review, and final approval. TW, study design, interpretation of data, critical review, revising, final approval, and accountability for all aspects of the work. All authors contributed to the article and approved the submitted version.

FUNDING

This study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Moalem J, Suh I, Duh QY. Treatment and Prevention of Recurrence of Multinodular Goiter: An Evidence-Based Review of the Literature. *World J Surg* (2008) 32(7):1301–12. doi: 10.1007/s00268-008-9477-0
2. Simó R, Nixon IJ, Vander Poorten V, Quer M, Shaha AR, Sanabria A, et al. Surgical Management of Intrathoracic Goitres. *Eur Arch Otorhinolaryngol* (2019) 276(2):305–14. doi: 10.1007/s00405-018-5213-z
3. Phitayakorn R, Super DM, McHenry CR. An Investigation of Epidemiologic Factors Associated With Large Nodular Goiter. *J Surg Res* (2006) 133(1):16–21. doi: 10.1016/j.jss.2006.02.044
4. Fiore E, Tonacchera M, Vitti P. Influence of Iodization Programmes on the Epidemiology of Nodular Goitre. *Best Pract Res Clin Endocrinol Metab* (2014) 28(4):577–88. doi: 10.1016/j.beem.2014.04.002
5. Carlé A, Krejbjerg A, Laurberg P. Epidemiology of Nodular Goitre. Influence of Iodine Intake. *Best Pract Res Clin Endocrinol Metab* (2014) 28(4):465–79. doi: 10.1016/j.beem.2014.01.001
6. Nankee L, Chen H, Schneider DF, Sippel RS, Elfenbein DM. Substernal Goiter: When Is a Sternotomy Required? *J Surg Res* (2015) 199(1):121–5. doi: 10.1016/j.jss.2015.04.045
7. Ríos A, Rodríguez JM, Balsalobre MD, Tebar FJ, Parrilla P. The Value of Various Definitions of Intrathoracic Goiter for Predicting Intra-Operative and Postoperative Complications. *Surgery* (2010) 147(2):233–8. doi: 10.1016/j.surg.2009.06.018
8. Wong WK, Shetty S, Morton RP, McIvor NP, Zheng T. Management of Retrosternal Goiter: Retrospective Study of 72 Patients at Two Secondary Care Centers. *Auris Nasus Larynx* (2019) 46(1):129–34. doi: 10.1016/j.anl.2018.06.012
9. Patel KN, Yip L, Lubitz CC, Grubbs EG, Miller BS, Shen W, et al. The American Association of Endocrine Surgeons Guidelines for the Definitive Surgical Management of Thyroid Disease in Adults. *Ann Surg* (2020) 271(3):e21–93. doi: 10.1097/SLA.0000000000003580
10. Cooper DS, Doherty GM, Haugen BR, Kloos RT, Lee SL, Mandel SJ, et al. Revised American Thyroid Association Management Guidelines for Patients With Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* (2009) 19(11):1167–214. doi: 10.1089/thy.2009.0110
11. White ML, Doherty GM, Gauger PG. Evidence-Based Surgical Management of Substernal Goiter. *World J Surg* (2008) 32(7):1285–300. doi: 10.1007/s00268-008-9466-3
12. Chen AY, Bernet VJ, Carty SE, Davies TF, Ganly I, Inabnet WB 3rd, et al. American Thyroid Association Statement on Optimal Surgical Management of Goiter. *Thyroid* (2014) 24(2):181–9. doi: 10.1089/thy.2013.0291
13. Agarwal A, Agarwal S, Tewari P, Gupta S, Chand G, Mishra A, et al. Clinicopathological Profile, Airway Management, and Outcome in Huge Multinodular Goiters: An Institutional Experience From an Endemic Goiter Region. *World J Surg* (2012) 36(4):755–60. doi: 10.1007/s00268-012-1447-x
14. de Rienzo-Madero B, Sabra JP, Gand E, Donatini G, Kraimps JL. Unilateral Benign Multinodular Versus Solitary Goiter: Long-Term Contralateral Reoperation Rates After Lobectomy. *Surgery* (2019) 165(1):75–9. doi: 10.1016/j.surg.2018.04.074
15. Hegedüs L, Bonnema SJ, Bennedbaek FN. Management of Simple Nodular Goiter: Current Status and Future Perspectives. *Endocr Rev* (2003) 24(1):102–32. doi: 10.1210/er.2002-0016
16. Dauksiene D, Petkeviciene J, Klumbiene J, Verkauskiene R, Vainikonyte-Kristapone J, Seibokaite A, et al. Factors Associated With the Prevalence of Thyroid Nodules and Goiter in Middle-Aged Euthyroid Subjects. *Int J Endocrinol* (2017) 2017:8401518. doi: 10.1155/2017/8401518
17. Machado NO, Grant CS, Sharma AK, al Sabti HA, Kolidyan SV. Large Posterior Mediastinal Retrosternal Goiter Managed by a Transcervical and Lateral Thoracotomy Approach. *Gen Thorac Cardiovasc Surg* (2011) 59(7):507–11. doi: 10.1007/s11748-010-0712-x
18. de Perrot M, Fadel E, Mercier O, Farhamand P, Fabre D, Mussot S, et al. Surgical Management of Mediastinal Goiters: When Is a Sternotomy Required? *Thorac Cardiovasc Surg* (2007) 55(1):39–43. doi: 10.1055/s-2006-924440
19. Cichoń S, Anielski R, Konturek A, Baczyński M, Cichoń W, Orlicki P. Surgical Management of Mediastinal Goiter: Risk Factors for Sternotomy. *Langenbecks Arch Surg* (2008) 393(5):751–7. doi: 10.1007/s00423-008-0338-y
20. Sancho JJ, Kraimps JL, Sanchez-Blanco JM, Larrad A, Rodríguez JM, Gil P, et al. Increased Mortality and Morbidity Associated With Thyroidectomy for Intrathoracic Goiters Reaching the Carina Tracheae. *Arch Surg* (2006) 141(1):82–5. doi: 10.1001/archsurg.141.1.82
21. Ozbas S, Kocak S, Aydintug S, Cakmak A, Demirkiran MA, Wishart GC. Comparison of the Complications of Subtotal, Near Total and Total Thyroidectomy in the Surgical Management of Multinodular Goitre. *Endocr J* (2005) 52(2):199–205. doi: 10.1507/endocrj.52.199
22. Barczyński M, Konturek A, Hubalewska-Dydejczyk A, Gołkowski F, Cichoń S, Nowak W. Five-Year Follow-Up of a Randomized Clinical Trial of Total Thyroidectomy Versus Dunhill Operation Versus Bilateral Subtotal Thyroidectomy for Multinodular Nontoxic Goiter. *World J Surg* (2010) 34(6):1203–13. doi: 10.1007/s00268-010-0491-7

23. Lin YS, Wu HY, Lee CW, Hsu CC, Chao TC, Yu MC. Surgical Management of Substernal Goitres at a Tertiary Referral Centre: A Retrospective Cohort Study of 2,104 Patients. *Int J Surg* (2016) 27:46–52. doi: 10.1016/j.ijsu.2016.01.032
24. Staubitz JI, Watzka F, Poplawski A, Riss P, Clerici T, Bergenfelz A, et al. Effect of Intraoperative Nerve Monitoring on Postoperative Vocal Cord Palsy Rates After Thyroidectomy: European Multicentre Registry-Based Study. *BJS Open* (2020) 4(5):821–9. doi: 10.1002/bjs5.50310
25. Knobel M. Etiopathology, Clinical Features, and Treatment of Diffuse and Multinodular Nontoxic Goiters. *J Endocrinol Invest* (2016) 39(4):357–73. doi: 10.1007/s40618-015-0391-7
26. Moten AS, Thibault DP, Willis AW, Willis AI. Demographics, Disparities, and Outcomes in Substernal Goiters in the United States. *Am J Surg* (2016) 211 (4):703–9. doi: 10.1016/j.amjsurg.2015.11.022
27. Testini M, Gurrado A, Avenia N, Bellantone R, Biondi A, Brazzarola PC, et al. Does Mediastinal Extension of the Goiter Increase Morbidity of Total Thyroidectomy? A Multicenter Study of 19,662 Patients. *Ann Surg Oncol* (2011) 18(8):2251–9. doi: 10.1245/s10434-011-1596-4

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.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 03 June 2022
doi: 10.3389/fendo.2022.850231

The Effect of Early Life Exposure to Triclosan on Thyroid Follicles and Hormone Levels in Zebrafish

*Ning Tang[1], Pianpian Fan[1], Li Chen[2], Xiaogang Yu[1], Wenjuan Wang[1], Weiye Wang[1] and Fengxiu Ouyang[1]**

[1] Ministry of Education and Shanghai Key Laboratory of Children's Environmental Health, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] Guangxi Key Laboratory of Tumor Immunology and Microenvironmental Regulation, Guilin Medical University, Guilin, China

OPEN ACCESS

Edited by:
Maaike Van Gerwen, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Monia Perugini, University of Teramo, Italy
Caroline Serrano-Nascimento, Federal University of São Paulo, Brazil

***Correspondence:**
Fengxiu Ouyang
ouyangfengxiu@xinhuamed.com.cn; ouyangfengxiu@126.com

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *07 January 2022*
Accepted: *02 May 2022*
Published: *03 June 2022*

Citation:
Tang N, Fan P, Chen L, Yu X, Wang W, Wang W and Ouyang F (2022) The Effect of Early Life Exposure to Triclosan on Thyroid Follicles and Hormone Levels in Zebrafish. Front. Endocrinol. 13:850231. doi: 10.3389/fendo.2022.850231

Triclosan (TCS) is an antimicrobial chemical widely used in personal care products. Most of the TCS component is discharged and enters the aquatic ecosystem after usage. TCS has a similar structure as thyroid hormones that are synthesized by thyroid follicular epithelial cells, thus TCS has a potential endocrine disrupting effect. It is still not clear how the different levels of the environmental TCS would affect early development *in vivo*. This study examines the effects of TCS on thyroid hormone secretion and the early development of zebrafish. The fertilized zebrafish eggs were exposed to TCS at 0 (control), 3, 30, 100, 300, and 900 ng/mL, and the hatching rate and the larvae mortality were inspected within the first 14 days. The total triiodothyronine (TT_3), total thyroxine (TT_4), free triiodothyronine (FT_3), and free thyroxine (FT_4) were measured at 7, 14, and 120 days post-fertilization (dpf). The histopathological examinations of thyroid follicles were conducted at 120 dpf. TCS exposure at 30-300 ng/mL reduced the hatching rate of larvae to 34.5% to 28.2 % in the first 48 hours and 93.8 .7 % to 86.8 % at 72 h. Extremely high TCS exposure (900 ng/mL) strongly inhibited the hatching rate, and all the larvae died within 1 day. Exposure to TCS from 3 to 300 ng/mL reduced the thyroid hormones production. The mean TT_3 and FT_3 levels of zebrafish decreased in 300 ng/mL TCS at 14 dpf (300 ng/mL TCS vs. control : TT_3 , 0.19 ± 0.08 vs. 0.39 ± 0.06; FT_3, 19.21 ± 3.13 vs. 28.53 ± 1.98 pg/mg), and the FT_4 decreased at 120 dpf (0.09 ± 0.04 vs. 0.20 ± 0.14 pg/mg). At 120 dpf , in the 300 ng/mL TCS exposure group, the nuclear area and the height of thyroid follicular epithelial cells became greater, and the follicle cell layer got thicker. This happened along with follicle hyperplasia, nuclear hypertrophy, and angiogenesis in the thyroid. Our study demonstrated that early life exposure to high TCS levels reduces the rate and speed of embryos hatching, and induces the histopathological change of thyroid follicle, and decreases the TT_3, FT_3, and FT_4 production in zebrafish.

Keywords: triclosan, early exposure, thyroid hormone, thyroid follicles, zebrafish

INTRODUCTION

Thyroid hormones are synthesized and secreted by the thyroid follicular epithelial cells, and are critical for fetal growth and neuro-development in early life (1, 2). The thyroid gland is sensitive to environmental endocrine disruptor chemicals (EDCs) during early life (3). Triclosan (TCS) is a widely used antimicrobial agent in personal care products with a similar molecular structure as the thyroid hormone, thus it is a potential EDC. TCS has been widely used in personal care products, such as soaps, toothpastes, shampoos, and cosmetics for more than 40 years (4, 5). Most of TCS is discharged into residential drains after usage, and contaminates the aquatic ecosystem (6, 7). TCS can be detected in rivers, lakes (8), and drinking water (9). The highest TCS concentrations can reach up to 5370 ng/L in effluent water of wastewater treatment plants (10) and 1023 ng/L in rivers (11). TCS is bio-accumulated in fish and other aquatic species (12–14), and can also be detected in human urine, blood, fetal cord blood, and breast milk (15–17), suggesting there is a wide exposure to TCS of the general population in different regions of the world. For example, TCS has been detected in pregnant women with the median urinary concentration ranging from 0.4 μg/L to 26.5 μg/L (18–22).

Concerns have been raised on the potential risk for TCS to perturb thyroid endocrine functioning, in part, due to the structural similarities of TCS and thyroid hormones (23). Several animal studies had examined the potential thyroid-disrupting effect of TCS in rodents (24, 25). Most of the rodent studies found that TCS exposure decreased the serum total thyroxine (T_4) and triiodothyronine (T_3) levels (26, 27) at the doses ranging from 1 to 300 mg/kg/d. Considering TCS is a persistent and ubiquitous pollutant in the aquatic environment, the thyroid disturbing effect of TCS at different aquatic relevant concentrations needs to be studied. But few studies have directly evaluated the effect of TCS early exposure in fish (28, 29). Moreover, the histopathologic examination is considered the gold standard for evaluating the pathological changes in tissues and organs (30), but few studies have explored the impact of TCS exposure on thyroid follicular pathological changes in fish.

Zebrafish has been a good model for studies of thyroid function, since the development of its thyroid system is comparable to human (31). None has examined the effect of early exposure to TCS from the embryo stage at the environment-related level or the bio-sample concentrations, on the thyroid gland and the related hormone levels. In this study, by using a zebrafish model, we examined the impacts of early exposure to TCS at environmentally relevant concentrations on the histopathology of thyroid follicles and the thyroid hormones levels.

MATERIALS AND METHODS

TCS

TCS (Irgasan, 5-chloro-2-(2, 4-dichlorophenoxy) phenol, ≥ 97.0 % purity (HPLC), CAS no. 3380-34-5) and dimethyl sulfoxide (DMSO, 99 % purity) were purchased from Sigma-Aldrich (St. Louis, MO).

Zebrafish Maintenance and TCS Exposure

Zebrafish strains of AB wild-type line were used in the study. The male and female zebrafish at 5-months-old were acclimated in tanks containing dechlorinated tap water for 4 weeks before mating under a photoperiod of 14:10 h light/dark cycle. The fish were fed with brine shrimp twice per day.

Fertilized eggs were collected within 30 min after natural mating and unfertilized eggs were discarded. Fertilized eggs were randomly assigned to 0 (0.01 % DMSO as solvent control), 3, 30, 100, 300, and 900 ng/mL TCS (600 eggs per group) and raised up to 7 and 14 days at 28.0 ± 0.5°C under 14:10 h light/dark photoperiod cycle (32). These TCS concentrations were set based on the environmental exposure levels of both humans and wildlife (17, 33). There was 0.01% DMSO used as a solvent to enhance TCS solubility (34), as the control group . The zebrafish embryos were tolerant to low concentrations (0.01 %) of DMSO (35).

There were 200 fertilized eggs with a density of one embryo per 2 mL medium put into a culture dish until 72 h post-fertilization (hpf). The larvae were transferred into glass tanks with a density of one larva per 60 mL medium until 14 days post-fertilization (dpf) (36). After 14 dpf, the zebrafish were transferred into larger glass tanks with a density of one fish per 200 mL medium (36). Half of the medium was changed twice per day to ensure TCS concentration stable. The number of larvae hatched were inspected and recorded twice per day in the first 3 dpf (i.e., 72 hpf). The larvae were fed with paramecium twice per day until 14 dpf. Starting from 15 dpf, larvae were fed brine shrimp. The pH of raising water was maintained at 7.5 ± 0.5, conductivity was maintained at 550 ± 50 μS, temperature was maintained at 28 ± 0.5°C , and the dissolved oxygen of the raising water was monitored (32).

Hatching Rate and Mortality

The hatching rate was calculated as the number of larvae hatched during the first 3 days divided by the total number of fertilized eggs. The death of larvae was inspected and recorded twice per day for first 14 days, and the dead larvae were removed from dishes/tanks. The mortality was calculated as the number of dead larvae divided by the total number of hatched larvae.

Thyroid Hormone Measurements

At 7, 14, and 120 dpf, the zebrafish were euthanized by immersing in ice-cold water for 40 min (37, 38), and thyroid hormones (THs) were measured by ELISA (enzyme-linked immunosorbent assay) kits. Specifically, at 7 dpf, 50 larvae were homogenated and put together for one measurement. Three replicates (50 larvae each replicate) were conducted. At 14 dpf, 100 larvae were gathered for each replicate for THs measurements. For the zebrafish at 120 dpf, the heads were removed for thyroid histological analysis, and the rest of fish body was used for thyroid hormones measurement.

The free triiodothyronine (FT_3), free thyroxine (FT_4), total triiodothyronine (TT_3), and total thyroxine (TT_4) levels were

measured by ELISA (Labor Diagnostika Nord commercial kit, Nordhorn, Germany) (39). The larvae samples (at 7 and 14 dpf) and the body of the fish (with head, viscera, and gut removed, at 120 dpf) were sonicated in cold 0.01 M phosphate buffer saline (PBS) at w/v of 1mg/5μL (w: wet weight of samples, v: volume of 0.01 M PBS, mg/μL), put on intermittent sonic oscillation for 5 min, vortexed vigorously for 10 min, then centrifuged at 12,000 × g at 4°C for 5 min. The supernatant was collected to measure thyroid hormones and total protein concentration. Total protein concentration was measured with the Pierce© BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL) to normalize thyroid hormone concentration (40). The limit of detection (LOD) was 0.1 ng/mL for TT_3, 8 nmol/L for TT_4, 0.3 pg/mL for FT_3, and 1 pg/mL for FT_4. Values below the limit of detection were replaced with values equal to the LOD divided to 2.

Hematoxylin and Eosin (H&E) Staining of Thyroid Gland and Histological Analysis

The histological analysis of the thyroid gland was conducted in zebrafish at 120 dpf. The heads of zebrafish were fixed in 10% neutral formalin (Zhongshan Beijing Biotechnology Co., Ltd., Beijing, China) for at least 12 h, and transferred to 70% ethanol, all at room temperature. Each zebrafish head was placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in the wax blocks.

Serial transverse cross-sections were cut using a microtome 5 μm and dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS. The sections were then stained with hematoxylin for 4–5 min and with eosin for 1–2 min (Zhongshan Beijing Biotechnology Co., Ltd.). Thyroid follicles were located according to their being dispersed among the afferent branchial arterioles (for example, ventral aorta) in the subpharyngeal region (41). Stained thyroid follicle sections were photographed under light microscopy (BX53–p; Olympus Corporation, Tokyo, Japan).

The Nuclear Size and Height of Follicular Epithelial Cells

Photographs of each follicle were taken at the largest follicle diameter (determined by observing serial sections) with an Olympus digital camera (BX53–p; Olympus Corporation). The height of follicular epithelial cells and the long and short diameters of their cell nuclei were quantitatively analyzed. The nuclear size was calculated as the long diameter × short diameter × π/4 (41). At least 3-5 histological tissue sections per one fish sample and three different follicles (or three separate areas) per one section were selected for measuring in cases where the follicular structure was unclear or absent. The nuclear size was calculated in at least 50 thyroid follicular cell nuclei per fish at a magnification of ×1000. The height of a follicular epithelial cell was calculated by the mean of five measures along the follicle perimeter at equal intervals, and 40-80 follicular epithelial cells were measured for each fish (42). Image Pro–Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD) was used to analyze the photos.

Statistical Analysis

The ANOVA F-test was used to compare the differences in thyroid hormone levels, the height of thyroid follicle epithelial cells and nuclear size, and chi square test was used to compare the difference in hatching rate and mortality of larvae among TCS exposure groups. We used linear regression models to evaluate the associations of TCS exposures with thyroid hormones and thyroid follicle histopathological changes. The level of significance was two-sided P value < 0.05. All analyses were performed using the SAS 9.3 software (SAS Institute, Inc., Cary, NC).

RESULTS

TCS Exposure and Hatching Rate of Larva

All the hatched larvae presented a well-developed head, body, and tail within 72 h. At 48 hpf, the hatching rate was 42% in the control group, and 47.8 % in 3 ng/ml TCS exposure group . But with the increase of TCS exposure concentrations , the hatching rate significantly dropped from 34.5 % to 4.3 % in 30 - 900 ng/mL TCS exposure (P < 0.01. **Table 1**). At 72 hpf, the hatching rate was 95.8 % in the control group, while the hatching rates in 3-300 ng/mL TCS exposure groups slightly reduced as TCS dose increased, but the differences were not statistically significant compared to the control. However, 900 ng/mL of TCS exposure strongly inhibited the hatching rate: only 4.3 % of fertilized eggs hatched at 48 hpf, and 18.3 % at 72 hpf (**Table 1**).

TCS Exposure and Mortality of Zebrafish Larvae

All the larvae died shortly after hatching in 900 ng/mL TCS exposure group, and the mortality reached 100 % at 3 dpf. But in the TCS exposure 3 to 300 ng/mL groups, the mortalities of larvae were comparable to the control group at 7 dpf and 14 dpf, respectively (**Table 2**).

TCS Exposure and Thyroid Hormone Levels in Larvae and Zebrafish

We further measured the thyroid hormone levels in larvae after exposure to different concentrations of TCS at different time

TABLE 1 | The association of TCS exposure concentration and the hatching rate of zebrafish embryos within 48 and 72 h.

Treatment	Fertilized eggs	Number of larvae hatched	
TCS exposure levels (ng/mL)	n	Up to 48 hpf n (%)	Up to 72 hpf n (%)
0 (control)	600	252 (42.0%)	575 (95.8%)
3	600	287 (47.8%)	580 (96.7%)
30	600	207 (34.5%)	563 (93.8%)
100	600	141 (23.5%)	561 (93.5%)
300	600	169 (28.2%)	521 (86.8%)
900	600	26 (4.3%)	110 (18.3%)
P		**<0.01***	**<0.01***

Chi-square test was used to test the difference; hpf, hours post fertilization.*
Bold values mean statistical significant.

TABLE 2 | The influence of TCS exposure on the larvae mortality in 14 days post fertilization.

Treatment TCS exposure levels (ng/mL)	Larvae hatched within 3 dpf n	Larvae death within 7 dpf n (%)	Number of larvae alive at the beginning of 8 dpf[#] n	Larvae death during 8 to 14 dpf n (%)
0 (control)	575	36 (6.3%)	389	16 (4.1%)
3	580	41 (7.1%)	389	18 (4.6%)
30	563	50 (8.9%)	363	18 (5.0%)
100	561	72 (12.8%)	339	9 (2.7%)
300	521	45 (8.6%)	326	9 (2.8%)
900	110	110 (100%)	——	——
P		**< 0.01***		0.16

dpf, days post fertilization.

** Chi-square test was used.*

[#] number of larvae at the beginning of 8 dpf = total hatched larvae in 3 dpf number of larvae death up to 7 dpf – 150 larvae used for measuring thyroid hormones at the 7th day (i.e., 50 larvae/measure × 3 measures = 150 larvae for each exposure group).

Bold values mean statistical significant.

points. At 7 dpf, the TT_3 and FT_3 levels were comparable as control in 0 to 300 ng/mL TCS exposure groups; while the TT_4 and FT_4 levels were reduced with TCS exposure doses from 0 to 300 ng/mL, but the difference was not statistically significant (**Supplementary Table S1**).

At 14 dpf, the TT_3 and FT_3 levels in larvae were significantly lower in all the TCS exposure groups when compared to the control group. TT_3 levels were on average 0.20 to 0.21 ng/mg lower in 3-300 ng/mL TCS exposure groups than that of the control group (all $P < 0.05$, **Table 3**), and the FT_3 levels were 9.49, 10.74, and 9.32 pg/mg lower in TCS 30, 100, and 300 ng/mL groups, respectively ($P < 0.01$, **Table 3**). The FT_4 level tended to decrease with the increase of TCS exposure doses (P trend < 0.05, **Table 3**); while TT_4 levels had no statistically significant changes in 0-300 ng/mL TCS exposure groups (**Table 3**).

At 120 dpf, the FT_4 levels were 0.14, 0.12, and 0.11 pg/mg lower in 30, 100, and 300 ng/mL TCS groups when compared to the control group, respectively (**Figure 1** and **Supplementary Table S2**, all $P < 0.01$). The TT_3, TT_4, and FT_3 levels were comparable among the five TCS exposure groups. After adjusted for sex, the FT_4 levels were still lower in TCS 30, 100, and 300 ng/mL groups than that in the control group (all $P < 0.01$, **Supplementary Table S3**).

TCS Exposure and Thyroid Histopathological Change in Zebrafish

We further examined the histological change of the thyroid gland in 120 dpf zebrafish. Ventral aorta was used to locate the dispersed thyroid follicles. In 3-300 ng/mL TCS exposure groups, the thyroid follicular epithelial cells became hyperplasia (increased number of follicular cell) and hypertrophy (enlarged follicular cell size and nuclear size), shown as increased nuclear area and cell height of thyroid follicle epithelial cells (**Figures 2** and **3**, **Supplementary Table S4**). At the same time, fish had oval thyroid follicles composed of a uniform monolayer of cuboidal thyrocytes and lumen filled with pink-stained colloid in most of the follicles in the control group (**Figures 3A, F**). But after exposure to TCS for 120 days, colloid depletion of follicles and angiogenesis (the development of new blood vessels from an existing vasculature) were observed (**Figures 3B–E** at 400×; **G, H, I**, and **J** at 1000×). TCS exposure induced follicle hyperplasia (**Figures 3C–E**), and nuclear hypertrophy (**Figures 3G–J**), and the thickening of the layer of follicle epithelial cell in those follicles (**Figures 3C–E**). In the zebrafish exposed to high TCS levels at 100 ng/mL and 300 ng/mL, the thyroid follicle cells had obvious morphological alterations, for example, the follicle cells became significantly larger and follicular interstitial hyperplasia (**Figures 3C–E, G**).

DISCUSSION

In this study, we examined the disrupting effects of early exposure to TCS on the thyroid hormones in larvae and adult zebrafish, and the pathological changes of thyroid follicles. We found that the hatching rate of zebrafish embryos reduced whin 48 hpf with high TCS exposure, while the hatching rate became similar as the control when exposing to TCS at 0 - 300 ng/mL. TCS exposure did not change the mortality of larvae at 0 - 300 ng/mL, either. But in the extremely high TCS exposure (900 ng/mL), both the hatching rate and the survival of larvae were severely reduced. As to the effects of TCS on thyroid hormones, the levels of TT_3 and FT_3 were lower in larvae at 14 dpf in 30, 100, and 300 ng/mL TCS exposure, and the FT_4 level was lower at 120 dpf in 30, 100, and 300 ng/mL TCS exposure. Moreover, with increasing concentration of TCS exposure, the epithelial height and nuclear size (area) of thyroid follicular cell turned larger in the thyroid gland of adult zebrafish at 120 dpf.

Zebrafish is an ideal animal model for study of chemical pollutants in water and thyroid hormone-disruption, partly due to its high (71 %) genetic similarity to humans (43). We set 2, 3, 7, and 14 days for larvae phase TCS exposure, 120 days (long-time) for adult phase TCS exposure (36). In this study, TCS exposure duration time was chosen according to the physiological development characteristics of zebrafish. The thyroid hormone level of zebrafish became stable after 7 dpf (44), and zebrafish reach sexual maturity at 120 dpf (45). Series of comparable TCS concentrations with natural and human exposure concentration were set in this study. Similar to our results, a previous study also found that significantly delayed hatchability of fertilized eggs and increased mortality of larvae in TCS 500 ng/mL exposure for 6 days (33). The influence of EDCs on zebrafish's thyroid histopathology has been explored in many other related EDCs, such as perchlorate (46), arsenate (47),

TABLE 3 | The influence of TCS exposure on the thyroid hormone level of zebrafish larvae at 14 days post fertilization.

Treatment	TT_3 (ng/mg)		TT_4 (nmol/g)		FT_3 (pg/mg)		FT_4 (pg/mg)	
TCS exposure levels (ng/mL)	mean ± SD	β (95% CI)	mean ± SD	β (95% CI)	mean ± SD	β (95% CI)	mean ± SD	β (95 % CI)
0 (control)	0.39 ± 0.06	Reference	2.24 ± 0.38	Reference	28.53 ± 1.98	Reference	0.72 ± 0.20	Reference
3	0.19 ± 0.08	-0.199 (-0.347, -0.050)*	1.74 ± 0.67	-0.493 (-1.253, 0.267)	25.82 ± 0.24	-2.708 (-8.626, 3.210)	0.63 ± 0.20	-0.084 (-0.436, 0.268)
30	0.18 ± 0.11	-0.206 (-0.355, -0.058)*	1.84 ± 0.36	-0.396 (-1.156, 0.363)	19.04 ± 5.10	-9.495 (-15.413, -3.577)**	0.43 ± 0.20	-0.287 (-0.639, 0.065)
100	0.18 ± 0.08	-0.203 (-0.352, -0.054)*	2.05 ± 0.36	-0.188 (-0.948, 0.572)	17.79 ± 3.63	-10.745 (-16.663, -4.827)**	0.55 ± 0.22	-0.172 (-0.524, 0.180)
300	0.19 ± 0.08	-0.192 (-0.341, -0.044)*	1.61 ± 0.13	-0.629 (-1.389, 0.130)	19.21 ± 3.13	-9.326 (-15.244, -3.408)**	0.38 ± 0.14	-0.341 (-0.693, 0.012)
P for trend	**0.04***		0.23		**0.0013****		**0.04***	

*100 larvae in each exposure group for one measurement (n=3, with 100 larvae per n). *P < 0.05 **P < 0.01.*
Bold values mean statistical significant.

fluoride (48), and microcystin-LR (49). To date, there was only one study focused on the TCS exposure to adult zebrafish and thyroid histopathology (29). Consistent with our results, it reported that the thyroid follicle epithelium was changed in the sub-chronic (21 days) TCS treated fish (29). The reduction or increase in height of thyroid follicle cells are both the evidence of pathological diagnosis in thyroid dysfunction.

In previous studies, Schnitzler et al. (28) found that 20, 50, and 100 ng/mL TCS exposure to embryos decreased T_4 and T_3 levels at 9 days post-hatching (dph) and 12 dph, and increased T_4 levels at 15 dph in *Cyprinodon variegatus*. Pinto et al. (29) assessed the effect and possible mechanism of TCS on adult zebrafish. They found that TCS exposure to adult zebrafish (about 100 μg/g per day for 21 days *via* diet) induced a reduction in circulating thyroid hormones, hyperplasia of follicles and the thyrocyte height. In our experiments, the TT_4 and FT_4 levels tended to be decreased with higher TCS exposure after 7 days, although they were not significant. The obvious thyroid disrupting effect by TCS shown at 14 dpf, and the FT_4 decreased significantly with the increasing TCS exposure concentration both at 14 and 120 dpf. The impact of TCS exposure on thyroid hormone levels (TT_3 and FT_3) at 14 dpf provides the causal support for the findings in the human population (17). In the fish models, only three studies have examined the influence of TCS exposure on thyroid disruption (28, 29). Similar to our results, one study found that TCS exposure delayed the hatching of 6-13 h in medaka fish (28). A recent study found that the offspring of zebrafish exposed to TCS decreased the survival rate and delayed maturation (50), which might be partially explained by decreased thyroid hormone resulting from TCS exposure (50). The adverse effect of long-term exposure to TCS on thyroid hormone levels might be due to the cumulative effect of TCS on the development of zebrafish thyroid glands.

Most of the TT_4 and a few of the TT_3 are synthesized by thyroid follicular cells directly and then are transported to blood and be functioned in tissues (51). FT_3 is the most active free form which comes from FT_4 deiodination. FT_3 and FT_4, TT_3 and TT_4 can be transformed into each other and maintain dynamic balance in the blood (51). TT_4 and TT_3 are usually used as an indicator of the reserve capacity of the thyroid gland. In this study, under the high TCS exposure levels, TT_3 and FT_3, rather than TT_4 or FT_4, decreased statistically significantly in larvae at 14 dpf. Continuous exposing to 120 dpf, only the FT_4 level decreased significantly. More studies about the mechanism still need to be explored.

In our study, since the larvae all died by day 3 after they were hatched, thyroid hormone levels were not measured in the 900 ng/mL TCS exposure group. We used the whole body of larvae fish at 7 and 14 dpf and the body without head, viscera, and gut of adult fish at 120 dpf to measure the thyroid hormone levels as most previous studies. In zebrafish, thyroid hormone is expressed in both hepatic and muscle (52). If adult zebrafish were used as models, plasma or body are generally used as biological samples (53). Thyroid hormone levels in muscle can reflect the growth and development of the body (52), and in our

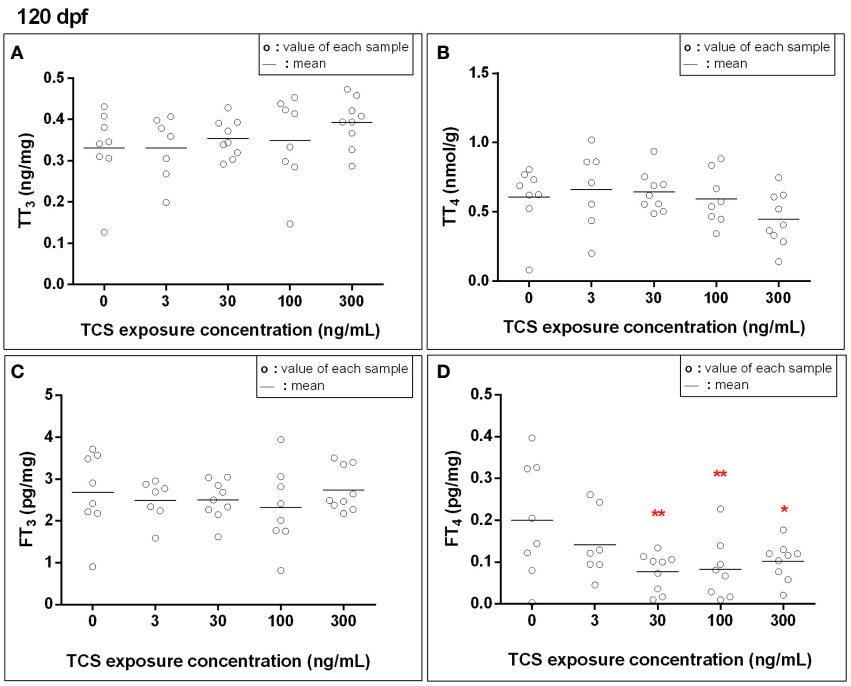


FIGURE 1 | The thyroid hormone levels [**(A)**: total triiodothyronine, **(B)**: total thyroxine, **(C)**: free triiodothyronine, **(D)**: free thyroxine] in zebrafish exposed to TCS for 120 days after fertilizing. Thyroid hormone concentrations in fish at 120 dpf were measured for each exposure group, with each sample homogenized from every single fish at 120 dpf. *P* trend for FT_4 = 0.01. **P* < 0.05, ***P* < 0.01.

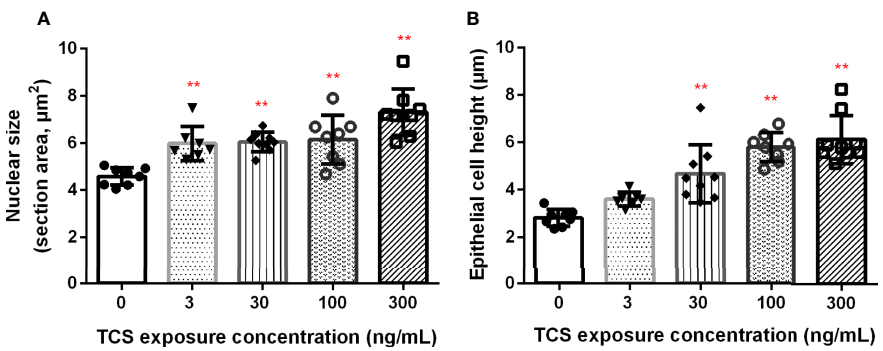


FIGURE 2 | The association between TCS exposure concentrations and nuclear area **(A)** and the height **(B)** of thyroid follicle cells. *P* trend < 0.0001 both for nuclear size and epithelial height of the thyroid. ** *P*< 0.01 in the TCS exposure group vs. the control group.

study, the thyroid hormone concentration in the muscle was found related to the TCS exposure level and therefore presumably affects the body growth of the zebrafish.

In this study, we selected the TCS exposure (3, 30, 100, 300, and 900 ng/mL) based on the environmental TCS levels, including wildlife and general population TCS exposure levels in a natural environment. The 3-100 ng/mL TCS is the range of urinary TCS concentrations in pregnant women (17), and 3-30 ng/mL is the TCS range in waste water (10). TCS was found in ranges from 5370 ng/L to 86,161 ng/L in wastewater treatment plants (10), and 1.85 ng/L to 9650 ng/L in rivers and surface water worldwide (9–11, 54, 55). The median urinary TCS concentration is 2.52 ng/mL (17, 19), while the highest urinary TCS concentrations in pregnant women was close to 100 ng/mL (17). It is about 25% of the lethal concentration (LC50) for zebrafish (33). In zebrafish, LC50 values of TCS were 420 μg/L (95% CI, 380 - 450 ng/mL) for embryos at 96 hpf, and 340 μg/L for adult zebrafish within 96 h (33).

The toxic effects of TCS were well recognized on bacterial resistance, reproductive toxicity, and cytotoxicity in aquatic organisms (56–58), but the results on thyroid hormone disruption were inconsistent (59, 60). Most of the animal studies

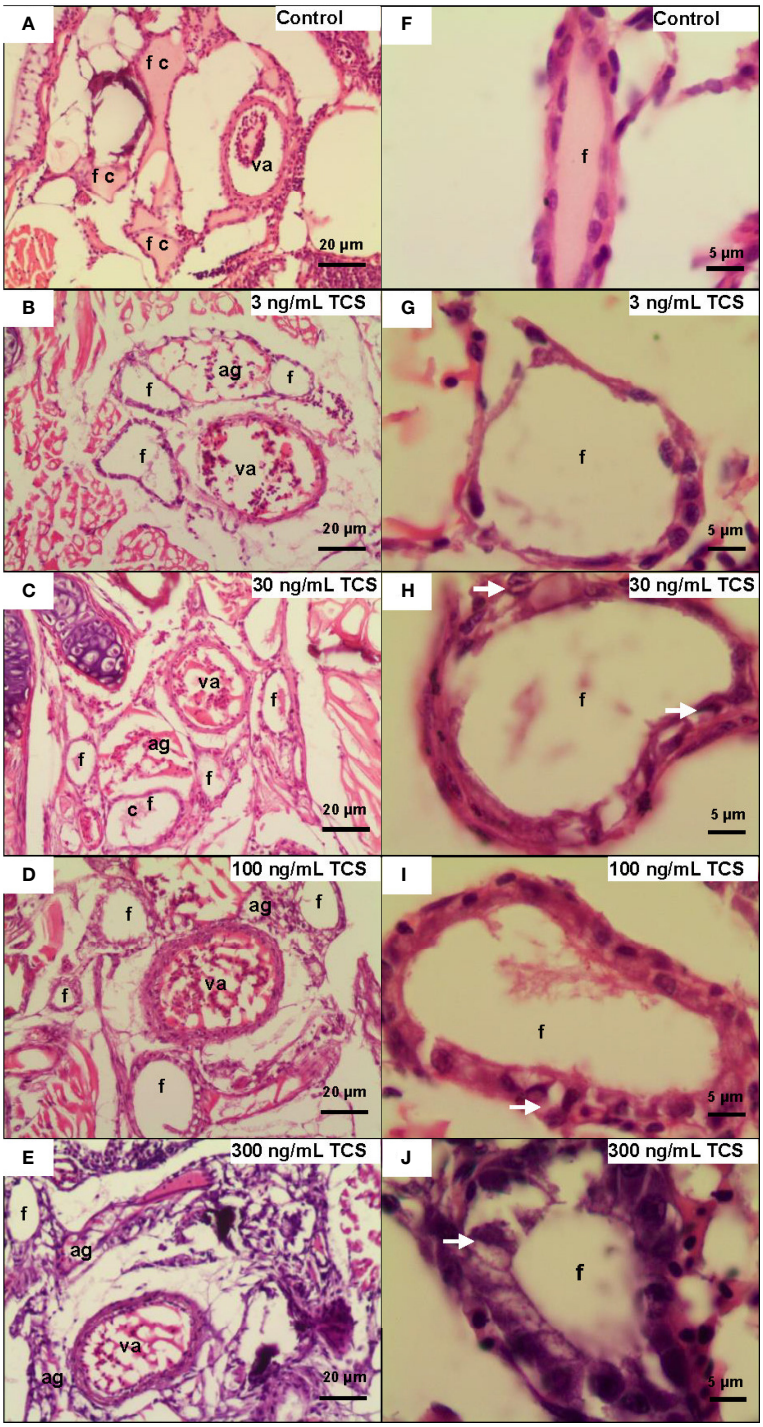


FIGURE 3 | The morphological changes of thyroid follicles in different TCS concentration groups. **(A)** In the control group, the zebrafish follicle squamous to cuboidal follicular epithelium, with colloid in follicle at 400× and 1000× **(F)**. **(B–E)** Follicle hyperplasia with decreased colloid in the lumen and angiogenesis in TCS 3, 30, 100, and 300 ng/mL exposure at 400×. **(G–J)** Hypertrophy and hyperplasia of follicle cells in TCS 3, 30, 100, and 300 ng/mL exposure at 1000×. (va = ventral aorta; c = colloid; f = thyroid follicle; ag = angiogenesis; white arrows = hypertrophy).

mainly focused on rodents (24, 61). In the adult rat, TCS treatment reduced the levels of blood TT_4 and FT_4, but no change was found in the levels of blood thyroid stimulating hormone (TSH) (25, 62–64). Another study found that maternal TCS (300 mg/kg/day) exposure during pregnancy decreased approximately 30% of TT_4 in dams at the postpartum day 22 in mice (64). Previous studies found that TCS exposure decreased thyroid hormone levels and inhibited metamorphosis (59, 65), or neither changed the thyroid hormone level nor altered the metamorphosis in *Xenopus laevis* (60).

We speculate that TCS may inhibit thyroid hormones secretion by acting as a disruptor of the hypothalamic-pituitary-thyroid (HPT) axis or due to the increasing thyroid hormones clearance. Some mechanisms have been studied but the data were controversial. TCS exposure may promote (29) or inhibit (66) the sodium-iodide symporter (NIS)-mediated iodide uptake in animals' thyroid, then increase or decrease the thyroid hormone levels. TCS decreased the thyroid hormones in the circulation of zebrafish but induced TSH gene transcription by a negative feedback regulation (29). TCS was reported to inhibit the activity of thyroid peroxidase (TPO) and thyroid hormone synthesis at concentrations of 50 ng/mL in rat (66) , and increased T_4 metabolism and decreased T_4 bioavailability by increasing the hepatic enzymes activity of glucuronyltransferase and pentoxyresorufin-O-deethylase (PROD) in the liver (67–69), or by activating the nuclear receptor, pregnane X receptor (PXR) (24, 61). It was also found that TCS caused hypothyroidism in rats through p38/TRHr-dependent pathway (57). Therefore, the mechanism about TCS inhibiting the thyroid hormones is still unclear and needs further investigation.

The result in larvae of this study is consistent with our previous study in humans, in which we observed an inverse association between maternal urinary TCS and cord blood FT_3 level in Chinese newborns (17). It is established that thyroid hormone is critical for intrauterine neurodevelopment, due to the regulation of migration, proliferation, and differentiation of fetal neuronal cells, as well as synaptogenesis and myelination (1, 2). The nervous system is highly thyroid hormone sensitive in prenatal life, especially in the early weeks of embryonic development (70).

Our study found that zebrafish exposed with TCS for 120 days (from embryo to adult phase) caused thyroid histopathology changes in 3, 30, 100, and 300 ng/mL TCS exposure, and disturbed the thyroid hormone level (FT_4) in 30, 100, and 300 ng/mL TCS exposure. This is the first study to explore the thyroid disturbed effect by long-term TCS exposure starting from the early life in zebrafish. It has been reported that the formation, growth, and differentiation of thyroid follicles in zebrafish appear to be independent of TSH (71), although the TSH receptor is responsible for thyroid gland differentiation in zebrafish (72). Future study with measurements on TSH level can lead to exploring the potential disruptive effect of TCS on the feedback mechanism of HPT axis. Considering an environmental TCS exposure, we set various TCS concentrations in water from very low to high levels in our experiment but did not measure the direct TCS concentration in the fish.

We found that exposure to TCS delayed the hatching of zebrafish embryos, decreased the thyroid hormone (FT_3 and TT_3) levels in the zebrafish larvae and the FT_4 level in adult zebrafish (even adjusted for age). Moreover, TCS exposure affected the structure of thyroid follicles by increasing nuclear area and epithelial cell height of zebrafish thyroid follicles. Considering the reduced level of thyroid hormones which are crucial for neurodevelopment (73), our study raises the concern that early TCS exposure (from conception) may profoundly influence child neurobehavioral development at such a highly sensitive window.

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 animals were reviewed and approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

AUTHOR CONTRIBUTIONS

FO conceptualized the study. FO, NT, and PF implemented the study, FO and NT interpretated data and drafted the manuscript. FO, NT, PF, WYW, WJW, and XY contributed to acquisition and analysis of research data. LC had intensively revised the manuscript. All authors have reviewed and approved the manuscript as submitted.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China [grant number No. 81961128023; 81673178], Shanghai Municipal Education Commission - Gaofeng Clinical Medicine Grant [grant number 20152518], and in part by the Collaborative Innovation Program of Shanghai Municipal Health Commission [grant number 2020CXJQ01].

ACKNOWLEDGMENTS

We thank Mr. Yangou Du (McGill University, Canada) for the reviewing and editing of our manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.850231/full#supplementary-material

REFERENCES

1. Lavado-Autric R, Auso E, Garcia-Velasco JV, Arufe Mdel C, Escobar del Rey F, Berbel P, et al. Early Maternal Hypothyroxinemia Alters Histogenesis and Cerebral Cortex Cytoarchitecture of the Progeny. *J Clin Invest* (2003) 111:1073–82. doi: 10.1172/JCI16262
2. Bernal J. Thyroid Hormones and Brain Development. *Vitam Horm* (2005) 71:95–122. doi: 10.1016/S0083-6729(05)71004-9
3. Brouwer A, Morse DC, Lans MC, Schuur AG, Murk AJ, Klasson-Wehler E, et al. Interactions of Persistent Environmental Organohalogens With the Thyroid Hormone System: Mechanisms and Possible Consequences for Animal and Human Health. *Toxicol Ind Health* (1998) 14:59–84. doi: 10.1177/074823379801400107
4. Witorsch RJ. Critical Analysis of Endocrine Disruptive Activity of Triclosan and its Relevance to Human Exposure Through the Use of Personal Care Products. *Crit Rev Toxicol* (2014) 44:535–55. doi: 10.3109/10408444.2014.910754
5. Jones RD, Jampani HB, Newman JL, Lee AS. Triclosan: A Review of Effectiveness and Safety in Health Care Settings. *Am J Infect Control* (2000) 28:184–96. doi: 10.1067/mic.2000.102378
6. Reiss R, Mackay N, Habig C, Griffin J. An Ecological Risk Assessment for Triclosan in Lotic Systems Following Discharge From Wastewater Treatment Plants in the United States. *Environ Toxicol Chem* (2002) 21:2483–92. doi: 10.1002/etc.5620211130
7. Dann AB, Hontela A. Triclosan: Environmental Exposure, Toxicity and Mechanisms of Action. *J Appl Toxicol* (2011) 31:285–311. doi: 10.1002/jat.1660
8. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, et al. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999-2000: A National Reconnaissance. *Environ Sci Technol* (2002) 36:1202–11. doi: 10.1021/es011055j
9. Li X, Ying GG, Su HC, Yang XB, Wang L. Simultaneous Determination and Assessment of 4-Nonylphenol, Bisphenol A and Triclosan in Tap Water, Bottled Water and Baby Bottles. *Environ Int* (2010) 36:557–62. doi: 10.1016/j.envint.2010.04.009
10. Kumar KS, Priya SM, Peck AM, Sajwan KS. Mass Loadings of Triclosan and Triclocarbon From Four Wastewater Treatment Plants to Three Rivers and Landfill in Savannah, Georgia, USA. *Arch Environ Contam Toxicol* (2010) 58:275–85. doi: 10.1007/s00244-009-9383-y
11. Peng X, Yu Y, Tang C, Tan J, Huang Q, Wang Z. Occurrence of Steroid Estrogens, Endocrine-Disrupting Phenols, and Acid Pharmaceutical Residues in Urban Riverine Water of the Pearl River Delta, South China. *Sci Total Environ* (2008) 397:158–66. doi: 10.1016/j.scitotenv.2008.02.059
12. Coogan MA, Edziyie RE, La Point TW, Venables BJ. Algal Bioaccumulation of Triclocarban, Triclosan, and Methyl-Triclosan in a North Texas Wastewater Treatment Plant Receiving Stream. *Chemosphere* (2007) 67:1911–8. doi: 10.1016/j.chemosphere.2006.12.027
13. Arnot JA, Pawlowski S, Champ S. A Weight-of-Evidence Approach for the Bioaccumulation Assessment of Triclosan in Aquatic Species. *Sci Total Environ* (2018) 618:1506–18. doi: 10.1016/j.scitotenv.2017.09.322
14. Lyndall J, Fuchsman P, Bock M, Barber T, Lauren D, Leigh K, et al. Probabilistic Risk Evaluation for Triclosan in Surface Water, Sediments, and Aquatic Biota Tissues. *Integr Environ Assess Manag* (2010) 6:419–40. doi: 10.1897/IEAM_2009-072.1
15. Hovander L, Malmberg T, Athanasiadou M, Athanassiadis I, Rahm S, Bergman A, et al. Identification of Hydroxylated PCB Metabolites and Other Phenolic Halogenated Pollutants in Human Blood Plasma. *Arch Environ Contam Toxicol* (2002) 42:105–17. doi: 10.1007/s002440010298
16. Adolfsson-Erici M, Pettersson M, Parkkonen J, Sturve J. Triclosan, a Commonly Used Bactericide Found in Human Milk and in the Aquatic Environment in Sweden. *Chemosphere* (2002) 46:1485–9. doi: 10.1016/s0045-6535(01)00255-7
17. Wang X, Ouyang F, Feng L, Wang X, Liu Z, Zhang J. Maternal Urinary Triclosan Concentration in Relation to Maternal and Neonatal Thyroid Hormone Levels: A Prospective Study. *Environ Health Perspect* (2017) 125:067017. doi: 10.1289/EHP500
18. Liu H, Li J, Xia W, Zhang B, Peng Y, Li Y, et al. Blood Pressure Changes During Pregnancy in Relation to Urinary Paraben, Triclosan and Benzophenone Concentrations: A Repeated Measures Study. *Environ Int* (2019) 122:185–92. doi: 10.1016/j.envint.2018.11.003
19. Philippat C, Mortamais M, Chevrier C, Petit C, Calafat AM, Ye X, et al. Exposure to Phthalates and Phenols During Pregnancy and Offspring Size at Birth. *Environ Health Perspect* (2012) 120:464–70. doi: 10.1289/ehp.1103634
20. Arbuckle TE, Marro L, Davis K, Fisher M, Ayotte P, Belanger P, et al. Exposure to Free and Conjugated Forms of Bisphenol A and Triclosan Among Pregnant Women in the MIREC Cohort. *Environ Health Perspect* (2015) 123:277–84. doi: 10.1289/ehp.1408187
21. Watkins DJ, Ferguson KK, Anzalota Del Toro LV, Alshawabkeh AN, Cordero JF, Meeker JD. Associations Between Urinary Phenol and Paraben Concentrations and Markers of Oxidative Stress and Inflammation Among Pregnant Women in Puerto Rico. *Int J Hyg Environ Health* (2015) 218:212–9. doi: 10.1016/j.ijheh.2014.11.001
22. Lassen TH, Frederiksen H, Kyhl HB, Swan SH, Main KM, Andersson AM, et al. Prenatal Triclosan Exposure and Anthropometric Measures Including Anogenital Distance in Danish Infants. *Environ Health Perspect* (2016) 124:1261–8. doi: 10.1289/ehp.1409637
23. Haggard DE, Noyes PD, Waters KM, Tanguay RL. Phenotypically Anchored Transcriptome Profiling of Developmental Exposure to the Antimicrobial Agent, Triclosan, Reveals Hepatotoxicity in Embryonic Zebrafish. *Toxicol Appl Pharmacol* (2016) 308:32–45. doi: 10.1016/j.taap.2016.08.013
24. Paul KB, Hedge JM, DeVito MJ, Crofton KM. Short-Term Exposure to Triclosan Decreases Thyroxine *In Vivo via* Upregulation of Hepatic Catabolism in Young Long-Evans Rats. *Toxicol Sci* (2010) 113:367–79. doi: 10.1093/toxsci/kfp271
25. Crofton KM, Paul KB, Devito MJ, Hedge JM. Short-Term *In Vivo* Exposure to the Water Contaminant Triclosan: Evidence for Disruption of Thyroxine. *Environ Toxicol Pharmacol* (2007) 24:194–7. doi: 10.1016/j.etap.2007.04.008
26. Axelstad M, Boberg J, Vinggaard AM, Christiansen S, Hass U. Triclosan Exposure Reduces Thyroxine Levels in Pregnant and Lactating Rat Dams and in Directly Exposed Offspring. *Food Chem Toxicol* (2013) 59:534–40. doi: 10.1016/j.fct.2013.06.050
27. Rodriguez PE, Sanchez MS. Maternal Exposure to Triclosan Impairs Thyroid Homeostasis and Female Pubertal Development in Wistar Rat Offspring. *J Toxicol Environ Health A* (2010) 73:1678–88. doi: 10.1080/15287394.2010.516241
28. Schnitzler JG, Frederich B, Dussenne M, Klaren PH, Silvestre F, Das K. Triclosan Exposure Results in Alterations of Thyroid Hormone Status and Retarded Early Development and Metamorphosis in *Cyprinodon Variegatus*. *Aquat Toxicol* (2016) 181:1–10. doi: 10.1016/j.aquatox.2016.10.019
29. Pinto PIS, Guerreiro EM and Power DM. Triclosan Interferes With the Thyroid Axis in the Zebrafish (*Danio Rerio*). *Toxicol Res* (2013) 2:60–9. doi: 10.1039/c2tx20005h
30. Watermann A, Gieringer R, Bauer AM, Kurch S, Kiesslich R, Tremel W, et al. Fluorescein- and Egfr-Antibody Conjugated Silica Nanoparticles for Enhancement of Real-Time Tumor Border Definition Using Confocal Laser Endomicroscopy in Squamous Cell Carcinoma of the Head and Neck. *Nanomater (Basel)* (2019) 9:1378. doi: 10.3390/nano9101378
31. Wendl T, Lun K, Mione M, Favor J, Brand M, Wilson SW, et al. Pax2.1 is Required for the Development of Thyroid Follicles in Zebrafish. *Development* (2002) 129:3751–60. doi: 10.1242/dev.129.15.3751
32. Avdesh A, Chen M, Martin-Iverson MT, Mondal A, Ong D, Rainey-Smith S, et al. Regular Care and Maintenance of a Zebrafish (*Danio Rerio*) Laboratory: An Introduction. *J Vis Exp* (2012) 69:e4196. doi: 10.3791/4196
33. Oliveira R, Domingues I, Koppe Grisolia C, Soares AM. Effects of Triclosan on Zebrafish Early-Life Stages and Adults. *Environ Sci Pollut Res Int* (2009) 16:679–88. doi: 10.1007/s11356-009-0119-3
34. Goldsmith P. Zebrafish as a Pharmacological Tool: The How, Why and When. *Curr Opin Pharmacol* (2004) 4:504–12. doi: 10.1016/j.coph.2004.04.005
35. Lantz-McPeak S, Guo X, Cuevas E, Dumas M, Newport GD, Ali SF, et al. Developmental Toxicity Assay Using High Content Screening of Zebrafish Embryos. *J Appl Toxicol* (2015) 35:261–72. doi: 10.1002/jat.3029
36. Altenhofen S, Nabinger DD, Bitencourt PER, Bonan CD. Dichlorvos. alters morphology and behavior in zebrafish (*Danio rerio*) larvae. *Environ Pollut* (2019) 245:1117–23. doi: 10.1016/j.envpol.2018.11.095
37. Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, et al. American Veterinary Medical Association for the Euthanasia of Animals. In:

AVMA Guidelines 2013 Edition. Schaumburg, Illinois, USA: American Veterinary Medical Association.
38. Strykowski JL, Schech JM. Effectiveness of Recommended Euthanasia Methods in Larval Zebrafish (Danio Rerio). *J Am Assoc Lab Anim Sci* (2015) 54:81–4.
39. Tang T, Yang Y, Chen Y, Tang W, Wang F, Diao X. Thyroid Disruption in Zebrafish Larvae by Short-Term Exposure to Bisphenol AF. *Int J Environ Res Public Health* (2015) 12:13069–84. doi: 10.3390/ijerph121013069
40. Campion EM, Loughran ST, Walls D. Protein Quantitation and Analysis of Purity. *Methods Mol Biol* (2017) 1485:225–55. doi: 10.1007/978-1-4939-6412-3_12
41. Patino R, Wainscott MR, Cruz-Li EI, Balakrishnan S, McMurry C, Blazer VS, et al. Effects of Ammonium Perchlorate on the Reproductive Performance and Thyroid Follicle Histology of Zebrafish. *Environ Toxicol Chem* (2003) 22:1115–21. doi: 10.1002/etc.5620220520
42. Liu FJ, Wang JS, Theodorakis CW. Thyrotoxicity of Sodium Arsenate, Sodium Perchlorate, and Their Mixture in Zebrafish. *Danio Rerio Environ Sci Technol* (2006) 40:3429–36. doi: 10.1021/es052538g
43. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The Zebrafish Reference Genome Sequence and its Relationship to the Human Genome. *Nature* (2013) 496:498–503. doi: 10.1038/nature12111
44. Chang J, Wang M, Gui W, Zhao Y, Yu L, Zhu G. Changes in Thyroid Hormone Levels During Zebrafish Development. *Zoolog Sci* (2012) 29:181–4. doi: 10.2108/zsj.29.181
45. Liu Y, Wang J, Fang X, Zhang H, Dai J. The Thyroid-Disrupting Effects of Long-Term Perfluorononanoate Exposure on Zebrafish (*Danio Rerio*). *Ecotoxicology* (2011) 20:47–55. doi: 10.1007/s10646-010-0555-3
46. Schmidt F, Schnurr S, Wolf R, Braunbeck T. Effects of the Anti-Thyroidal Compound Potassium-Perchlorate on the Thyroid System of the Zebrafish. *Aquat Toxicol* (2012) 109:47–58. doi: 10.1016/j.aquatox.2011.11.004
47. Liu F, Gentles A, Theodorakis CW. Arsenate and Perchlorate Toxicity, Growth Effects, and Thyroid Histopathology in Hypothyroid Zebrafish. *Danio Rerio Chemosphere* (2008) 71:1369–76. doi: 10.1016/j.chemosphere.2007.11.036
48. Jianjie C, Wenjuan X, Jinling C, Jie S, Ruhui J, Meiyan L. Fluoride Caused Thyroid Endocrine Disruption in Male Zebrafish (*Danio Rerio*). *Aquat Toxicol* (2016) 171:48–58. doi: 10.1016/j.aquatox.2015.12.010
49. Liu Z, Tang R, Li D, Hu Q, Wang Y. Subacute Microcystin-LR Exposure Alters the Metabolism of Thyroid Hormones in Juvenile Zebrafish (*Danio Rerio*). *Toxins (Basel)* (2015) 7:337–52. doi: 10.3390/toxins7020337
50. Stenzel A, Wirt H, Patten A, Theodore B, King-Heiden T. Larval Exposure to Environmentally Relevant Concentrations of Triclosan Impairs Metamorphosis and Reproductive Fitness in Zebrafish. *Reprod Toxicol* (2019) 87:79–86. doi: 10.1016/j.reprotox.2019.05.055
51. Fujita H. Functional Morphology of the Thyroid. *Int Rev Cytol* (1988) 113:145–85. doi: 10.1016/s0074-7696(08)60848-7
52. Yang X, Xie J, Wu T, Yue G, Chen J, Zhao R. Hepatic and Muscle Expression of Thyroid Hormone Receptors in Association With Body and Muscle Growth in Large Yellow Croaker, Pseudosciaena Crocea (*Richardson*). *Gen Comp Endocrinol* (2007) 151:163–71. doi: 10.1016/j.ygcen.2007.01.005
53. Yamano K. The Role of Thyroid Hormone in Fish Development With Reference to Aquaculture. *JARQ* (2005) 39:161–68. doi: 10.6090/jarq.39.161
54. Ma X, Wan Y, Wu M, Xu Y, Xu Q, He Z, et al. Occurrence of Benzophenones, Parabens and Triclosan in the Yangtze River of China, and the Implications for Human Exposure. *Chemosphere* (2018) 213:517–25. doi: 10.1016/j.chemosphere.2018.09.084
55. Nag SK, Das Sarkar S, Manna SK. Triclosan - an Antibacterial Compound in Water, Sediment and Fish of River Gomti, India. *Int J Environ Health Res* (2018) 28:461–70. doi: 10.1080/09603123.2018.1487044
56. James MO, Li W, Summerlot DP, Rowland-Faux L, Wood CE. Triclosan is a Potent Inhibitor of Estradiol and Estrone Sulfonation in Sheep Placenta. *Environ Int* (2010) 36:942–9. doi: 10.1016/j.envint.2009.02.004
57. Zhang P, Yang M, Zeng L, Liu C. P38/TRHr-Dependent Regulation of TPO in Thyroid Cells Contributes to the Hypothyroidism of Triclosan-Treated Rats. *Cell Physiol Biochem* (2019) 45:1303–15. doi: 10.1159/000487558
58. Hirota R, Ohya Y, Yamamoto-Hanada K, Fukutomi IY, Muto G, Ngatu-Nlandu R, et al. Triclosan-Induced Alteration of Gut Microbiome and Aggravation of Asthmatic Airway Response in Aeroallergen-Sensitized Mice. *Allergy* (2018) 74:996–9. doi: 10.1111/all.13639
59. Veldhoen N, Skirrow RC, Osachoff H, Wigmore H, Clapson DJ, Gunderson MP, et al. The Bactericidal Agent Triclosan Modulates Thyroid Hormone-Associated Gene Expression and Disrupts Postembryonic Anuran Development. *Aquat Toxicol* (2006) 80:217–27. doi: 10.1016/j.aquatox.2006.08.010
60. Fort DJ, Rogers RL, Gorsuch JW, Navarro LT, Peter R, Plautz JR. Triclosan and Anuran Metamorphosis: No Effect on Thyroid-Mediated Metamorphosis in *Xenopus Laevis*. *Toxicol Sci* (2010) 113:392–400. doi: 10.1093/toxsci/kfp280
61. Zorrilla LM, Gibson EK, Jeffay SC, Crofton KM, Setzer WR, Cooper RL, et al. The Effects of Triclosan on Puberty and Thyroid Hormones in Male Wistar Rats. *Toxicol Sci* (2009) 107:56–64. doi: 10.1093/toxsci/kfn225
62. Stoker TE, Gibson EK, Zorrilla LM. Triclosan Exposure Modulates Estrogen-Dependent Responses in the Female Wistar Rat. *Toxicol Sci* (2010) 117:45–53. doi: 10.1093/toxsci/kfq180
63. Paul KB, Hedge JM, Bansal R, Zoeller RT, Peter R, DeVito MJ, et al. Developmental Triclosan Exposure Decreases Maternal, Fetal, and Early Neonatal Thyroxine: A Dynamic and Kinetic Evaluation of a Putative Mode-of-Action. *Toxicology* (2012) 300:31–45. doi: 10.1016/j.tox.2012.05.023
64. Paul KB, Hedge JM, Devito MJ, Crofton KM. Developmental Triclosan Exposure Decreases Maternal and Neonatal Thyroxine in Rats. *Environ Toxicol Chem* (2010) 29:2840–4. doi: 10.1002/etc.339
65. Hinther A, Bromba CM, Wulff JE, Helbing CC. Effects of Triclocarban, Triclosan, and Methyl Triclosan on Thyroid Hormone Action and Stress in Frog and Mammalian Culture Systems. *Environ Sci Technol* (2011) 45:5395–402. doi: 10.1021/es1041942
66. Wu Y, Beland FA, Fang JL. Effect of Triclosan, Triclocarban, 2,2',4,4'-Tetrabromodiphenyl Ether, and Bisphenol A on the Iodide Uptake, Thyroid Peroxidase Activity, and Expression of Genes Involved in Thyroid Hormone Synthesis. *Toxicol In Vitro* (2016) 32:310–9. doi: 10.1016/j.tiv.2016.01.014
67. Zhou Z, Yang J, Chan KM. Toxic Effects of Triclosan on a Zebrafish (*Danio Rerio*) Liver Cell Line, ZFL. *Aquat Toxicol* (2017) 191:175–88. doi: 10.1016/j.aquatox.2017.08.009
68. Schuur AG, Legger FF, van Meeteren ME, Moonen MJ, van Leeuwen-Bol I, Bergman A, et al. *In Vitro* Inhibition of Thyroid Hormone Sulfation by Hydroxylated Metabolites of Halogenated Aromatic Hydrocarbons. *Chem Res Toxicol* (1998) 11:1075–81. doi: 10.1021/tx9800046
69. Wang LQ, Falany CN, James MO. Triclosan as a Substrate and Inhibitor of 3'-Phosphoadenosine 5'-Phosphosulfate-Sulfotransferase and UDP-Glucuronosyl Transferase in Human Liver Fractions. *Drug Metab Dispos* (2004) 32:1162–9. doi: 10.1124/dmd.104.000273
70. Nicholson JL, Altman J. The Effects of Early Hypo- and Hyperthyroidism on the Development of Rat Cerebellar Cortex. I. Cell Proliferation and Differentiation. *Brain Res* (1972) 44:13–23. doi: 10.1016/0006-8993(72)90362-9
71. Alt B, Reibe S, Feitosa NM, Elsalini OA, Wendl T, Rohr KB. Analysis of Origin and Growth of the Thyroid Gland in Zebrafish. *Dev Dyn* (2006) 235:1872–83. doi: 10.1002/dvdy.20831
72. Opitz R, Maquet E, Zoenen M, Dadhich R, Costagliola S. TSH Receptor Function is Required for Normal Thyroid Differentiation in Zebrafish. *Mol Endocrinol* (2011) 25:1579–99. doi: 10.1210/me.2011-0046
73. Morreale de Escobar G, Obregon MJ, Escobar del Rey F. Role of Thyroid Hormone During Early Brain Development. *Eur J Endocrinol* (2004) 151 Suppl 3:U25–37. doi: 10.1530/eje.0.151u025

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 08 July 2022
doi: 10.3389/fendo.2022.911487

The Thyroid Condition and Residual Clinical Signs in 31 Existing Endemic Neurological Cretins After 42 Years of Iodine Supplementation in China

Jianshuang Li[1,2,3,4], *Yanhong He*[1,3,4], *Bingxuan Ren*[1,3,4], *Zhaojun Zhang*[1,3,4], *Fangang Meng*[1,3,4], *Xiaoye Zhang*[1,3,4], *Zheng Zhou*[1,3,4], *Baoxiang Li*[1,3,4], *Fan Li*[1,3,4], *Lixiang Liu*[1,3,4*] *and Hongmei Shen*[1,3,4*]

[1] Centre for Endemic Disease Control, Chinese Centre for Disease Control and Prevention, Harbin Medical University, Harbin City, China, [2] College of Medical Laboratory Science and Technology, Harbin Medical University (Daqing), Daqing, China, [3] National Health Commission and Education Bureau of Heilongjiang Province, Key Laboratory of Etiology and Epidemiology, Harbin Medical University, Harbin, China, [4] Heilongjiang Provincial Key Laboratory of Trace Elements and Human Health, Harbin Medical University, Harbin, China

OPEN ACCESS

Edited by:
Janete Maria Cerutti,
Federal University of São Paulo, Brazil

Reviewed by:
Creswell John Eastman,
The University of Sydney, Australia
Ileana G. Rubio,
Federal University of São Paulo, Brazil
Inés Velasco,
Hospital Germans Trias i Pujol, Spain

***Correspondence:**
Hongmei Shen
shenhm119@hrbmu.edu.cn
Lixiang Liu
liulixiang@hrbmu.edu.cn

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *02 April 2022*
Accepted: *08 June 2022*
Published: *08 July 2022*

Citation:
Li J, He Y, Ren B, Zhang Z, Meng F, Zhang X, Zhou Z, Li B, Li F, Liu L and Shen H (2022) The Thyroid Condition and Residual Clinical Signs in 31 Existing Endemic Neurological Cretins After 42 Years of Iodine Supplementation in China. Front. Endocrinol. 13:911487. doi: 10.3389/fendo.2022.911487

Backgroud: Endemic cretinism is the most severe manifestation among the iodine deficiency-related disorders. The clinical status of the cretins may be modified subsequently by the duration and severity of the disease. We aimed to reassess the clinical status and thyroid function of 31 surviving "neurological cretins" after 42 years of iodine supplementation in a historically severely iodine deficiency area of China.

Methods: It was a cross-sectional study in design and we investigated all 31 surviving neurological cretins and 85 controls. A detailed neurological examination was conducted on each patients. All the participants were given a questionnaire and underwent B-mode ultrasonography of the thyroid. The serum levels of thyroid hormones, thyroid antibodies, serum iodine concentration (SIC) and urine iodine concentration (UIC) were measured.

Results: The neurological cretins had shorter stature than that of the control. Neurological damage is still present in patients with cretinism. The prevalence of subclinical hypothyroidism and thyroid nodule in the cretins was significantly higher (χ^2 = 4.766, P=0.029 and χ^2 = 17.077, P<0.0001, respectively) compared with the control. After adjusting for confounding factors, endemic neurocretinism was found to be an independent risk factor for subclinical hypothyroidism (OR=4.412; 95% CI: 1.358–14.334; P=0.014) and thyroid nodule (OR=6.433; 95% CI: 2.323–17.816; P<0.0001).

Conclusions: Iodine supplementation after birth does not reverse the neurological damage that results from maternal/foetal hypothyroidism *in utero* and is subsequently manifested as neurological cretinism. There is a cross-sectional association between endemic neurocretinism and subclinical hypothyroidism and thyroid nodule.

Keywords: endemic neurological cretin, iodine-deficient area, subclinical hypothyroidism, nodules, neurological signs, thyroid function

INTRODUCTION

Iodine is an essential micronutrient for thyroid hormone synthesis (1). Thyroid hormone is particularly critical for normal fetal and infant neurodevelopment, therefore, adequate maternal iodine nutrition is essential during pregnancy (2), and severe iodine deficiency during pregnancy may result in fetal hypothyroidism as well as serious neurologic and cognitive defificits in children (3).

Endemic cretinism is the most severe manifestation among the iodine deficiency-related disorders (4). As early as 1908, two types of cretins are distinguished by McCarrison (5): neurological cretins are characterized by profound neurologic dysfunction, deaf-mutism, cerebral diplegia but clinical euthyroidism. Neurological abnormalities occurred *in utero* due to both maternal and fetal hypothyroxinemia caused by severe iodine deficiency. Postnatally, the persistence of hypothyroidism entails the development of myxedematous cretinism (6). Consequently, myxedematous cretins are traditionally characterized by stunted growth and signs and symptoms of hypothyroidism (7). Later studies have demonstrated that this overlap is greater than previously thought (8–10). In 1993, Boyages & Halpern (6) have proposed that both neurological cretins and myxedematous cretins are associated with severe iodine deficiency, with a time-dependent effect. For example, whatever the cause of thyroid destruction in myxedematous cretins, it appears not operative at birth but becomes an important factor with increasing age. Considering that the clinical status of the myxedematous cretins were subsequently modified by the duration length and severity of hypothyroidism (11, 12), we were curious that whether the neurological cretins also have thyroid function or clinical status changes with increasing age.

Therefore, we investigated all 31 surviving cretins in a historically severe iodine deficiency area, Jixian Village, Heilongjiang Province, China. Our institute had conducted a survey in this village in 1979, finding that the goitre rate was estimated to be 74% and the prevelence of cretinism was as high as 11.04% (145 people in 1313) (13, 14). Now, 42 years have passed, we surveyed this village again, the aim of our study is to evaluate the thyroid function and clinical signs of the endemic cretins after salt iodine supplementing with increasing age.

MATERIALS AND METHODS

Survey Areas

Our study was performed in a district named Jixian Village located in the Huachuan County, Heilongjiang Province, which lies on the west side of Sanjiang Plain in the northeast of the People's Republic of China. In this sparsely populated region with low terrain, inhabitants mainly rely on subsistence agriculture for their livelihood.

Survey Subjects

This was a cross-sectional study in design and we have evaluated all the surviving cretins (n=31) who have been diagnosed as neurological cretins with marked intellectual disability in 1979 by expert physicians according to the diagnosis criteria of cretinism. All the 31 cretins have been living in Jixian Village since they were born. According to the inclusion and exclusion criteria, we surveyed 85 adults as the control group, who were from the same village as the cretins, with similar diet and socioeconomic status. The inclusion criteria were as follows: age ranges from 43 to 79 (the same age range as the cretins); and resided locally since they were born. The exclusion criteria were as follows: pregnant or lactating women; those with no blood or urine samples for determination or with missing information on key sociodemographic and lifestyle characteristics; Individuals taking medications (amiodarone etal.) that interfere with thyroid function; participants with family history of cretinism.

The project was approved by the Ethics Review Committee of Harbin Medical University (No. hrbmuecdc20201201). Participants in the control group signed informed consent before data collection and the families or guardians of the cretins also consented to participate in the study voluntarily.

Survey Methods

Detailed neurologic examination of each cretin patient was performed by two expert physicians.

Intelligence Testing

Subjects were given the Raven's Progressive Matrices (RPM) for intelligence tests. The RPM is regarded as the most well researched of all the nonverbal measures (15). It is most valuable for use with people whose test performance may be confounded by language, hearing, or motor impairments or those who are non-English speaking (16). The RPM is the recommended method of intelligence test for diagnosing cretinism in China, and the type of intelligence disability was classified as severe for IQ <25, moderate for 25-39 and mild for 40-54.

Audiometry

Audiometric assessment was carried out on 29 patients, the mean hearing threshold (dB) is given as the average of the hearing thresholds at 500Hz, 1000Hz and 2000 Hz (17).

A neurological examination was undertaken, and signs of paralysis and motor spasticity were particularly sought. Special attention was focused on the gait.

We have performed several physical examinations for all the participants in this study, variables were height; weight; body mass index (BMI; height/weight^2 [kg/m^2]) (18). A standard questionnaire was designed to acquire demographic characteristics.

Laboratory Test and Clinical Diagnosis

5 samples in total from east, south, west, north, and central locations were collected from the Jixian Village. Each water sample was at least 15 ml. Water samples were stored at 4°C until analysis. The As^{3+}-Ce^{4+} catalytic spectrophotometry method was used for the determination of the iodine concentration of drinking water (19).

Salt samples were collected from all of the participants. Each participant provided at least 50g of household use of table salt in a clean, labeled ziplock bag. The iodine content in the salt samples was determined using the general test method of the

salt industry (20). The standard salt iodine content in the village of our study was 25 mg/kg (± 30%) (21). From each participant, a single spot urine sample was collected in the morning in clean plastic tubes and stored at 4°C, and measured within four months of collection. UIC was measured according to the standard procedure method for determination of iodine in urine by As^{3+}-Ce^{4+} catalytic spectrophotometry (22). The reference values of iodine deficiency in adults was as a median urinary iodine (MUI) <100μg/L, iodine adequate 100–299 μg/L, iodine excess ≥300 μg/L (23, 24).

Venous blood samples were collected from each subject after fasting for 8h. The supernatant was centrifuged and stored in a low-temperature refrigerator at −80°C until analysis to measure serum iodine concentration (SIC) and thyroid function. SIC was measured using the As3+-Ce4+ catalytic spectrophotometry method (25). The reference range of SIC in general population determined by the World Health Organization (WHO) is 45–90 μg/L (26).

The serum levels of FT_3, FT_4, TSH, Tg, TPOAb, TGAb were determined through electrochemiluminescent immunoassays using a Cobas Elesys 601 instrument (Roche Diagnostics Ltd., Switzerland). The reference values were 3.1–6.8 pmol/L FT_3, 12–22pmol/L FT_4, 0.27–4.2 mIU/L TSH, 0–34 IU/ml TPOAb, 0–115 IU/ml TgAb, and 3.5–77 ng/ml Tg (obtained from the manufacturer). The diagnostic criteria for thyroid disease were as follows (27): hypothyroxinaemia, FT_4 < 12 pmol/l and TSH within the normal range; overt hypothyroidism, TSH > 4.20 mIU/L and FT_4 < 12 pmol/l; subclinical hypothyroidism, TSH > 4.20 mIU/L and FT_4 within the normal range; overt hyperthyroidism, TSH < 0.27 mIU/L, FT_4 > 22 pmol/l, and FT_3 > 6.8 pmol/l; subclinical hyperthyroidism, TSH < 0.27 mIU/L, and FT_3 and FT_4 within the normal range.

In addition, all participants underwent thyroid ultrasonography by experienced radiologists, using a portable instrument (LOGIQ 100 PRO, GE, Milwaukee, WI, USA with 7.5 MHz linear transducers). Subjects were examined in a sitting position with the neck hyperextended to fully expose the thyroid (28). Thyroid volume was calculated with the following formula: V (mL) = 0.479×d (mm)×w (mm)×l (mm)×0.001 (29). The diagnostic criteria for goiter, thyroid volume was > 25 ml (male) and > 18 ml (female) (30, 31); and thyroid nodule, one or more nodule (> 5mm) without goiter (27).

Statistical Analysis

SPSS V.23.0 was used for statistical analysis. Normally distributed variables were expressed as a mean and standard deviation ($\bar{x} \pm s$), and the difference between the cretins and control were compared using the independent-samples *t*-test. Non-normally distributed variables were expressed as a median and inter-quartile range, and the *Mann-Whitney U* test was performed to compare the difference between the groups. Categorical variables and ordinal variables were expressed as a number (%), and were compared the difference between the cretins and control using the χ^2 test and the *Mann-Whitney U* test, respectively. Logistic regression was performed to analyze the risk factors associated with the subclinical hypothyroidism and thyroid nodule. With versus without the subclinical hypothyroidism and thyroid nodule were taken as dependent variables. The stepwise method was used to filter independent variables. The test level of alpha was set at 0.05 (two-sided), and $P<0.05$ was considered statistically significant.

RESULTS

In this study, the median water iodine concentration in Jixian Village was 4.5μg/L. A median water iodine concentration ≤ 10 μg/L is defined as an iodine deficiency area according to the Chinese national standard (32). The iodine content of salt samples was 26.76 ± 3.24mg/kg. In addition, the coverage of household use of qualified iodized salt in this village was 98.3%, which is in agreement with the evaluation content and criteria for the elimination of iodine deficiency diseases in China (coverage of household use of qualified iodized salt > 90.0%) (33).

Demographic Characteristics and Clinical Status for the Neurological Cretin Patients

The basic demographic characteristics and clinical signs of the cretins were described in **Table 1**, including degree of intellectual disability, deafmutism, neurological signs, facial features, thyroid ultrasonography and individual TSH levels. In addition, the IQ level of the local control population was 88 (79~95).

Of the 31 neurological cretins, 17 were male and 14 were female. The average age was 59.52 ± 9.63 years. 27 of 31 neurological cretins with ataxic gait, with marked motor spasticity, walked with bent knees, and their arms tended to be held in flexion (**Figure 1**). In 2 patients, standing up was impossible.

Audiologic examination revealed deafmutism or moderate neurologic hypoacusia in 23 of the 29 subjects examined, while for the remaining 2 patients, the audiometry was unobtainable because of the presence of defective attention or severe intellectual disability. It is worth mentioning that, 6 were deemed to have no hearing or speech difficulties. Most of the patients, to some extent, had typical cretinism facial features, such as laughing stupidly, ocular hypertelorism, flat nose or salivation. We did not find similar physical neurological abnormalities in the control group.

Basic Demographic Characteristics in the Neurological Cretin Patients and Control

Most of the neurological cretins, whether males or females, had short stature, and the average height of the male cretins (163.18 ± 5.60) was significantly lower ($p<0.05$) than that of the control group (167.70 ± 5.45) living in the same endemic area; and of the female cretins the average height (151.07 ± 6.28) was significantly lower ($p<0.01$) than that of the control group (156.44 ± 6.26) (**Table 2**).

Iodine Nutrition Status and Thyroid Function in the Neurological Cretin Patients and Control

The UIC, SIC and thyroid function in the neurological cretins and control are shown in **Table 3**. There were no statistically

TABLE 1 | Clinial findings in the endemic neurological cretins.

Patient No.	Sex F/M	Age (yr)	Height (cm)	Intellectual disability +Slight ++Moderate +++Severe	Deafmutism +Deafness mutism/ ± Moderate impaired/ Normal	Neurological signs Paralysis▲/Motor spasticity and Gait disorder #	Facial features A:Laugh stupidly B:ocular hypertelorism C:flat nose D:salivation	thyroid ultrasonography Thyroid Volumes (GoiterØ)	Thyroid nodule※	TSH mIU/L (0.27–4.2)
1	M	73	162	+++	[1]NA	▲	A B C D	10.57	_	2.21
2	M	44	160	+++	+	#	B C	14.13	_	3.95
3	M	51	176	+	+	#	B C	5.65	_	4.53
4	M	71	156	+++	[1]NA	▲	A B C	7.74	※	4.80
5	M	66	164	+	Normal	#	Normal	6.13	_	0.96
6	M	68	165	++	±	#	A B	9.49	※	5.68
7	M	65	169	+	±	#	B C	14.13	_	2.75
8	M	62	156	++	±	#	B C D	18.84	※	5.60
9	M	43	162	+	Normal	#	B C	5.66	_	3.35
10	M	46	166	++	±	#	A B D	9.34	_	3.10
11	M	71	163	++	±	#	B	7.86	※	20.97
12	M	67	153	++	Normal	#	B C	8.92	※	25.44
13	M	57	165	++	+	#	A B	8.63	※	3.45
14	M	64	164	++	±	#	B C	8.39	※	1.42
15	M	48	158	++	±	#	B C	4.93	※	3.15
16	M	48	166	+	±	#	B C	5.06	_	2.47
17	M	43	169	+	Normal	#	B C	4.93	※	0.69
Total (M)	M=17	58.06 ± 11.04	163.18 ± 5.60	+ = 6 ++ = 8 +++ = 3	NA=2 +=3 ±=8 Normal=4	▲ = 2 # = 15	A=5 B=16 C=12 D=3 Normal=1	8.85 ± 3.83	※=9 - =8	5.56 ± 6.84
1	F	54	155	+	±	#	BC	5.13	_	4.59
2	F	61	145	+++	+	#	A B C	6.32	※	2.57
3	F	58	156	++	±	#	B C	8.05	_	1.52
4	F	61	154	++	Normal	#	B C	12.50	※	1.08
5	F	50	160	++	+	#	B C	7.00	_	4.28
6	F	74	145	+	±	#	B C	(Ø)	※	1.15
7	F	58	155	+	+	#	B C	9.31	_	1.15
8	F	56	151	++	±	#	B C	8.40	_	5.10
9	F	60	141	+	+	#	A B C D	6.99	※	30.66
10	F	70	144	++	+	#	A B C	8.28	※	0.70
11	F	71	157	+++	Normal	#	A B C	15.47	_	0.75
12	F	64	159	+	±	#	A B C	10.37	※	0.10
13	F	70	148	+	±	#	B C	15.03	※	3.70
14	F	51	145	+++	+	#	A B C	10.10	※	5.92
Total (F)	F=14	61.29 ± 7.61	151.07 ± 6.28	+ = 6 ++ = 5 +++ = 3	+=6 ±=6 Normal=2	#=14	A=6 B=14 C=14 D=1	9.46 ± 3.20	※=8 - =6	4.52 ± 7.76
Total	31	59.52 ± 9.63	157.71 ± 8.45	+ = 12 ++ = 13 +++ = 6	NA=2 +=9 ±=14 Normal=6 [1]NA: Not Assessed	▲ = 2 # = 29	A=11 B=30 C=26 D=4 Normal=1	9.11 ± 3.52	※=17 - =14	5.09 ± 7.16

Intellectual disability: +Mild (IQ, 40-54); ++Moderate (IQ, 25-39); +++Severe (IQ <25).
Ø: Goiter, beyond the measuring range of the instrument.

significant differences in both the median UIC (168.92 Vs 175.86μg/L) and the median SIC levels (62.39 Vs 63.82μg/L) between the neurological cretins and the control.

Compared with the control group, the TSH levels of the neurological cretins were significantly higher (3.35 μIU/ml vs. 2.06μIU/ml, P<0.01). No statistical difference was detected in median FT_3 concentrations, median FT_4 concentrations, and TgAb and TPOAb-positive rates between the neurological cretins and control. There were no differences in Tg levels between the neurological cretins and control.

The prevalence of subclinical hypothyroidism and thyroid nodule in the neurological cretins was significantly higher (P = 0.029 and P < 0.0001, respectively) compared with the control. Nevertheless, we observed that there was no significant

FIGURE 1 | An endemic neurological cretin patient (Female, 60 years old, 141 cm, 45.9 kg). Characterized by severe intellectual impairment, deaf mutism and motor spasticity. Showing characteristic postural abnormality of endemic neurological cretin cretinism. There is knock knee, but stance is moderately wide-based. The arms are held with the shoulders abducted, and the elbows flexed. Huachuan, Heilongjiang Province, PRC, 2020.

difference in the prevalence of other thyroid diseases between the two groups (**Table 4**).

Logistic Regression Analysis Between Neurocretinism and Subclinical Hypothyroidism and Thyroid Nodule

Because of the high prevalence of subclinical hypothyroidism and thyroid nodule in neurocretinism in single-factor analysis. To further determine whether the prevalences of subclinical hypothyroidism and thyroid nodule were associated with endemic neurocretinism in multivariate analysis, binary logistic regression models were used. After adjusting for confounding factors, endemic neurocretinism was found to be an independent risk factor for subclinical hypothyroidism (OR=4.412; 95% CI: 1.358–14.334; P=0.014) and thyroid nodule (OR=6.433; 95% CI: 2.323–17.816; P<0.0001). Refer to **Table 5** for details.

DISCUSSION

The Jixian Village, as a typical area we selected to investigate is once a severe iodine deficiency area. Due to a large number of cretinis, Jixian Village was once called "Village of Fools" and thus became famous throughout China in the last century. Now it is

TABLE 2 | Demographic characteristics in the neurological cretins and control.

Characteristics	Total	Endemic cretins (*n*=31)	Control (*n*=85)	*P* Value
Gender *n* (%)				
Male	47 (40.5)	17 (54.8)	30 (35.3)	0.058
Female	69 (59.5)	14 (45.2)	55 (64.7)	
Age(years) *n* (%)				
41–50	31 (26.7)	7 (22.6)	24 (28.2)	0.127
51–60	42 (36.2)	8 (25.8)	34 (40.0)	
61–70	29 (25.0)	11(35.5)	18 (21.2)	
71–79	14 (12.1)	5 (16.1)	9 (10.6)	
Height (cm) $\bar{x} \pm s$				
Total	159.62 ± 8.21	157.71 ± 8.45	160.39 ± 8.05	0.126
Male	165.95 ± 5.89	163.18 ± 5.60	167.70 ± 5.45	0.013*
Female	155.27 ± 6.61	151.07 ± 6.28	156.44 ± 6.26	0.006*
P Value	<0.0001*	<0.0001*	<0.0001*	
Weight (kg) $\bar{x} \pm s$				
Total	61.95 ± 11.08	59.01 ± 10.92	63.13 ± 10.99	0.080
Male	66.08 ± 11.41	61.72 ± 9.60	68.82 ± 11.77	0.043*
Female	59.11 ± 9.98	55.71 ± 11.86	60.06 ± 9.30	0.151
P Value	0.001*	0.130	0.001*	
BMI (kg/m^2) $\bar{x} \pm s$				
Total	24.25 ± 3.53	23.69 ± 3.74	24.47 ± 3.44	0.299
Male	23.92 ± 3.46	23.15 ± 3.02	24.41 ± 3.68	0.245
Female	24.47 ± 3.58	24.35 ± 4.50	24.51 ± 3.33	0.882
P Value	0.427	0.384	0.903	

BMI, boy mass index; A t-test was used for height, weight and BMI; the Mann-Whitney U test was adopted for age; a χ^2 test was used for gender.
**P < 0.05 and the difference between groups was statistically significant.*

one of a few villages with so many existing endemic cretin patients for study in China. All the 31 cretins were born before the introduction of iodine prophylaxis, the IQ level of the local control population was 88 (79~95) which matched the moderate IQ level according to the national Chinese standards. Consequently, it is admirable that the introduction of iodized salt into Jixian village in 1978 prevented the occurrence of any further neurological cretinism from that date onwards. Since the investigation in 1979 of endemic goiter and endemic cretinism, all patients diagnosed with endemic goiter have been treated with oral potassium iodide. The thyroid goiter rate decreased from 74% in 1979 to 22.38% in 1982, according to a census taken three years later (34). During the second census conducted in the same area in 2002, the rate of thyroid goiter declined from 74% to 18.6% (35). In this survey, the thyroid goiter rate in cretin patients and control population was 3.2% and 3.5%, respectively. According to a nationally representative cross-sectional study with 78,470 participants from all 31 provincial regions of China, the weighted prevalence of goiter in adults was 1.17% (27). By comparison, the thyroid goiter rate in Jixian is the same as other regions, which indicates that the long-term mandatory USI program is effective in preventing iodine deficiency disorders.

UIC is a reliable indicator for the iodine status assessment because 90% of dietary iodine is excreted in urine within 24 hours after consumption (36). Due to the sensitivity of serum iodine in evaluating individual iodine nutrition status, the stability of population iodine nutrition status, and the significance of screening in people at high risk for thyroid disease (37), our study included SIC as well as the UIC for the iodine status assessment. According to the recommendations of the WHO/UNICEF/ICCIDD (24, 26, 38), whether from the

TABLE 3 | The iodine status and thyroid function in the neurological cretins and control.

Characteristics	Total	Endemic cretins (*n*=31)	Control (*n*=85)	*P* Value
UIC (μg/l) [M, Q]	169.90 (123.65,243.89)	168.92 (123.95,202.39)	175.86 (123.65,263.35)	0.378
SIC (μg/l) [M, Q]	63.60 (54.03,71.69)	62.39 (54.63,71.70)	63.82 (53.85,71.68)	0.830
FT_3 (pmol/l) [M, Q]	5.40 (4.95,5.82)	5.60 (5.34,5.90)	5.29 (4.89,5.80)	0.070
FT_4 (pmol/l) [M,Q]	16.27 (14.97,18.30)	15.94 (14.96,18.09)	16.39 (14.92,18.57)	0.518
TSH (μlU/ml) [M, Q]	2.33 (1.26,3.67)	3.35 (1.42,5.10)	2.06 (1.24,3.25)	0.009*
Tg (ng/ml) [M, Q]	11.84 (5.57,34.05)	23.97 (7.77,58.39)	10.68 (5.07,25.53)	0.060
TPOAb (+), *n* (%)	13 (11.2)	5 (16.1)	8 (9.4)	0.495
TgAb (+), *n* (%)	12 (10.3)	5 (16.1)	7 (8.2)	0.373

[M, Q], [median, inter-quartile range]; UIC, urinary iodine concentration; SIC, serum iodine concentration; FT_3, free triiodothyronine; FT_4, free thyroxine; TSH, thyroid stimulating hormone; Tg, thyroglobulin; TPOAb (+), thyroid peroxidase antibody-positive; TgAb (+), thyroglobulin antibody-positive.
Mann-Whitney U test was adopted for UIC, SIC, FT_3, FT_4, TSH, Tg; χ^2 test was used for TPOAb (+) and TgAb (+). The normal reference ranges were obtained from the manufacturer: FT3, 3.10-6.80 pmol/L; FT4, 12.00-22.00 pmol/L; TSH, 0.27-4.20 mIU/L; TPOAb, 0-34 IU/mL; TgAb, 0-115 IU/mL; Tg, 3.5–77 ng/ml.
**P < 0.05 was considered significant.*

TABLE 4 | Prevalence of thyroid disease between neurological cretins and control (Number of thyroid disease and percentages).

	Endemic cretins (N=31) *n* (%)	Control (N=85) *n* (%)	*P* Value
Hypothyroxinaemia			
Yes	0 (0.0)	1 (1.2)	1.000
No	31 (100.0)	84 (98.8)	
Overt hypothyroidism			
Yes	3 (9.7)	1 (1.2)	0.100
No	28 (90.3)	84 (98.8)	
Subclinical hypothyroidism			
Yes	8 (25.8)	7 (8.2)	0.029*
No	23 (74.2)	78 (91.8)	
Overt hyperthyroidism			
Yes	1 (3.2)	2 (2.4)	1.000
No	30 (96.8)	83 (97.6)	
Subclinical hyperthyroidism			
Yes	0 (0.0)	1 (1.2)	1.000
No	31 (100.0)	84 (98.8)	
Autoimmune thyroid disease			
Yes	7 (22.6)	10 (11.8)	0.246
No	24 (77.4)	75 (88.2)	
Thyroid nodule			
Yes	17 (54.8)	14 (16.5)	<0.0001*
No	14 (45.2)	71 (83.5)	
Goiter			
Yes	1 (3.2)	3 (3.5)	0.720
No	30 (96.8)	82 (96.5)	

**P < 0.05 and the difference between groups was statistically significant.*

perspective of the median UIC or the median SIC, the population iodine status was adequate both in the cretins and the control, which indicates that the policy of salt iodization is well implemented and successful in this area. However, it is worth noting that no statistically significant differences were found in both the median UIC and SIC levels between the neurological cretins and the control. The result shows that there is no abnormal iodine metabolism in neurological cretins after salt iodization, compared with control.

The neurological cretins, male or female, had shorter stature than that of the control group, which indicated that with increasing age, the height of neurocretinisms may also be affected. However this finding is not consistent with previously published data which have shown that growth retardation was found at a much greater frequency in myxedematous cretins (15, 39–41), rather than in neurological cretins, while for neurological cretins, they may have postnatal growth retardation, but may eventually be normal or approximately normal when they be adults (42). It seems that there is no reversal for the deaf-mutism and neurological damage for these neurological cretins even after receiving iodine supplement. This reinforces the conclusion of previous publications that treatment after delivery may improve brain growth and developmental achievement slightly, but it does not reverse the neurological damage that results *in utero* from maternal/foetal hypothyroidism and is subsequently manifested as neurological cretinism (43). The surviving neurological cretins are more in signs of mild intellectual disability and neurological signs, and they can still participate in family activities to some extent. The reason for this variability in signs of neurologic involvement among different surveys may be explained by the age differences of the subjects or by the different methods used to detect neurologic and audiological deficits (44). Furthermore,

TABLE 5 | Risk factors associated with subclinical hypothyroidism and thyroid nodule.

	Model	β	S_x	Wald χ^2	OR (95%CI)	*P* Value
Subclinical hypothyroidism						
Endemic cretinism						
No	Ref					
Yes	1	2.123	0.757	7.864	8.353 (1.895, 36.823)	0.005
	2	1.484	0.601	6.097	4.412 (1.358, 14.334)	0.014
Thyroid nodule						
Endemic cretinism						
No	Ref					
Yes	1	2.056	0.557	13.637	7.812 (2.624, 23.260)	<0.0001
	2	1.861	0.520	12.825	6.433 (2.323, 17.816)	<0.0001

Binary logistic regression analysis was performed to estimate odds ratio for endemic cretinism for subjects in two models: model 1 (unadjusted) and model 2 (adjusted for age, gender). OR, odds ratio; 95% CI, 95% confidence interval.

researches have shown that the variability of neurologic clinical signs among the types of cretinism may be explained both by environmental factors and by the severity and duration of fetal and postnatal hypothyroidism, which could modify the phenotypic expression of the disorder (9, 11).

In this study, high prevalence of subclinical hypothyroidism and thyroid nodule was found in neurological cretin patients. Although the cross-sectional study which we used makes it difficult to determine the causal relationship between the neurological cretins and subclinical hypothyroidism and thyroid nodule, the historical data from previous investigation of the village shows that all cretins were clinical euthyroidism at the time of diagnosis in 1979 (FT_4: 9.83 ± 1.91μg/dl; TSH: 6.14 ± 3.49mIU/L, measured by radioimmunoassay with a reference value of 0-10 mIU/L) (13). Although endemic neurological cretins are caused by maternal/fetal hypothyroidism *in utero*, and they may have only transient hypothyroidism in the postnatal period, we speculate these cretin patients are more likely to be affected by iodine deficiency or other mechanisms affecting thyroid development. Also, they are more prone to thyroid dysfunction and morphological abnormalities in the future, such as subclinical hypothyroidism and thyroid nodules in our study. Biondi B point that subclinical hypothyroidism is common and most individuals can be observed without treatment, also there is no evidence that levothyroxine therapy is beneficial to persons aged 65 or older (45). Nevertheless, the endemic neurocretinism patients also should be followed up regularly to prevent the development of overt hypothyroidism.

In our study, the prevalence of thyroid nodules in the neurological cretins (54.8%) was much higher than normal, while the condition of the control group (16.5%) was similar to that of the normal populations. Numerous studies suggest a prevalence of 19-35% with ultrasound data (46). According to the epidemiological evidence from 31 provinces of mainland China, the weighted prevalence of thyroid nodules in adults was 20.43%, and iodine deficiency was significantly associated with higher odds of most thyroid disorders (27). Gharib also reported that iodine deficiency seem to increase the risk of thyroid nodules (47). The high prevalence of nodules in the cretins did still so high even after iodine supplementation, because studies show that the thyroid gland before birth and in the first few years of life (<4 years old) is able to respond physiologically to iodine deficiency and supplementation, while in patients with endemic cretinism there is a reduction in response to iodine supplementation with increasing age (11, 12, 48). Fortunately, very few of these lesions ultimately prove to be malignant (about 5%) (49). Nonetheless, as recognized risk factors for thyroid malignancy, nodules that are firm, fixed, or rapidly growing require prompt evaluation (50). It also requires more regular follow-up checks for the existing endemic neurocretinism patients.

This study had some limitations. First, the survey was confined to a unique village, although 31 cretins were all cases we investigated as hard as we could, while the results might not be representative because of the relatively small sample size, which may affect the authenticity of the results. It is expected that this problem can be solved by a larger sample or multi-center validation in other areas. Second, survivor bias may exist due to the since most of the 31 existing cretin patients surveyed had mild clinical status because of the death of patients with severe conditions.

CONCLUSION

In conclusion, in this historically severe iodine-deficient area, under the implementation of a comprehensive salt iodization policy, endemic neurological cretins had normal iodine nutrition. The institution of iodine supplementation after birth does not reverse the neurological damage that results *in utero* from maternal/foetal hypothyroidism and is subsequently manifested as neurological cretinism. We found a cross-sectional association between endemic neurological cretins and subclinical hypothyroidism and thyroid nodule.

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 project was approved by the Ethics Review Committee of Harbin Medical University (No. hrbmuecdc20201201). Harbin Medical University is the authors' affiliation. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HS, designed the study. LL, project administration. JL, data curation and writing- original draft. HS, LL, ZZha, FM, XZ, JL, YH, BR, ZZho, BL, FL, investigation. All authors revised the report and approved the final version before submission.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant no. 82073490) and the Fundamental Research Funds for the Provincial Universities (grant no. JFXN201909).

ACKNOWLEDGMENTS

The work on the field was with assistance from the Centers for Disease Control and Prevention(CDC), Huachuan County, Heilongjiang Province. The authors are grateful for the contributions and support from all participants and their families.

REFERENCES

1. Velasco I, Bath SC, Rayman MP. Iodine as Essential Nutrient During the First 1000 Days of Life. *Nutrients* (2018) 10:290. doi: 10.3390/nu10030290
2. Lee SY. Editorial. Consequences of Iodine Deficiency in Pregnancy. *Front Endocrinol (Lausanne)* (2021) 12:740239. doi: 10.3389/fendo.2021.740239
3. de Escobar GM, Obregón MJ, del Rey FE. Iodine Deficiency and Brain Development in the First Half of Pregnancy. *Public Health Nutr* (2007) 10:1554–70. doi: 10.1017/S1368980007360928
4. Hetzel BS. The Story of Iodine Deficiency, an International Challenge in Nutrition. New Delhi: Oxford University Press (1989). 36–51.
5. McCarrison R. Observations on Endemic Cretinism in the Chitral and Gilgit Valleys. *Ind Med Gaz* (1908) 43:441–9. doi: 10.1016/S0140-6736(01)62987-8
6. Boyages SC, Halpern JP. Endemic Cretinism: Toward a Unifying Hypothesis. *Thyroid* (1993) 3:59–69. doi: 10.1089/thy.1993.3.59
7. Stanbury JB. The Pathogenesis of Endemic Cretinism. *J Endocrinol Invest* (1984) 7:409–19. doi: 10.1007/bf03351027
8. Delong R. Neurological Involvement in Iodine Deficiency Disorders. In: BS Hetzel, JT Dunn and JB Stanbury, editors. *The Prevention and Control of Iodine Deficiency Disorders*. Amsterdam: Elsevier (1986). p. 49–63.
9. Boyages SC, Halpern JP, Maberly GF, Eastman CJ, Morris J, Collins J, et al. A Comparative Study of Neurological and Myxedematous Endemic Cretinism in Western China. *J Clin Endocrinol Metab* (1988) 67:1262–71. doi: 10.1210/jcem-67-6-1262
10. Halpern JP, Boyages SC, Maberly GF, Collins JK, Eastman CJ, Morris JG. The Neurology of Endemic Cretinism. *A Stud Two Endemias Brain* (1991) 114:825–41. doi: 10.1093/brain/114.2.825
11. Vanderpas JB, Rivera-Vanderpas MT, Bourdoux P, Luvivila K, Lagasse R, Perlmutter-Cremer N, et al. Reversibility of Severe Hypothyroidism With Supplementary Iodine in Patients With Endemic Cretinism. *N Engl J Med* (1986) 315:791–5. doi: 10.1056/nejm198609253151302
12. Boyages SC, Halpern JP, Maberly GF, Collins J, Jupp J, Eastman CJ, et al. Supplementary Iodine Fails to Reverse Hypothyroidism in Adolescents and Adults With Endemic Cretinism. *J Clin Endocrinol Metab* (1990) 70:336–41. doi: 10.1210/jcem-70-2-336
13. Li JQ, He QY, Wei Z. Investigation of Endemic Goiter and Endemic Cretinism in Jixian Village. *Beijing: China Acad J Electronic Publish House* (1979) 1:1–7.
14. Hetzel BS, Pandav CS. *S.O.S. For a Billion: The Conquest of Iodine Deficiency Disorders*. Delhi: Oxford University Press (1994). 17p.
15. Strauss E, Sherman EMS, Spreen OA. *Compendium of Neuropsychological Tests*. New York: Oxford University Press (2006).
16. Leavitt VM, Weber E. *Encyclopedia of Clinical Neuropsychology*. Germany: Springer International Publishing Ag (2017) p. 1–3p.
17. Wang YY, Yang SH. Improvement in Hearing Among Otherwise Normal Schoolchildren in Iodine-Deficient Areas of Guizhou, China, Following Use of Iodized Salt. *Lancet* (1985) 2:518–20.
18. Chen CM, Zhao WH, Yang XG. Criteria of Weight for Adults (Ws/T 428-2013). Beijing: Chinese Specification Press (2013).
19. Wang HY, Li L, Li SH. Study on the Method of Water Iodine Detection in Areas Lacking Iodine and High Iodine. *Chin J Endemiol* (2007) 26:333–6.
20. Tong YK, Huo JF. General Test Method in Salt Industry Determination of Iodine. Beijing: Chinese Specification Press (2012).
21. Chinese Ministry of Health. *National Standard on Food Safety-Iodine Content of Edible Salt: GB26878-2011* (2012). Available at: http://www.chinafic.org/uploadfile/2016/0504/20160504103253932.pdf.
22. Zhang YP, Yan YQ, Liu LJ. *Method for Determination of Iodine in Urine By As3+ - Ce4+ Catalytic Spectrophotometry (Ws/T 107.1-2016)*. Beijing: Health Standard of China (2016).
23. Zimmermann MB, Aeberli I, Andersson M, Assey V, Yorg JA, Jooste P, et al. Thyroglobulin Is a Sensitive Measure of Both Deficient and Excess Iodine Intakes in Children and Indicates No Adverse Effects on Thyroid Function in the Uic Range of 100-299 Mg/L: A Unicef/Iccidd Study Group Report. *J Clin Endocrinol Metab* (2013) 98:1271–80. doi: 10.1210/jc.2012-3952
24. United Nations Children's Fund (UNICEF). *Guidance on the Monitoring of Salt Iodization Programmes and Determination of Population Iodine Status* (2018). Available at: https://www.unicef.org/nutrition.
25. Shen HM, Ji XH, Zhang YP. *Determination of Iodine in Serum- As3+-Ce4+ Catalytic Spectrophotometry (WS/T 107.1-2016)*. Beijing: Health Standard of China (2017).
26. Han JH, Wu L, Yu SL, Fang HL, Kang WM, Cheng XQ, et al. Values of Iodine Metabolism Biomarkers in Assessing the Iodine Nutrition Status in Surgically Treated Patients With Thyroid Disease. *Acta Acad Med Sin* (2015) 37:221–5.
27. Li Y, Teng D, Ba J, Chen B, Du J, He L, et al. Efficacy and Safety of Long-Term Universal Salt Iodization on Thyroid Disorders: Epidemiological Evidence From 31 Provinces of Mainland China. *Thyroid* (2020) 30:568–79. doi: 10.1089/thy.2019.0067
28. Hess SY, Zimmermann MB. Thyroid Volumes in a National Sample of Iodine-Sufficient Swiss School Children: Comparison With the World Health Organization/International Council for the Control of Iodine Deficiency Disorders Normative Thyroid Volume Criteria. *Eur J Endocrinol* (2000) 142:599–603. doi: 10.1530/eje.0.1420599
29. Wang Z, Luo B, Zang J, Shi Z, Cui X, Song Q, et al. Appropriate Range of Median Urinary Iodine Concentration in 8- to 10-Year-Old Children Based on Generalized Additive Model. *Thyroid* (2020) 30:1535–40. doi: 10.1089/thy.2019.0813
30. Liu SJ, Chen ZH, Jia QZ. *Diagnostic Criteria for Endemic Goiter: WS276-2007*. Beijing: Health Standards of China (2017).
31. Carlé A, Krejbjerg A, Laurberg P. Epidemiology of Nodular Goitre. Influence of Iodine Intake. *Best Pract Res Clin Endocrinol Metab* (2014) 28:465–79. doi: 10.1016/j.beem.2014.01.001
32. Shen HM, Su XH, Ge XG, Wei HC, Qi Q. *Delimitation for the Endemic Areas of Iodine Deficiency Disorders (IDD) (GB 16005-2009)*. Beijing: Chinese Specification Press (2009).
33. Zhang YX, Chen ZP, Ge PF. *Criteria for the Elimination of Iodine Deficiency Disorders in China (GB 16006-2008)*. Beijing: Chinese Specification Press (2008).
34. Li JQ, He QY, Wei Z. Summary of Three Years' Effort on Prevention and Treatment of Endemic Goiter and Endemic Cretinism in Jixian Area. *Heilongjiang Med Sci* (1982) 4:40–5.
35. Su XH, Yu J, Li Y, Zhang SB, Wang WH, Su M, et al. Epidemiological Investigation of Endemic Goiter and Endemic Cretinism in Jixian Village. *Chin J Endemiol* (2003) 4:67–8.
36. Jabbar A, Pingitore A, Pearce SH, Zaman A, Iervasi G, Razvi S. Thyroid Hormones and Cardiovascular Disease. *Nat Rev Cardiol* (2017) 14:39–55. doi: 10.1038/nrcardio.2016.174
37. Jin X, Jiang P, Liu L, Jia Q, Liu P, Meng F, et al. The Application of Serum Iodine in Assessing Individual Iodine Status. *Clin Endocrinol (Oxf)* (2017) 87:807–14. doi: 10.1111/cen.13421
38. WHO/UNICEF/ICCIDD. Assessment of Iodine Deficiency Disorders and Monitoring Their Elimination. In: *A Guide For Program Managers, 3rd ed*. Geneva: WHO, WHO/ NDH/ (2007). p. 01.1.
39. Rajatanavin R, Chailurkit L, Winichakoon P, Mahachoklertwattana P, Soranasataporn S, Wacharasin R, et al. Endemic Cretinism in Thailand: A Multidisciplinary Survey. *Eur J Endocrinol* (1997) 137:349–55. doi: 10.1530/eje.0.1370349
40. Donati L, Antonelli A, Bertoni F, Moscogiuri D, Andreani M, Venturi S, et al. Clinical Picture of Endemic Cretinism in Central Apennines (Montefeltro). *Thyroid* (1992) 2:283–90. doi: 10.1089/thy.1992.2.28
41. Antonelli A, Boyages SC, Halpern JP. Clinical Neurophysiological, CT and RM Finding in Endemic Cretinism (Abstract). *Thyroid* (1995) 1:S–86.
42. Ma T, Lu TZ, Yu ZH. *Iodine Deficiency Disorder—Endemic Goitre and Cretinism*. China: People's sanitary publishing press (1993). 89p.
43. Cao XY, Jiang XM, Dou ZH, Rakeman MA, Zhang ML, O'Donnell K, et al. Timing of Vulnerability of the Brain to Iodine Deficiency in Endemic Cretinism. *N Engl J Med* (1994) 331:1739–44. doi: 10.1056/nejm199412293312603
44. DeLong GR, Stanbury JB, Fierro-Benitez R. Neurological Signs in Congenital Iodine-Deficiency Disorder (Endemic Cretinism). *Dev Med Child Neurol* (1985) 27:317–24. doi: 10.1111/j.1469-8749.1985.tb04542.x
45. Biondi B, Cappola AR, Cooper DS. Subclinical Hypothyroidism: A Review. *Jama* (2019) 322:153–60. doi: 10.1001/jama.2019.9052
46. Dean DS, Gharib H. Epidemiology of Thyroid Nodules. *Best Pract Res Clin Endocrinol Metab* (2008) 22:901–11. doi: 10.1016/j.beem.2008.09.019
47. Gharib H. Changing Concepts in the Diagnosis and Management of Thyroid Nodules. *Endocrinol Metab Clin North Am* (1997) 26:777–800. doi: 10.1016/s0889-8529(05)70282-6
48. Liu JL, Tan YB, Zhuang ZJ, Shi ZF, Wang YL, Li XT, et al. Morphologic Studies of Therapeutically Aborted Fetus Thyroid in an Endemic Goiter Region of Guizhou Province. *Chin Med J (Engl)* (1982) 95:347–54.

49. Durante C, Grani G, Lamartina L, Filetti S, Mandel SJ, Cooper DS. The Diagnosis and Management of Thyroid Nodules: A Review. *Jama* (2018) 319:914–24. doi: 10.1001/jama.2018.0898
50. Schwetschenau E, Kelley DJ. The Adult Neck Mass. *Am Fam Phys* (2002) 66:831–8.

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 29 July 2022
DOI 10.3389/fendo.2022.904437

OPEN ACCESS

EDITED BY
Maaike Van Gerwen,
Icahn School of Medicine at Mount Sinai, United States

REVIEWED BY
Laura Croce,
University of Pavia, Italy
Andrea Claudia Freitas Ferreira,
Federal University of Rio de Janeiro, Brazil
Cinzia La Rocca,
Center for Gender-specific Medicine, National Institute of Health (ISS), Italy

*CORRESPONDENCE
Laura Sterian Ward
ward@unicamp.br

SPECIALTY SECTION
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 25 March 2022
ACCEPTED 05 July 2022
PUBLISHED 29 July 2022

CITATION
Dal' Bó IF, Teixeira ES, Rabi LT, Peres KC, Nascimento M, Chiamolera MI, Máximo V, Bufalo NE and Ward LS (2022) Alternation between toxic and proliferative effects of Roundup® on human thyroid cells at different concentrations. *Front. Endocrinol.* 13:904437. doi: 10.3389/fendo.2022.904437

Alternation between toxic and proliferative effects of Roundup® on human thyroid cells at different concentrations

Izabela Fernanda Dal' Bó[1], Elisângela Souza Teixeira[1], Larissa Teodoro Rabi[1], Karina Colombera Peres[1], Matheus Nascimento[1], Maria Izabel Chiamolera[2], Valdemar Máximo[3,4,5], Natássia Elena Bufalo[1,6] and Laura Sterian Ward[1*]

[1]Laboratory of Cancer Molecular Genetics, School of Medical Sciences, University of Campinas, Campinas, Brazil, [2]São Leopoldo Mandic School of Medicine, Campinas, Brazil, [3]Department of Medicine, Federal University of São Paulo, São Paulo, Brazil, [4]Institute for Research and Innovation in Health (i3S), University of Porto, Porto, Portugal, [5]Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup), Porto, Portugal, [6]Department of Pathology, Faculty of Medicine of the University of Porto (FMUP), Porto, Portugal

Endocrine-disrupting and carcinogenic effects of glyphosate have long been suspected, but little is known about the effect of compounds used in real life at different concentrations, neither in normal nor in thyroid tumor cells. As cancer cells may have different sensitivities and the effect of the product containing glyphosate may be different from that produced by the active ingredient alone, including the Acceptable Occupational Exposure Level (AOEL=160µg/L) and the Acceptable Daily Intake (ADI=830µg/L) determined by ANVISA, we used two human thyroid-derived cell lines, Nthy-ori 3-1 (from normal follicular cells) and TPC-1 (from papillary carcinoma), to test 15 different concentrations of Roundup® Original DI. Trypan blue (TB), CCK-8 and BrdU assays were used to evaluate cytotoxicity, metabolic activity and proliferation with 24h and 48h exposures in technical and biological triplicates. TB showed an important toxic effect, especially after 24h of exposure, in both cell lines. The AOEL concentration caused the death of 43% and 50% of the Nthy-ori and TPC-1 cells, respectively, in 24 h, while ADI resulted in 35% and 58% of cell death. After 48h of exposure, AOEL and ADI caused a lower number of dead Nthy-ori (33% and 18%) and TPC-1 (33% and 37%) cells, respectively, suggesting that the toxic effect of the product disappears and/or both strains have repair mechanisms that protect them from longer exposures. On the other hand, the CCK-8 assay showed that small concentrations of Roundup have a proliferative effect: 6.5µg/L increased the number of both Nthy-ori and TPC-1 cells at 24h, and the BrdU assay confirmed the stimulatory effect with a 321% increase in the absorbance of Nthy-ori cells at 48h. The herbicide produced even more frequent increases in the BrdU absorbance of TPC-1 cells, mainly at 24h. We conclude that thyroid cells exposed to Roundup present a nonmonotonic dual dose–response curve. Low concentrations of the pesticide, considered acceptable, cause significant cell death but also have an important proliferative effect, especially on TPC-1 cells. This herbicide, widely used around the world, may

play a role in the increased incidence rate of thyroid nodules and cancer that has been observed in recent decades.

KEYWORDS

thyroid, endocrine disruptor, glyphosate, pesticide, cytotoxicity, proliferation

Introduction

The incidence rate of thyroid cancer (TC) has increased dramatically in recent decades, making it one of the most frequent neoplasms and the fastest growing cancer in women almost all over the world (1). Although most of this increase is certainly due to the use of sensitive and accessible diagnostic methods such as cervical ultrasound, there is solid evidence that other factors may contribute. Indeed, large tumors have also been detected more frequently, and there have been major changes in the histological profile (2) and genetic landscape of currently diagnosed tumors (3).

Endocrine disruptors (EDs) have long been considered among those environmental factors most likely to be involved in thyroid tumorigenesis. These chemicals affect the endocrine system at the cellular and molecular levels (4, 5), and many EDs persist in the environment and become organic pollutants (6, 7). Because ED may be used in our routine in different environments, contact with low doses and exposure from a very early age, even in intrauterine life, may influence disease outbreaks, including thyroid diseases (4, 5).

ED impairs the action and activity of triiodothyronine (T3) and thyroxine (T4), which can interfere with several pathways of thyroid hormone metabolism (8). Furthermore, alterations in thyroid hormone homeostasis modify the hypothalamic-pituitary-thyroid axis, affecting thyrotrophic hormone (TRH) and thyroid stimulating hormone (TSH) (9, 10). Exposure to ED has been linked to hypothyroidism and the appearance of goiter (11). However, little is known about the concentrations at which chemical compounds have a disruptive effect on the thyroid and/or their mechanism of action.

Glyphosate-based herbicides have been widely used for over 4 decades thanks to the introduction of glyphosate-resistant genetically modified crops in most of the world's major food producers (12). Glyphosate (N-(phosphonomethyl)glycine) is an organophosphate considered by the International Agency for Research on Cancer (IARC) to be a class 2A carcinogen (probably carcinogenic) (13), but although glyphosate-based herbicide toxic effects as endocrine disruptors have been demonstrated in several cell lines and experimental *in vivo* models, their effect in the real world, especially concerning humans, is still a matter of debate.

One of the main commercial glyphosate-based products is Roundup®, first patented by Monsanto. Glyphosate was classified as a low toxic product by the Brazilian National Health Surveillance Agency (ANVISA) (14), but the potential harmful effects of the various commercial formulations available as Roundup®, which contain various adjuvants added with the aim of improving the product's competence, are still scarce (15). In fact, in certain formulations, the adjuvants are at higher concentrations than the active ingredient, and some of these adjuvants have been reported to cause different types of damage (16–20).

Key cellular processes, such as proliferation, cell mobility, apoptosis and differentiation, are regulated by hormones, including thyroid hormones, which have been clearly demonstrated to be affected by glyphosate (21). Therefore, an effect on thyroid cell proliferation and consequent goiter or even on thyroid malignancy is plausible. In addition, thyroid tumor cells could be more susceptible to the effects of the chemical (22). Furthermore, since the dose–response curve of ED is nonmonotonic, it is essential to evaluate the effects of different doses, including those considered safe for humans, such as the AOEL and ADI, available in Brazilian Technical Note (Process No. 25351.056754/2013-17) of ANVISA (23).

The present study was designed to verify the effects of different doses of Roundup® Original DI in normal and papillary carcinoma thyroid lines.

Abbreviations: ADI, Acceptable Daily Intake; AOEL, Acceptable Occupational Exposure Level; CCK-8, Cell Counting Kit – 8; ED, Endocrine disruptors; EFSA, European Food Safety Authority; GBH, Glyphosate-based herbicides; IARC, International Agency for Research on Cancer; ANVISA, National Health Surveillance Agency; OD, Optical density; REL, Residential exposure level; TC, Thyroid cancer; TRH, Thyrotrophic hormone; TSH, Thyroid stimulating hormone; T4, Thyroxine; T3, Triiodothyronine; TB, Trypan Blue; WHO, World Health Organization.

Materials and methods

Cell culture

We used the Nthy-ori 3–1-cell line obtained from Sigma–Aldrich, St. Louis, USA (90011609) and the TPC-1-cell line, kindly provided by Prof. Dr. Valdemar de Jesus Conde Maximo, from the Institute of Pathology and Molecular Immunology -

Ipatimup, University of Porto, Portugal. Both cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin–streptomycin and 250 mg/ml fungizone (Sigma–Aldrich, St. Louis, USA). Cells were kept at 37°C in a humidified environment with 5% CO_2.

Roundup® Original DI preparation

To expose thyroid cell lines to different concentrations of the herbicide from 6.5 µg/L to 6500 µg/L, in conditions as close as possible to real life such as in food cultures (24, 25); doses to which farm workers are exposed (26); levels found in food and in water (12, 27). We used Roundup® Original DI (Monsanto, São Paulo, Brazil), whose formulation contains 445 g/L (44.5% w/v (phosphonomethyl) glycine diammonium salt (glyphosate), 370 g/L (37.0% w/v) equivalent to N-(phosphonomethyl)glycine acid (glyphosate) and 751 g/L (75.1% w/v) other unspecified ingredients with a concentration of 1,566 g/L.

The herbicide was diluted in water, and treatment doses were prepared at different concentrations, including the doses referenced by ANVISA. We used the following formulas for the dilution (23, 28):

$$\frac{Acceptable\ \ daily\ \ intake}{0,5\ \ mg/kg\ \ (ADI)\ \ x\ \ 70\ \ kg\ \ (\text{average adult body weight})\ \ /\ \ 42\ \ L\ \ (\text{total body weight water})}$$

$$\frac{Acceptable\ \ level\ \ of\ \ occupational\ \ exposure}{0,1\ \ mg/kg\ \ (AOEL)\ \ x\ \ 70\ \ kg\ \ (average\ \ adult\ \ body\ \ weight)\ \ /\ \ 42\ \ L\ \ (\text{total body weight water})}$$

Trypan blue exclusion test

TPC-1 and Nthy-ori 3-1 cells were seeded at $3.7x10^4$ cells in 12-well culture plates and incubated for 24 hours for complete cell adhesion. After 24 hours, the cells were treated with Roundup® Original DI ranging from 6.5 µg/L to 6500 µg/L. Exposures lasted 24 and 48 hours. We used a 1:1 dilution (10 µl cell suspension of each concentration in 10 µl Trypan Blue 0.4%) (Sigma–Aldrich, St. Louis, USA) for cell counting, which was performed with Countess® II FL (Thermo Fisher Scientific). The results are expressed as the percentage of viable cells compared to controls. All tests were performed in triplicate. We used the trypan blue (TB) assay to choose the concentrations used in the following cytotoxicity assay.

Cell counting kit – 8 test

Cytotoxicity was detected by CCK-8 assay, performed according to the Sigma–Aldrich protocol (St. Louis, USA). A total of 100 µl of cell suspension (5000 cells/well) of the TPC-1 and Nthy-ori 3-1 strains was dispensed in 96-well plates and incubated for 24 hours in a humidified incubator at 37°C and 5% CO_2 for complete cell adhesion. After 24 hours, Roundup® Original DI different dilutions were added: 6.5 µg/L; 65 µg/L; 160 µg/L (AOEL); 830 µg/L (ADI); 6500 µg/L upon trypan blue pretest selection. Cells were incubated for 24 and 48 hours. After that, 10 µl of CCK-8 solution (Sigma–Aldrich, St. Louis, USA) was added to each well of the plate and incubated for 3 hours in a CO2 incubator. Then, the absorbance or optical density (OD) at 450 nm was measured using a microplate reader (ELx808, Biotek, Winooski, VT, USA). The assay was performed in technical and biological triplicate, and the cellular sensitivity to the chemical was expressed as the percentage of viable cells compared to control cells using the following equation:

$$\text{Cell viability}\ \ (\%) = \frac{OD\ \ (Roundup)\ \ -OD\ \ (White)}{OD\ \ (Control) - OD\ \ (White)} x\ \ 100$$

BrdU cell proliferation assay

Cell proliferation was measured by the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology®) according to the manufacturer's instructions. A total of 100,000 cells/well of the TPC-1 and Nthy-ori 3-1 strains were dispensed in 96-well plates and incubated for 24 hours in a humidified incubator at 37°C and 5% CO2 for complete cell adhesion. Then, Roundup® Original DI treatments were added, and the lines were incubated at 24 and 48 hours of exposure. A 10X BrdU solution was added to the wells that were incubated for 2 hours. After incubation, 100 µl/well of the fixation/denaturation solution was added, and the plates were maintained at room temperature for 30 minutes. Then, 100 µl of 1X detection antibody solution was added and maintained at room temperature for 1 hour. After washing the plates 3 times with 1X Wash Buffer, 1X HRP-conjugated secondary antibody solution was added, and again, the plates were kept at room temperature for 30 minutes and washed 3 times with 1X Wash Buffer. After that, 100 µl of TMB substrate was added and incubated for 30 minutes at room temperature. At this stage, the color change of the plates was already observed; we added 100 µl of STOP solution to each well, and a microplate reader (ELx808, Biotek, Winooski, VT, USA) with absorbance at 450 nm was used to read the plates. The assay was performed in technical triplicate. Cell proliferation was expressed by absorbance and corrected with the blank result (control reaction).

Statistical analysis

Statistical analysis was performed using the SAS System for Windows (Statistical Analysis System), version 9.4. SAS

Institute Inc, 2002-2012, Cary, North Carolina, USA. In all analyzes non-parametric techniques were applied. To compare numerical measurements between concentration and its respective control, the Wilcoxon test was used for related samples.

To compare numerical measurements between concentrations, times and cell lines, ANOVA was used for repeated measurements with transformation by ranks. The significance level adopted for the statistical tests was 5%.

Results

Effect of Roundup® on cell viability

We determined viability using loss of membrane integrity by trypan blue dye exclusion assay and, as demonstrated in Figure 1, observed that the effect of Roundup® Original DI on TPC-1 thyroid cells was not dose-dependent but exhibited a nonmonotonic response within 24 h, which flattened out within 48 h.

Roundup® Original DI caused the death of up to 71% of TPC-1 cells exposed for 24 h at the tested concentrations, but this effect diminished after 48 h of exposure (Figure 1).

We selected five concentrations of Roundup® Original DI that were further applied to Nthy-ori 3-1 cells that Figure 2A, after 24 hours of exposure, also showed a decrease in viable cell numbers and, likewise TPC-1, became stable after 48 hours of exposure, as demonstrated in Figure 2B and Table 1.

We observed an important but limited cell injury rate, as detailed in Table 1. Exposure of Nthy-ori and TPC-1 cells to AOEL concentrations for 24 h caused the death of 43% and 50% of the cells, respectively, while ADI resulted in 35% and 58% cell death. After 48 h of exposure, AOEL and ADI caused a lower number of Nthy-ori (33% and 18%) and TPC-1 (30% and 37%) dead cells, respectively.

Cytotoxicity assessment

To further investigate the product cytotoxicity, we performed a CCK-8 assay in normal and thyroid cancer cells after 24 h and 48 h exposure to Roundup® Original DI at concentrations ranging from 6.5 µg/L to 6500 µg/L. Figure 3 shows that both TPC-1 and Nthy-ori 3-1 cell viability was maintained above 79% at all tested concentrations.

In fact, the impact on cell viability was relatively small in both thyroid lineages. On the other hand, we observed that treatment with Roundup® Original DI produced proliferation of Nthy-ori 3-1 and TPC-1 cells at certain concentrations at both 24h and 48h.

The number of viable cells at 24 h and 48 h of exposure to Roundup® Original DI at the same doses showed that the herbicide had similar effects on Nthy-ori 3-1 and TCP-1 cells (Figure 4; Table 2).

We used ANOVA comparison for repeated measures (concentrations and times) considering the following elements: the type of cells; the concentrations and exposure times; and the interactions between them. By specifying the results of the concentrations*cell interaction in the Nthy-ori 3-1 strain, we found differences in concentrations such that 65 µg/L presented values greater than 160, 830 and 6500 µg/L, and that 160 µg/L was less than 830 µg/L. A post-hoc (contrast profile test) was performed to identify this difference. However, in the TPC-1 strain, no difference was observed between the concentrations. Furthermore, there was no significant difference between cells (Nthy-ori 3-1 and TPC-1) at any concentration (Supplementary Table 1).

Influence of Roundup® on cell proliferation

To further explore the proliferative effect of Roundup® Original DI, we employed a BrdU assay on both thyroid cell

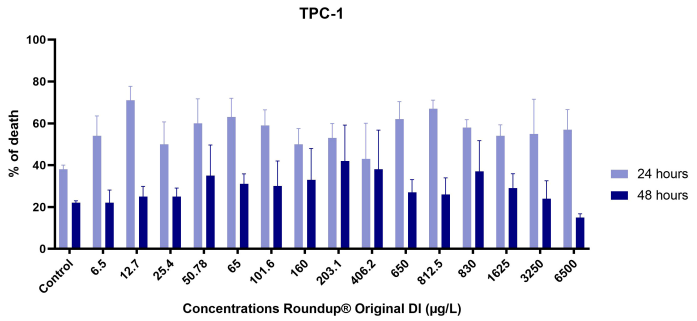


FIGURE 1
Percentage of dead TPC-1 cells after exposure to increasing concentrations of Roundup® Original DI for 24 h and 48 h measured by the trypan blue dye test. Results are presented as mean ± SE.

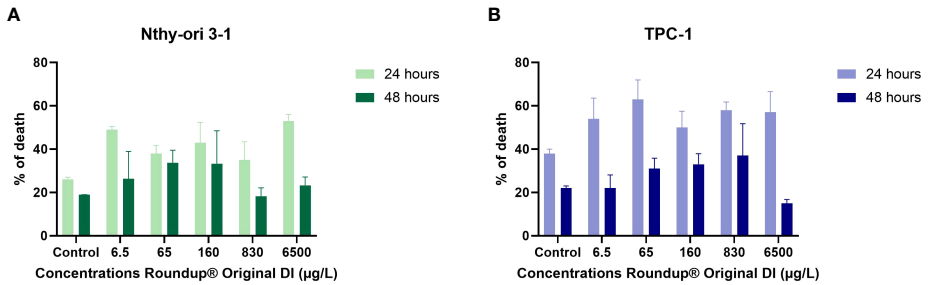


FIGURE 2
Comparison of cell mortality in Nthy-ori 3-1 **(A)** and TPC-1 **(B)** strains after exposure 216 to increasing concentrations of Roundup® Original DI for 24 h and 48 h by Trypan blue dye test.

lines. As shown in Figure 5, BrdU incorporation confirmed the results of the CCK-8 assay, revealing that 24 h exposure to Roundup® Original DI increased the number of Nthy-ori 3-1 cells, especially at low concentrations. Roundup® had an even more important proliferative effect on TPC-1 cells, and all concentrations, except 830 µg/L, increased the number of cells at 24 h; the proliferative effect persisted after 48 h, except at 65 µg/L and 160 µg/L.

In fact, as shown in Table 3, 6.5 µg/L of the herbicide had an important toxic destructive effect on Nthy-ori 3-1 exposed for 24 h but also produced a striking proliferative effect after 48 h of exposure. TPC-1 cells were even more sensitive to the proliferative effect of Roundup®, which was observed at diverse concentrations, especially at 24 h of exposure and at a concentration of 6500 µg/L after exposure for 48 h.

Comparing the results by ANOVA for repeated measures and using as factors the cell type; concentrations and exposure times; and the interactions between them, we found that the effect between cell types was significant, regardless of the exposure time and concentration. Evaluating the concentrations*times interaction that had a significant effect, at 48h the control was less than 6.5 ug/L, 6.5 ug/L was greater than 65, 160 and 830 ug/L and 65 ug/L was less than 6500 ug/L, in both cells. However, no significant differences were observed between the concentrations at 24 hours of exposure. The analysis of the times showed a significant difference just for one concentration (65 ug/L) that was greater at 24 h than at 48 h (Supplementary Table 2).

Discussion

In the present study, we demonstrated that exposure to different doses of Roundup® Original DI, including the Acceptable Occupational Exposure Level (AOEL) and the Acceptable Daily Intake (ADI), according to Brazilian National Health Surveillance Agency (ANVISA), produce a nonmonotone dose–response curve on normal and mutated thyroid cells with alternation between toxic and proliferative effects at different doses. This effect is a well-known characteristic of endocrine disruptors (29). Roundup® Original DI produced a greater maximal reduction in membrane integrity of TPC-1 cells (63%) than Nthy-ori 3-1 with 49% cell death, but both cell lines seemed to recover after 48 h of exposure. Confirming previous reports, we also demonstrated that damage and mortality are not proportional to Roundup® concentrations (5, 6). In fact, thyroid cell mortality occurred even at low doses, such as those recommended in agriculture; to which the operator or worker may be exposed daily; and those corresponding to the maximum amount of a substance that can

TABLE 1 Percent mortality of Nthy-ori 3-1 and TPC-1 cells after exposure to Roundup® Original DI for 24 h and 48 h at different concentrations, measured by Trypan Blue exclusion test.

	24 hours				48 hours			
	Nthy-ori 3-1	p value	TPC-1	p value	Nthy-ori 3-1	p value	TPC-1	p value
6.5 µg/L	49% ± 1.5	0.5000	54% ± 9.5	0.5000	26% ± 12.6	1.0000	22% ± 6.0	0.5000
65 µg/L	38% ± 3.7	0.2500	63% ± 8.9	0.5000	34% ± 5.7	0.5000	31% ± 4.8	0.5000
160 µg/L	43% ± 9.2	0.5000	50% ± 7.5	1.0000	33% ± 15.1	0.5000	33% ± 4.9	0.2500
830 µg/L	35% ± 8.4	1.0000	58% ± 3.7	0.2500	18% ± 3.8	0.2500	37% ± 14.7	0.5000
6500 µg/L	53% ± 3.0	0.2500	57% ± 9.6	0.5000	23% ± 3.8	0.5000	15% ± 1.7	1.0000

Values are presented as mean ± SE. The p value refers to the comparison between the number of viable cells exposed to the herbicide and the number of viable control nonexposed cells.

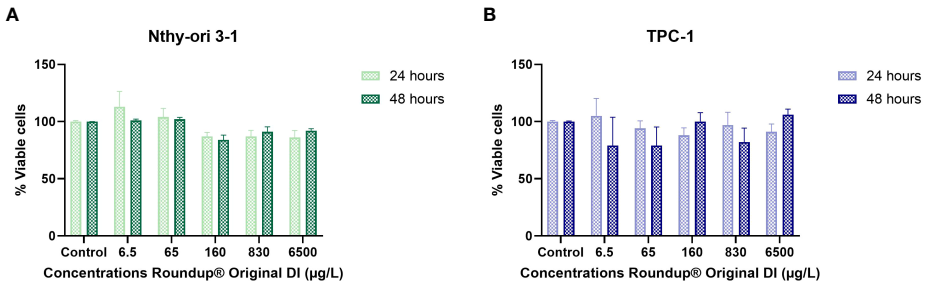


FIGURE 3
Comparison of cytotoxic effect on TPC-1 **(A)** and Nthy-ori 3-1 **(B)** strains after exposure to increasing concentrations of Roundup® Original DI for 24 h and 48 h, measured by the CCK-8 assay and presented as mean percentage of viable cells ± SE.

be ingested daily (12, 24–27). Conversely, other studies indicated a dose-dependent viability in human lymphocytes exposed to the pesticide (30). Chaufan et al found that a glyphosate formulation caused cytotoxicity in HepG2 cells depending on the dose and exposure time, even at dilutions below the recommended (31, 32). Another study, that used one of the formulated glyphosate-based products, also observed cytotoxic effects on HK-2 cells exposed for 24 hours at concentrations of 20-100 µM (equivalent to 3381.4-16907 µg/L), and the cell viability number was considerably reduced at concentrations above 40 µM (6762.8 µg/L). Furthermore, when the exposure times were extended to 36 and 48 hours, cell viabilities at concentrations of 40 and 60 µM (6762.8-10144.2 µg/L) were even more reduced (33). This difference could be related to a cell-specific sensitivity to the compound and to different effects of the active ingredient and commercially available formulations.

The important cell death rate observed in both normal and tumor thyroid cell lines exposed to low Roundup® doses, including those considered acceptable by regulatory agencies, is relevant since the herbicide can be frequently found in the environment at these concentrations. In fact, water samples collected from irrigated rice field canals and weirs in Brazil had glyphosate concentrations of 144 µg/L (34). In Campeche, Mexico, glyphosate concentrations in groundwater were 1.42 µg/L and were 0.47 µg/L in urine samples from farmers (35). The compound has also been found in a wide range of concentrations varying from 0.15 ppm to 13 ppm (equivalent to 150-13000 µg/L) in many foods. Fruits, fresh vegetables and processed products have 62 to 85 µg/L, a similar dose to the one we used in our experiments (36).

Interestingly, thyroid cells may adapt to the effects of the pesticide, since after 48 h, its harmful effect clearly diminishes. The activation of repair mechanisms may be responsible for damage control (31, 37). In fact, using *Anguilla anguilla* as an exposure model, Marques A et al showed that after the 7th and 14th days of exposure to Roundup®, DNA damage disappeared (38). Other mechanisms, including cell renewal, may justify the decrease in the number of cell deaths observed over time. Additionally, confirming our own data, a study involving the analysis of Roundup® toxicity in fish gill tissue demonstrated that the damage caused by the herbicide decreased as the exposure time increased (39). In addition, the effect of the pesticide may be tissue and/or cell specific. In fact, damage caused by Roundup®

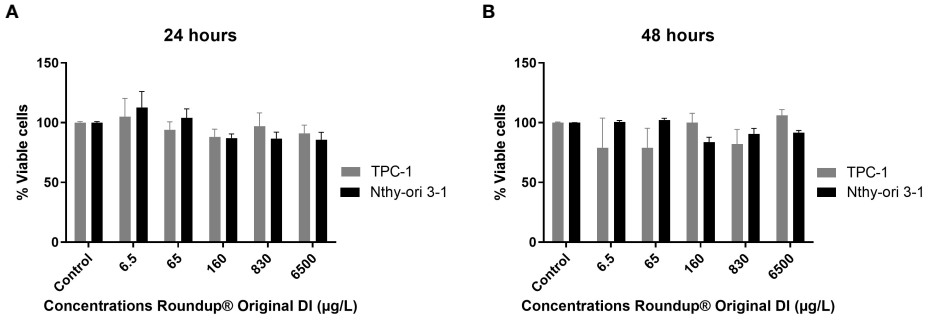


FIGURE 4
Comparison between the percentage of viable TPC-1 and Nthy-ori 3-1 cells at 24 h **(A)** and 48 h **(B)** of exposure to different concentrations of Roundup® Original DI using the CCK-8 assay and presented as mean percentage of viable cells ± SE.

10.3389/fendo.2022.904437

TABLE 2 Percentage of viable Nthy-ori 3-1 and TPC-1 cells after exposure to Roundup® Original DI for 24 h and 48 h at different concentrations, measured by CCK-8 assay.

	Nthy-ori 3-1		TPC-1	
	24 hours	48 hours	24 hours	48 hours
6.5 µg/L	113% ± 13.44	101% ± 1.20	105% ± 15.3	79% ± 24.8
65 µg/L	104% ± 7.55	102% ± 1.73	94% ± 6.6	79% ± 16.2
160 µg/L	87% ± 3.60	84% ± 4.18	88% ± 6.6	100% ± 7.83
830 µg/L	87% ± 5.36	91% ± 4.48	97% ± 11.1	82% ± 12.3
6500 µg/L	86% ± 6.23	92% ± 1.76	91% ± 6.9	106% ± 4.93

Values are presented as mean ± SE.

Transorb in fish erythrocytes was greater after 24 and 96 hours than after 6 hours of exposure. However, in the gill cells, the results were different: at 96 h, there was not much damage, but great destruction was observed after 24 h of exposure (40). Another important issue is that different adjuvants added to the many commercially available glyphosate-derived herbicides can produce different effects, either potentiating the damage produced by glyphosate or, conversely, mitigating its effects. Benachour et al showed that cells derived from human embryos and human placentas manifested greater time-and dose-dependent cytotoxicity and a more potent effect of Roundup® than of the pure glyphosate compound (41).

Remarkably, Roundup Original DI® also produced an increase in the viable cell rate, mainly in TPC-1 cells. This fact has been previously reported in human liver cancer (HepG2) cells after 4 hours of treatment at ADI and REL (residential exposure level) doses, equivalent to 2.91 µg/mL of the active ingredient (31).. Both glyphosate alone and some glyphosate-based herbicides, including Roundup®, have been reported to promote cell proliferation in breast cancer and adenocarcinoma cells (42). Lin and Garry reported 135% ± 3.5 and 126% ± 5.1 breast cancer cell proliferation rates with glyphosate and Roundup®, respectively (43). *In vitro* studies have also shown similar effects on HEC1A endometrial cancer cells (44). In fact, several studies have indicated that low concentrations of glyphosate and glyphosate-based herbicides effectively stimulate cell proliferation, especially in unstable and highly proliferating cells, such as tumor cells (43–46). Since TPC-1 cells are derived from papillary thyroid carcinoma, they harbor mutations, such as *RET/PTC* rearrangement, which confer greater resistance to apoptosis and cell death (47–51). This may explain the higher proliferation rate we observed in TPC-1 cells in comparison with the Nthy-ori 3-1-cell line, especially at 24 h (Table 3). Epidemiological data may be related, at least in part, with this proliferative effect. In fact, small thyroid carcinomas are frequently found in autopsies, and most of them are considered indolent (52). We hypothesize that exposure to endocrine disruptors contributes to the progression of these small lesions, contributing to the dramatic increase in thyroid cancer incidence rates, which parallels the increase in glyphosate-based herbicide usage rates.

It is worth remembering that these results are related to short-term exposure times to glyphosate, as in most studies involving this substance. In addition, the doses we used were limited and small compared to the broad spectrum that some regulatory agencies have established as tolerable for glyphosate in food and animal crops, ranging from 0 1 to 400 parts per million (or 100 to 400,000 ug/L) (53, 54). Also, it is possible that exposure to repeated doses of the chemical over a longer period may induce a cumulative effect with distinct consequences. A study carried out with rats exposed to 10 mg/kg glyphosate 3 times a week for 20 days observed a toxic effect of the chemical in

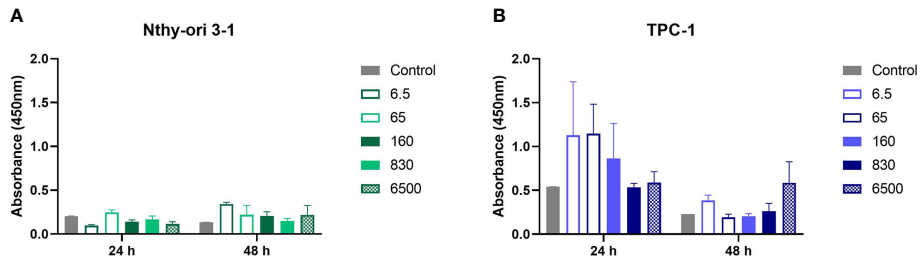


FIGURE 5
TPC-1 **(A)** and Nthy-ori 3-1 **(B)** cells were treated with increasing concentrations of Roundup® Original DI for 24 h and 48 h. Cell proliferation was measured by the BrdU absorbance, and readings were corrected by subtracting the blank results. Data are presented as the mean ± SE.

TABLE 3 Absorbance of BrdU and percentage of Nthy-ori 3-1 and TPC-1 cells after exposure to Roundup® Original DI for 24 h and 48 h at different concentrations.

	Nthy-ori-3-1				TPC-1			
	24 hours		48 hours		24 hours		48 hours	
[]	ABS	%	ABS	%	ABS	%	ABS	%
Control	0.204 ± 0.0	100	0.134 ± 0.0	100	0.541 ± 0.0	100	0.229 ± 0.0	100
6.5 µg/L	0.097 ± 0.01	34	0.341 ± 0.02	321	1.128 ± 0.6	210	0.384 ± 0.6	208
65 µg/L	0.248 ± 0.03	124	0.220 ± 0.10	192	1.146 ± 0.34	214	0.193 ± 0.03	75
160 µg/L	0.140 ± 0.02	60	0.206 ± 0.05	178	0.865 ± 0.39	159	0.206 ± 0.03	85
830 µg/L	0.166 ± 0.04	75	0.147 ± 0.03	114	0.536 ± 0.04	96	0.264 ± 0.09	125
6500 µg/L	0.116 ± 0.02	46	0.218 ± 0.10	190	0.587 ± 0.13	106	0.586 ± 0.24	349

Values are presented as mean ± SE.

liver tissue, which was able to induce cellular oxidative stress and activate apoptosis pathways (55). Another limitation to our study is the fact that we did not confirm our results with other similar assays, although the three assays we employed are robust and reliable. In addition, our data are specific to the cell types employed and may differ in other thyroid cells. They are also specific for the glyphosate-containing product tested (Roundup Original DI®) since other formulations have different adjuvants, which are not always well identified in the product leaflet.

The risk of cancer in humans upon use of glyphosate is still not conclusive, and although the IARC from the World Health Organization (WHO) established glyphosate as a probable human carcinogen (13), the European Food Safety Authority (EFSA) concluded that the herbicide does not prove to be carcinogenic or mutagenic (56). Our data highlight the importance of studying very carefully the implications of various dosages and co-formulants in the pesticide (57). In addition, further epidemiological evidence is urgently needed to evaluate the potential adverse effects of glyphosate products on sensitive human populations, particularly pregnant women, children and individuals with benign thyroid diseases.

Contribution to the field: Glyphosate-based herbicides, such as Roundup®, have environmental dispersion, and their accumulation can cause several effects on thyroid function, but their effects on normal and mutant thyroid cells are still poorly understood. This study shows that Roundup® Original DI acts on normal and thyroid papillary carcinoma cells at various concentrations, evidencing a dual toxic and proliferative effect. This herbicide, widely used around the world, may play a role in the increased incidence rate of thyroid nodules and cancer that has been observed in recent decades.

Data availability statement

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

Author contributions

All authors contributed to the concept and design of this study or to data acquisition and interpretation. All authors contributed to the review of the manuscript and read and approved the submitted version. All authors contributed to the article and approved the submitted version.

Acknowledgments

We are grateful for the contributions of agronomists Guilherme Guimarães and Luis Carlos Castanheira for important discussions, clarifications and technical information and to the biostatistics services of the Faculty of Medical Sciences at UNICAMP. The authors are also grateful to the American Journal Experts for the linguistic services provided and to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grant number 2020/02167-3; and to the Coordination for the Improvement of Higher Education Personnel (CAPES), grant number 88887.465269/2019-00, for the scholarship and financial support. LSW is a Category 1 Research Fellow at the National Council for Scientific and Technological Development (CNPq).

Conflict of interest

LSW is a Category 1 Research Fellow at the National Council for Scientific and Technological Development (CNPq).

The remaining authors declare that the research was carried out in the absence of any commercial or financial relationship that could be interpreted as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their

affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.904437/full#supplementary-material

References

1. Deng Y, Li H, Wang M, Li N, Tian T, Wu Y, et al. Global burden of thyroid cancer from 1990 to 2017. *JAMA network Open* (2020) 3(6):e208759. doi: 10.1001/jamanetworkopen.2020.8759

2. Xu B, Ghossein R. Evolution of the histologic classification of thyroid neoplasms and its impact on clinical management. *Eur J Surg Oncol J Eur Soc Surg Oncol Br Assoc Surg Oncol* (2018) 44(3):338–47. doi: 10.1016/j.ejso.2017.05.002

3. Tirro E, Martorana F, Romano C, Vitale SR, Motta G, Di Gregorio S, et al. Molecular alterations in thyroid cancer: From bench to clinical practice. *Genes* (2019) 10(9):1–33. doi: 10.3390/genes10090709

4. C. Castro-Correia MF. A influência da exposição ambiental a disruptores endócrinos no crescimento e desenvolvimento de crianças e adolescentes. *Rev Portuguesa Endocrinologia Diabetes e Metabolismo* (2015) 10(2):186–92. doi: 10.1016/j.rpedm.2014.10.002

5. Gore A CD, Doan LL, Merril ML, Patisaul H, Zota. (2014). Introduçaão aos disruptores endoícrinos (DEs): Um guia para governos e organizaçoães de interesse puíblico.

6. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: an endocrine society scientific statement. *Endocrine Rev* (2009) 30(4):293–342. doi: 10.1210/er.2009-0002

7. Ozen S, Darcan S. Effects of environmental endocrine disruptors on pubertal development. *J Clin Res Pediatr endocrinol* (2011) 3(1):1–6. doi: 10.4274/jcrpe.v3i1.01

8. Moraes NV. Exposição ambiental a desreguladores endócrinos: alterações na homeostase dos hormônios esteroidais e tireoideanos. *Rev Bras Toxicol* (2008) 21 (1):1–8.

9. Wilson AG, Thake DC, Heydens WE, Brewster DW, Hotz KJ. Mode of action of thyroid tumor formation in the male long-Evans rat administered high doses of alachlor. *Fundam Appl Toxicol Off J Soc Toxicol* (1996) 33(1):16–23. doi: 10.1006/faat.1996.0138

10. Axelstad M, Hansen PR, Boberg J, Bonnichsen M, Nellemann C, Lund SP, et al. Developmental neurotoxicity of propylthiouracil (PTU) in rats: relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes. *Toxicol Appl Pharmacol* (2008) 232(1):1–13. doi: 10.1016/j.taap.2008.05.020

11. Langer P, Tajtakova M, Kocan A, Trnovec T, Sebokova E, Klimes I. From naturally occurring goitrogens to the effects of anthropogenic endocrine disruptors on the thyroid in Slovakia. *Bratislavske lekarske listy* (2003) 104(3):101–7.

12. Myers JP, Antoniou MN, Blumberg B, Carroll L, Colborn T, Everett LG, et al. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. *Environ Health Global Access Sci source* (2016) 15:19. doi: 10.1186/s12940-016-0117-0

13. IARC. *IARC monographs volume 112: evaluation of five organophosphate insecticides and herbicides* (2015). Available at: https://www.iarc.who.int/wp-content/uploads/2018/07/MonographVolume112.pdf (Accessed 11/16/21).

14. ANVISA. *Monografias autorizadas (G01 – glifosato)*. Available at: http://antigo.anvisa.gov.br/registros-e-autorizacoes/agrotoxicos/produtos/monografia-de-agrotoxicos/autorizadas (Accessed 11/16/2021).

15. Vargas L, Roman ES. Available at: http://www.cnpt.embrapa.br/biblio/do/p_do56.htm (Accessed 09/17/2021). Conceitos e aplicações dos adjuvantes. (Embrapa Trigo. Documentos Online, 56). Passo Fundo: Embrap;, 2006. ISSN 1518-6512.

16. Amarante Junior O, Santos T, Brito NM, Ribeiro ML. Glifosato: propriedades, toxicidade, usos e legislação. *Química Nova* (2002) 25(4):589–93. doi: 10.1590/S0100-40422002000400014

17. Kim YH, Hong JR, Gil HW, Song HY, Hong SY. Mixtures of glyphosate and surfactant TN20 accelerate cell death *via* mitochondrial damage-induced apoptosis and necrosis. *Toxicol Vitro an Int J Published Assoc BIBRA* (2013) 27(1):191–7. doi: 10.1016/j.tiv.2012.09.021

18. Brausch JM, Smith PN. Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, thamnocephalus platyurus. *Arch Environ Contamination Toxicol* (2007) 52 (2):217–21. doi: 10.1007/s00244-006-0151-y

19. Mesnage R, Bernay B, Seralini GE. Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. *Toxicology* (2013) 313 (2-3):122–8. doi: 10.1016/j.tox.2012.09.006

20. Tsui MT, Chu LM. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. *Chemosphere* (2003) 52(7):1189–97. doi: 10.1016/S0045-6535(03)00306-0

21. Munoz JP, Bleak TC, Calaf GM. Glyphosate and the key characteristics of an endocrine disruptor: A review. *Chemosphere* (2021) 270:128619. doi: 10.1016/j.chemosphere.2020.128619

22. Li L, Li H, Zhang J, Gao X, Jin H, Liu R, et al. Bisphenol a at a human exposed level can promote epithelial-mesenchymal transition in papillary thyroid carcinoma harbouring BRAF(V600E) mutation. *J Cell Mol Med* (2021) 25(3):1739–49. doi: 10.1111/jcmm.16279

23. ANVISA. (2018). NOTA TÉCNICA N° 23/2018/SEI/CREAV /GEMAR/GGTOX/DIRE3/ANVISA.

24. Da Silva MD, Peralba MCR, Mattos MLT. Determinação de glifosato e acido aminometilfosfônico em aguas superficiais do arroio passo do pilão. *Pesticidas: Rev Ecotoxicol e Meio Ambiente* (2003) 2003(13):19–8. doi: 10.5380/pes.v13i0.3161

25. Peruzzo PJ, Porta AA, Ronco AE. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environ Pollut* (2008) 156(1):61–6. doi: 10.1016/j.envpol.2008.01.015

26. Connolly A, Basinas I, Jones K, Galea KS, Kenny L, McGowan P, et al. Characterising glyphosate exposures among amenity horticulturists using multiple spot urine samples. *Int J Hyg Environ Health* (2018) 221(7):1012–22. doi: 10.1016/j.ijheh.2018.06.007

27. Rubens Nodari e Sonia Hess. *PARECER TÉCNICO n. 02/2015*. São Paulo: Ministérios Públicos Federal, Estadual, e do Trabalho de Mato Grosso do Sul e de Santa Catarina (2015). Available at: https://wp.ufpel.edu.br/ccz/files/2018/05/Santo-Andre-agua-parecer-Sonia-Hess-03-outubro.pdf.

28. Guyton ACH, J E. *Tratado de fisiologia médica. 12ª Ed* Vol. 2011. R De Janeiro, editor. Rio de Jaeiro Elsevier (2011). p. 304.

29. Zoeller RT, Vandenberg LN. Assessing dose-response relationships for endocrine disrupting chemicals (EDCs): a focus on non-monotonicity. *Environ Health Global Access Sci source* (2015) 14:42. doi: 10.1186/s12940-015-0029-4

30. Mladinic M, Berend S, Vrdoljak AL, Kopjar N, Radic B, Zeljezic D. Evaluation of genome damage and its relation to oxidative stress induced by glyphosate in human lymphocytes in vitro. *Environ Mol Mutagenesis* (2009) 50 (9):800–7. doi: 10.1002/em.20495

31. Kasuba V, Milic M, Rozgaj R, Kopjar N, Mladinic M, Zunec S, et al. Effects of low doses of glyphosate on DNA damage, cell proliferation and oxidative stress in the HepG2 cell line. *Environ Sci pollut Res Int* (2017) 24(23):19267–81. doi: 10.1007/s11356-017-9438-y

32. Chaufan G, Coalova I, Rios de Molina Mdel C. Glyphosate commercial formulation causes cytotoxicity, oxidative effects, and apoptosis on human cells: differences with its active ingredient. *Int J Toxicol* (2014) 33(1):29–38. doi: 10.1177/1091581813517906

33. Gao H, Chen J, Ding F, Chou X, Zhang X, Wan Y, et al. Activation of the n-methyl-d-aspartate receptor is involved in glyphosate-induced renal proximal tubule cell apoptosis. *J Appl Toxicol JAT* (2019) 39(8):1096–107. doi: 10.1002/jat.3795

34. Mattos M PM, Dias S, Prata F, Camrgo L. Monitoramento ambiental do glifosato e do seu metabólito (ácido aminometilfosfônico) na água de lavoura de arroz irrigado. *Pesticidas: Rev Ecotoxicol e Meio Ambiente* (2002) 12:145–54. doi: 10.5380/pes.v12i0.3156

35. Rendon-von Osten J, Dzul-Caamal R. Glyphosate residues in groundwater, drinking water and urine of subsistence farmers from intensive agriculture localities: A survey in hopelchen, campeche, Mexico. *Int J Environ Res Public Health* (2017) 14(6):1–13. doi: 10.3390/ijerph14060595

36. Kolakowski BM, Miller L, Murray A, Leclair A, Bietlot H, van de Riet JM. Analysis of glyphosate residues in foods from the Canadian retail markets between 2015 and 2017. *J Agric Food Chem* (2020) 68(18):5201–11. doi: 10.1021/acs.jafc.9b07819

37. Moustacchi E. DNA Damage and repair: consequences on dose-responses. *Mutat Res* (2000) 464(1):35–40. doi: 10.1016/S1383-5718(99)00164-3

38. Marques A, Guilherme S, Gaivao I, Santos MA, Pacheco M. Progression of DNA damage induced by a glyphosate-based herbicide in fish (Anguilla anguilla) upon exposure and post-exposure periods–insights into the mechanisms of genotoxicity and DNA repair. *Comp Biochem Physiol Toxicol Pharmacol CBP* (2014) 166:126–33. doi: 10.1016/j.cbpc.2014.07.009

39. Guilherme S, Gaivao I, Santos MA, Pacheco M. DNA Damage in fish (Anguilla anguilla) exposed to a glyphosate-based herbicide – elucidation of organ-specificity and the role of oxidative stress. *Mutat Res* (2012) 743(1-2):1–9. doi: 10.1016/j.mrgentox.2011.10.017

40. Moreno NC, Sofia SH, Martinez CB. Genotoxic effects of the herbicide roundup transorb and its active ingredient glyphosate on the fish prochilodus lineatus. *Environ Toxicol Pharmacol* (2014) 37(1):448–54. doi: 10.1016/j.etap.2013.12.012

41. Benachour N, Sipahutar H, Moslemi S, Gasnier C, Travert C, Seralini GE. Time- and dose-dependent effects of roundup on human embryonic and placental cells. *Arch Environ Contamination Toxicol* (2007) 53(1):126–33. doi: 10.1007/s00244-006-0154-8

42. Mesnage R, Phedonos A, Biserni M, Arno M, Balu S, Corton JC, et al. Evaluation of estrogen receptor alpha activation by glyphosate-based herbicide constituents. *Food Chem Toxicol an Int J Published Br Ind Biol Res Assoc* (2017) 108 (Pt A):30–42. doi: 10.1016/j.fct.2017.07.025

43. Lin N, Garry VF. *In vitro* studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in red river valley, Minnesota. *J Toxicol Environ Health Part A* (2000) 60(6):423–39. doi: 10.1080/00984100050033494

44. De Almeida LKS, Pletschke BI, Frost CL. Moderate levels of glyphosate and its formulations vary in their cytotoxicity and genotoxicity in a whole blood model and in human cell lines with different estrogen receptor status. *3 Biotech* (2018) 8 (10):438. doi: 10.1007/s13205-018-1464-z

45. Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Seralini GE. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology* (2009) 262(3):184–91. doi: 10.1016/j.tox.2009.06.006

46. Thongprakaisang S, Thiantanawat A, Rangkadilok N, Suriyo T, Satayavivad J. Glyphosate induces human breast cancer cells growth *via* estrogen receptors. *Food Chem Toxicol an Int J Published Br Ind Biol Res Assoc* (2013) 59:129–36. doi: 10.1016/j.fct.2013.05.057

47. Saiselet M, Floor S, Tarabichi M, Dom G, Hebrant A, van Staveren WC, et al. Thyroid cancer cell lines: an overview. *Front endocrinol* (2012) 3:133. doi: 10.3389/fendo.2012.00133

48. Santoro M, Melillo RM, Carlomagno F, Fusco A, Vecchio G. Molecular mechanisms of RET activation in human cancer. *Ann New York Acad Sci* (2002) 963:116–21. doi: 10.1111/j.1749-6632.2002.tb04102.x

49. Prete A, Borges de Souza P, Censi S, Muzza M, Nucci N, Sponziello M. Update on fundamental mechanisms of thyroid cancer. *Front endocrinol* (2020) 11:102. doi: 10.3389/fendo.2020.00102

50. Haroon Al Rasheed MR, Xu B. Molecular alterations in thyroid carcinoma. *Surg Pathol Clinics* (2019) 12(4):921–30. doi: 10.1016/j.path.2019.08.002

51. Abdullah MI, Junit SM, Ng KL, Jayapalan JJ, Karikalan B, Hashim OH. Papillary thyroid cancer: Genetic alterations and molecular biomarker investigations. *Int J Med Sci* (2019) 16(3):450–60. doi: 10.7150/ijms.29935

52. Ramos AM, Sales Ade O, Barbalho de Mello LE, Cirino de Andrade M, Pinto Paiva F, Ramos CC, et al. Absence of peritumoral fibrosis or inflammatory infiltrate may be related to clinical progression of papillary thyroid microcarcinoma. *Int J Surg Pathol* (2009) 17(6):432–7. doi: 10.1177/1066896909333749

53. Cuhra M, Bøhn T, Cuhra P. Glyphosate: Too much of a good thing? *Front Environ Sci* (2016) 4. doi: 10.3389/fenvs.2016.00028

54. European Food Safety A. Review of the existing maximum residue levels for glyphosate according to article 12 of regulation (EC) no 396/2005 - revised version to take into account omitted data. *EFSA J Eur Food Saf Authority* (2019) 17(10): e05862. doi: 10.2903/j.efsa.2019.5862

55. Astiz M, de Alaniz MJ, Marra CA. Effect of pesticides on cell survival in liver and brain rat tissues. *Ecotoxicol Environ Safety* (2009) 72(7):2025–32. doi: 10.1016/j.ecoenv.2009.05.001

56. (EFSA) EFSA. Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. *EFSA J* (2015) 13:4302. doi: 10.2903/j.efsa.2017.4979

57. Lorenz V, Rossetti MF, Dallegrave E, Milesi MM, Varayoud J. Editorial: Glyphosate herbicide as endocrine disruptor and probable human carcinogen: Current knowledge and future direction. *Front endocrinol* (2021) 12:772911. doi: 10.3389/fendo.2021.772911

frontiers | Frontiers in Endocrinology

TYPE Systematic Review
PUBLISHED 23 September 2022
DOI 10.3389/fendo.2022.971213

OPEN ACCESS

EDITED BY
Janete Maria Cerutti,
Federal University of São Paulo, Brazil

REVIEWED BY
Rosario Le Moli,
University of Catania, Italy
Tereza Grimmichová,
Institute of Endocrinology (Prague), Czechia

*CORRESPONDENCE
Hao Zhang
haozhang@cmu.edu.cn

SPECIALTY SECTION
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 16 June 2022
ACCEPTED 05 September 2022
PUBLISHED 23 September 2022

CITATION
Dong W-w, Zhang D-L, Wang Z-H, Lv C-Z, Zhang P and Zhang H (2022) Different types of diabetes mellitus and risk of thyroid cancer: A meta-analysis of cohort studies. *Front. Endocrinol.* 13:971213. doi: 10.3389/fendo.2022.971213

Different types of diabetes mellitus and risk of thyroid cancer: A meta-analysis of cohort studies

Wen-wu Dong, Da-Lin Zhang, Zhi-Hong Wang, Cheng-Zhou Lv, Ping Zhang and Hao Zhang*

Department of Thyroid Surgery, The First Hospital of China Medical University, Shenyang, China

Objective: Sex-specific thyroid cancer risk exists in patients diagnosed with diabetes mellitus (DM). However, thyroid cancer risk in different types of DM is still unclear. This meta-analysis aims to identify the real correlation between different types of DM and thyroid cancer risk in both sexes.

Methods: Studies were identified by an electronic search of PubMed, EMBASE, and Cochrane Library on 16 January 2022. A random-effects model was used to estimate the relative risks (RRs). The Cochran's Q and I^2 statistics were computed to detect heterogeneity between studies.

Results: In comparison with non-DM counterparts, patients with DM had a 1.32-fold higher risk of thyroid cancer (95% CI, 1.22–1.44) with 1.26-fold (95% CI, 1.12–1.41) in men and 1.36-fold (95% CI, 1.22–1.52) in women, respectively. Subgroup analysis by the type of DM showed that the RR of thyroid cancer in patients with type 2 diabetes was 1.34 (95% CI, 1.17–1.53) in the study population with 1.32 (95% CI, 1.12–1.54) in men and 1.37 (95% CI, 1.12–1.68) in women, respectively; the RR of thyroid cancer was 1.30 (95% CI, 1.17–1.43) in patients with gestational diabetes; the risk of thyroid cancer in patients with type 1 diabetes was 1.51-fold in women but not in men. Although there were some heterogeneities, it did not affect the above results of this study.

Conclusion: This study indicates that, compared with non-DM individuals, patients with any type of DM have an elevated thyroid cancer risk. This positive correlation between type 2 diabetes and thyroid cancer risk exists in both men and women.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/, CRD42022304028.

KEYWORDS

diabetes mellitus, thyroid cancer, meta-analysis, cohort study, risk

Introduction

The incidence of thyroid cancer has increased greatly in the past 3 decades (1).Thyroid cancer is the most popular malignancy in endocrine system, accounting for an estimated 43,800 new cases with threefold higher overall incidence rates in American women in 2022 (2). On the basis of the latest Chinese cancer statistics, thyroid cancer is the fourth most frequent cancer in women with its incidence rising by 12.4% every year (3, 4). The rapidly increased incidence of thyroid cancer is considered to be predominantly owing to overdiagnosis. However, it is not yet clear whether the epidemic of thyroid cancer is also caused by exposure to certain risk factors (5).

Risk factors of thyroid cancer have not yet been established. Ionizing radiation and family history of thyroid cancer are the accepted risk factors of thyroid cancer. Additional potential risk factors include obesity and reproductive and environmental factors (6–8). The potential impact of other risk factors for thyroid carcinogenesis warrants further exploration. Here, we explore the possible impact of diabetes mellitus (DM) on subsequent thyroid cancer.

DM represents one of the most rapidly increasing global public health problems. The worldwide prevalence of DM is estimated to grow from 2.8% to 4.4% between 2000 and 2030 and projected to be one of the five leading contributors to disease burden by 2030 (9). China has the largest number of DM people in the world with the rise in prevalence from 0.9% in 1980 to 10.9% in 2013 and more than 90% of whom have type 2 diabetes (T2D) (10, 11). DM has been positively associated with the risk of cancer at different sites, including prostate, oral, breast, and pancreas (12–15). The prevalence of both DM and thyroid cancer has increased all over the world, providing theoretic support that DM may be a driver for thyroid cancer, although early studies did not find an association between them (16). The insufficient cancer cases among the exposed group might account for the lack of association. Furthermore, all of these studies were launched in the USA, possibly leading to the insufficiency of the heterogeneity of exposure and the power of statistics. Two recent meta-analyses have illustrated that the risk of thyroid cancer in female patients with DM has significantly increased in comparison with their non-diabetic counterparts (17, 18). However, there are still some shortcomings in these studies. First, the type of DM was not differentiated and thyroid cancer risk caused by various kinds of DM could not be highlighted. Second, the previous meta-analysis included patients with metabolic syndrome, which may lead to confounding bias. Furthermore, the correlation between DM and thyroid cancer in male patients is not clear, owing to the small sample size. Thus, this meta-analysis was designed to determine the association between various kinds of DM and the risk of thyroid cancer and whether the discrepancy exists in sex based on the available cohort studies.

Methods

This study was designed and carried out according to the guidelines for the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020) (19). The protocol has been registered in the International Prospective Register of Systematic Reviews platform. The registration number of this meta-analysis is CRD42022304028.

Search strategy

A literature search-up was conducted using Cochrane Library, EMBASE, and PubMed and without language restrictions from databases on 16 January 2022. Medical Subject Heading (MESH) terms and keywords were ("Diabetes Mellitus" OR "Diabetes") AND ("Thyroid Tumor" OR "Thyroid Neoplasm*" OR "Thyroid Adenoma*" OR "Thyroid Cancer*" OR "Thyroid Carcinoma*"). Furthermore, a manual screening of the reference lists of selected studies was also conducted to find extra potentially relevant studies. The integrated search strategy is shown in the Appendix (Table 1–3).

Eligibility criteria

Studies were included if the following criteria were met (1): cohort studies, whether prospective or retrospective (2); the exposed group could be patients with any type of DM, the most common being T2D, T1D, or gestational diabetes (GD), and the control group consisted of non-DM individuals (3); thyroid cancer risk as the outcome that expressed as a hazard ratio (HR), adjusted odds ratio (OR), relative risk (RR), or standardized incidence ratio (SIR); (4) thyroid cancer should be a risk occurring naturally under observation, and drug intervention to reduce thyroid cancer risk was not considered.

Exclusion criteria

The exclusion criteria were (1) conference abstracts or erratum, (2) duplicate published studies based on the same observation population, and (3) incomplete data or no interested outcome.

Research selection

The eligible studies were independently screened by two investigators (Wenwu Dong and Dalin Zhang). First, they excluded duplicate and irrelevant literatures by title and abstract. Then, each of them independently read the full text

of each potentially eligible article and finally identified all studies. Any disagreement was resolved by discussion with a third investigator (Hao Zhang) until consensus was achieved.

Data extraction

Two investigators (Wenwu Dong and Dalin Zhang) independently extracted data. The following pertinent information was included: name of first author, country, publication year, sample size, DM type, cases of thyroid cancer, mean age at baseline, follow-up years, diagnosis criteria of thyroid cancer, and adjusted confounders (20).

Risk-of-bias assessment

The quality of the selected studies was assessed on the basis of the Newcastle-Ottawa Quality Assessment Scale (NOS). The range of scores was from 0 to 9, and the higher the score, the higher the study quality. NOS scores ≥ 7, 4–6, and 0–3 mean high, medium, and low quality, respectively.

Statistical analysis

SIR, RR, HR, or adjusted OR and 95% confidence intervals (CI) were extracted to estimate thyroid cancer risk in different types of DM. Because of the low attack rate of thyroid cancer, RR is approximately equal to OR, HR, and SIR, so a pooled analysis can be performed. Heterogeneity was assessed using the chi-square test and I^2 value. *P*-value < 0.1 or I^2 > 50% was considered to indicate significant heterogeneity, and random-effects model was adopted. Otherwise, a fixed-effects model was employed. The sensitivity analysis was conducted to examine the robustness of our results. Subgroup analysis was conducted for different types of DM in different sex. Finally, funnel plots and Egger's regression test were conducted to assess publication bias. A two-sided *P*-value less than 0.05 was considered significant. Statistical analyses were performed using the Stata software (version 15.1).

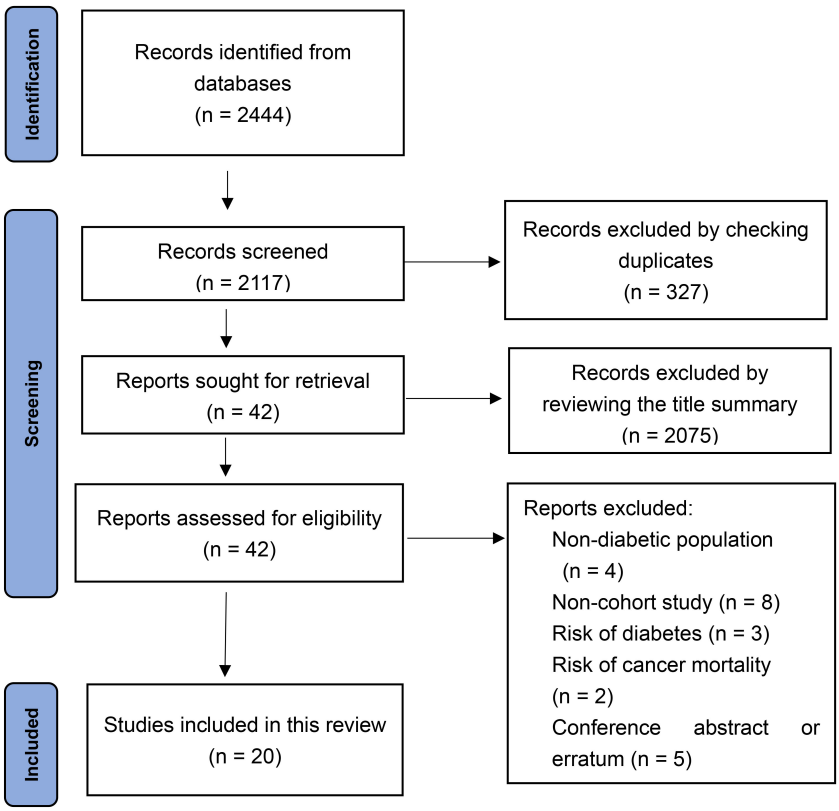


FIGURE 1
Flowchart of study screening.

Results

Literature search

From the electronic search, a total of 2,444 records were identified. A total of 327 articles were excluded by checking duplicates. Another 2,075 articles were excluded after reviewing the title summary. The 42 remaining articles were examined in full text. Finally, 20 cohort studies were included according to the inclusion criteria (16, 21–39). The literature search algorithm is shown in Figure 1.

Study characteristics

The included cohort studies (16, 21–39) were published between 1991 and 2019 from nine countries and regions, including Europe, Asia, and America, with the largest number of studies from Asia. Types of DM varied in these cohort studies. T2D (21, 24–26, 36–38) was the most frequently studied, followed by GD (22, 23, 27, 28). Three studies (34, 35, 39) did not identify the type of DM, and only one (29) reported thyroid cancer risk in patients with T1D. The sample size of the included studies was greater than 300,000, and a total of 11,091 cases of thyroid cancer occurred during follow-up. The mean follow-up time ranged from 3.0 to 20.8 years. Most studies on the diagnosis criteria of thyroid cancer were in accordance with International Classification of Diseases (ICD), and the confounding factors (e.g., sex and body mass index) were well controlled. The baseline characteristics of the included studies are presented in Table 1.

Quality assessment

Specific assessments with the NOS scores are shown in Table 1. Six studies with a score of 6 were deemed of moderate quality, and 14 studies with a score of ≥ 7 were classified as high quality. The mean score was seven points, suggesting a high overall quality.

Sex-specific risk of thyroid cancer in any type of DM

A total of 11 (16, 21, 24–26, 29, 32, 34, 35, 37, 39) and 15 (16, 21–29, 32, 34, 35, 37, 39) cohort studies reported that thyroid cancer risk in male and female patients with DM, respectively. The pooled analysis showed that thyroid cancer risk in male patients was [RR = 1.26, 95% CI (1.12, 1.41), I^2 = 36.7%, P = 0.000]; thyroid cancer risk in female patients was [RR = 1.36, 95% CI (1.22, 1.52), I^2 = 76.6%, P = 0.000]; and the total thyroid cancer risk in the overall study populations was [RR = 1.32, 95% CI (1.22, 1.44), I^2 = 68.9%, P = 0.000]. The risk of thyroid cancer in female patients was slightly higher than that in male patients. Forest plots of sex-specific thyroid cancer risk in any type of DM is shown in Figure 2. Because of some heterogeneity, sensitivity analyses were performed by sequentially excluding each study in the pooled analysis. The results did not affect the overall conclusions, suggesting that the conclusions of this study are reliable. Sensitivity analysis plot is in Appendix Figure 1.

Risk of thyroid cancer in T2D

A total of eight (16, 21, 24–26, 32, 37, 39) and seven (16, 21, 24–26, 31, 37) cohort studies reported thyroid cancer risk in male and female patients with T2D, respectively. The pooled analysis showed that thyroid cancer risk in male patients was [RR = 1.32, 95% CI (1.12, 1.54), I^2 = 41.0%, P = 0.001]; thyroid cancer risk in female patients was [RR = 1.37, 95% CI (1.12, 1.68), I^2 = 91.8%, P = 0.002]; and the total thyroid cancer risk in T2D patients was [RR = 1.34, 95% CI (1.17, 1.53), I^2 = 83.5%, P = 0.000]. In T2D, thyroid cancer risk in female patients was slightly higher than that in male patients. Forest plots of thyroid cancer risk in T2D is shown in Figure 3. Because of large heterogeneity, sensitivity analyses were performed by excluding each study sequentially from the pooled analysis, but the overall conclusion was not affected, suggesting that the results of our meta-analysis are robust. Sensitivity analysis plot is presented in Appendix Figure 2.

Risk of thyroid cancer in GD

Four cohort studies (22, 23, 27, 28) reported thyroid cancer risk in GD. The pooled analysis showed that thyroid cancer risk was [RR = 1.30, 95% CI (1.17, 1.43), I^2 = 0.0%, P = 0.000]. Forest plots of thyroid cancer risk in GD is shown in Figure 4.

Risk of thyroid cancer in T1D

Only one cohort study (29) reported that the risk of thyroid cancer increased in patients with T1D. Among them, no association was found between male patients and the risk of thyroid cancer. The risk of thyroid cancer in female patients was 1.51-fold.

Publication bias

No evidence of publication bias was observed in the visual distribution of the funnel plot (Figure 5). The result of Egger's regression tests is P = 0.108 ≥ 0.05, indicating no publication bias in this meta-analysis.

10.3389/fendo.2022.971213

TABLE 1 Basic characteristics of the included cohorts.

Author	Year	Country	Diabetes type	Sample	Thyroid cancer cases	Mean ageat baseline	Mean follow-up years	Diagnosis of thyroid cancer	Confounders adjusted	NOS scores
Linkeviciute (1)	2019	Lithuania	T2D	127,290	232	62.4	6.5	ICD-10	NR	6
Pace (2)	2019	Canada	GD	68,588	221	≤30	13.1	ICD-10	Race, BMI, maternal age at delivery, year of delivery, and ethnicity	7
Qi (3)	2019	China	T2D	410,191	510	61.8	3	ICD-10	Sex and age	7
Saarela (4)	2018	Finland	T2D	428,326	600	≥30	13	ICD-10	NR	6
Peng (5)	2019	China	GD	990,572	1,514	31.61/ 28.83	13	ICD-9	Age, infertility and kidney disease, dyslipidemia, liver disease, and hypertension	8
Fang (6)	2018	China	T2D	51,324	141	60	10	ICD-10	NR	7
Han (7)	2018	Korea	GD	102,900	1,953	28.25/ 27.28	10	ICD-10	Smoking, maternal age, FBG, and BMI before pregnancy	8
Carstensen (8)	2016	Denmark	T1D	3.9 million person-years	NR	≤40	20.8	ICD-10/ ICD-7	NR	6
Luo (9)	2016	USA	T2D	147,934	391	63.2/63	15.9	NR	Age, ethnicity, education, smoking status, recreational physical activity, alcohol intake, history of hormone therapy use, and previous thyroid disease	7
Dankner (10)	2016	Israel	T2D	1,152,122	833	21–89	11	ICD-Oncology	Age, ethnicity, and socioeconomic status	8
Bejaimal (11)	2016	Canada	GD	149,049	632	32	10	NR	Number of physician visits in 3 years before the index date and income	6
Xu (12)	2015	China	T2D	36,379	29	58.44/ 59.37	3.78	ICD-10	Age and sex	7
Lo (13)	2012	China	T2D	1,790,868	1,309	60.5/60.4	3.5	ICD-9	Age, sex, urbanization, hypertension, and hyperlipidemia	8
Kitahara (14)	2012	USA	T2D	674,491	818	60	10.5 (median)	ICD-Oncology	Race, education, sex, smoking, marital status, alcohol intake, BMI, cigarette, and cohort	8
Aschebrook-Kilfoy (15)	2011	USA	Untyped diabetes	496,548	525	62	10	ICD-Oncology	Age, sex, smoking status, education, BMI, family history of cancer, and race/ethnicity	7
Atchison (16)	2010	USA	Untyped diabetes	4,501,578	1,053	57.5/51.5	10.5–11.9	ICDA-8/ICD-9	Race, Age, latency, time, and alcohol-related conditions, number of visits, COPD, and obesity	8
Johnson (17)	2011	Canada	T2D	370,200	126	61	4.3	ICD-9	Sex, birth year, and index year	6
Chodick (18)	2010	Israel	T2D	100,595	114	47	8	ICD-9	Age, history of cardiovascular diseases, region, BMI, and SES level	7
Adami (19)	1991	Sweden	Untyped diabetes	51,008	19	≥20	5.2	ICD-7	Age and sex	6
Hemminki (20)	2010	Sweden	T2D	125,126	71	≥39	5	ICD-7~10	Obesity	7

ICD, International Classification of Diseases; T2D, type 2 diabetes; T1D, type 1 diabetes; GD, gestational diabetes; NR, no report; FBG, fasting blood glucose; BMI, body mass index; COPD, chronic obstructive pulmonary disease.

Discussion

This meta-analysis of 20 cohort studies included more than 300,000 individuals, providing evidence that thyroid cancer risk increased approximately 30% in DM for the entire study populations, with a 36% increase among female patients and a 26% increase among male patients. The increased thyroid cancer risk varies among patients with different kinds of DM with 34%, 30%, and 51% in patients with T1D, GD, and T2D, respectively. Unexpectedly, the correlation between T2D and thyroid cancer risk existed not only in female patients but also in male patients. This contradicts previous reports that speculated that this

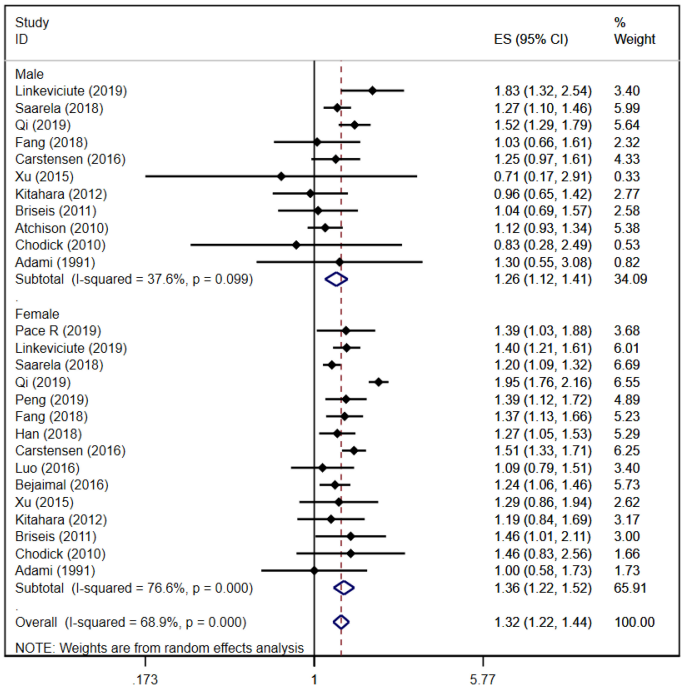


FIGURE 2
Forest plots of sex-specific thyroid cancer risk in any type of diabetes.

correlation between DM and thyroid cancer was prominent in female patients but not in male patients (17, 18). Our positive findings in men were mainly due to the more accurate patient enrolment, the subgroup analysis of different types of DM and the enlargement of sample size.

The global prevalence of DM in 2017 was 8.8% with a further increase expected to 9.9% by the year 2045, nearly 90% of which was T2D (40). Our findings indicated that T2D was a risk factor for thyroid cancer in both male and female patients, and therefore, male sex was not a protective factor for thyroid cancer in patients with T2D. However, the association between T1D and the risk of thyroid cancer was evident in female patients but not in male patients reported by only one cohort study (29). Thus, more research needs to be undertaken to confirm the findings and to explore the underlying molecular mechanisms. GD was also a highly prevalent condition affecting 9.3%–25.5% of pregnant women (41). Thus, pregnant women were potential susceptible population of thyroid cancer. Understanding potential sex discrepancy in risk of thyroid cancer is critical from both a population health and clinical perspective. From the perspective of public health, assessment of sex differences sends messages to targeted public health and is conducive to draw projections of the future disease burden of thyroid cancer and estimating relevant public health costs. Clinically, knowledge of sex differences may contribute to patient selection for thyroid cancer screening.

Some molecular biological mechanisms may explain this correlation. First, chronic elevated insulin levels observed in patients with DM may influence thyroid cancer risk, which was mediated by insulin receptors overexpressed in cancer cells and tissues (42). Insulin may activate insulin and insulin-like growth factor-1 (IGF-1) pathway to inhibit cell apoptosis and promote proliferation. Insulin may also activate mitogen-activated protein kinase (MAPK) and the phosphatidylinositide 3-kinase pathways by mimicking IGF-1 and binding to IGF-1 receptor to promote thyroid carcinogenesis (43). This also reflects that the presence of T2D is associated with insulin resistance, leptin resistance, increased oxidized low-density lipoprotein cholesterol, and obesity, which are present, but to a lesser extent, in T1D. Furthermore, the majority of tumors occurred in patients with T2D, postulating that cancer mainly exists in the older people, in which T2D is more frequent (44). Therefore, an increase in thyroid cancer incidence in T1D may be an artefact of diagnosis because clinicians may pay more medical attention to these patients than non-DM individuals (29). In addition, exogenous insulin is an absolute demand for patients with T1D because they lack endogenous insulin. Moreover, unlike patients with T2D, patients with T1D lack a long prediabetes and diabetes history with compensatory endogenous hyperinsulinemia. As hyperinsulinemia decreases IGF-binding protein–1 (IGFBP-1) and IGFBP-2 levels, insulin may reduce certain IGFBP levels and increase bioavailable IGF levels to indirectly enhance the

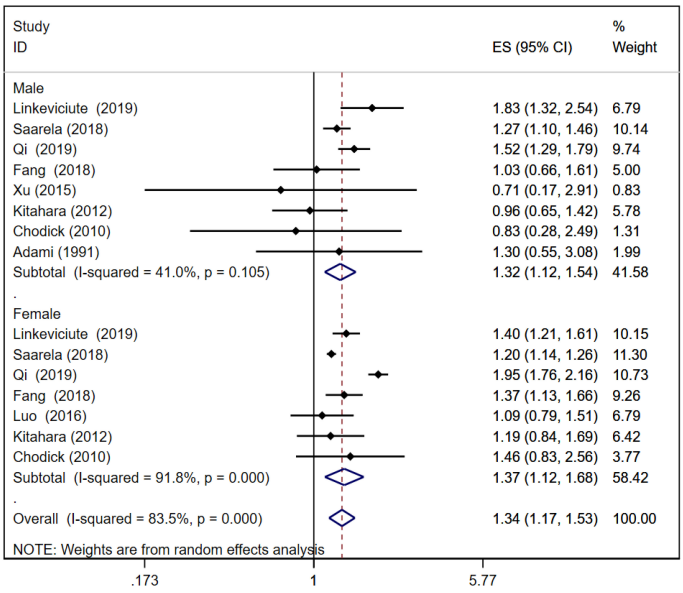


FIGURE 3
Forest plots of thyroid cancer risk in type 2 diabetes.

IGF–IGF-1R signaling pathway. In the situation of diabetes, hyperinsulinemia may therefore directly enhance tumor growth and progression or indirectly promote malignant transformation through IGF-1 signaling (45). Second, the increase of TSH levels is three times more common in patients with D2M than in those without DM (46). Chronic higher level of serum TSH was related to an elevated likelihood of differentiated thyroid cancer and more aggressive tumor stage (47). Interestingly, patients with malignancy may have normal TSH levels, whereas patients with benign tumors often have low TSH levels caused by toxic nodules, goiter, and Graves' disease. Thyroid hormones have been implicated in promoting thyroid inflammation and thyroid cancer through genomic and non-genomic (membrane integrin receptor) actions. Genomic action of thyroid hormones promotes thyroid carcinogenesis by binding to specific nuclear receptors, but accumulated evidence suggests that the activation of the MAPK signaling pathway is the pathophysiological mechanism, which has been noted in the pathogenesis of papillary thyroid carcinoma. Recently, a novel pathway mediated by a membrane receptor located in integrin αVβ3 has been revealed. The proliferative and angiogenic effects of THs have been postulated through this mechanism. It is not yet clear whether the tumorigenic action noted in other neoplasms may play a role on DTC (48). Third, increased oxidative stress caused by hyperglycemia influences tumor cell growth and proliferation (17). Patients with DM suffered from increased permanent pro-

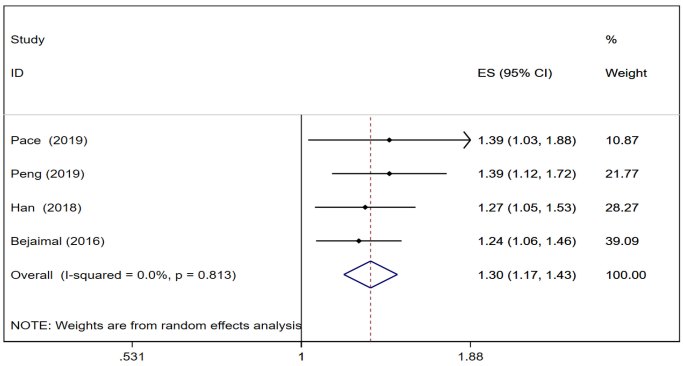


FIGURE 4
Forest plots of thyroid cancer risk in gestational diabetes.

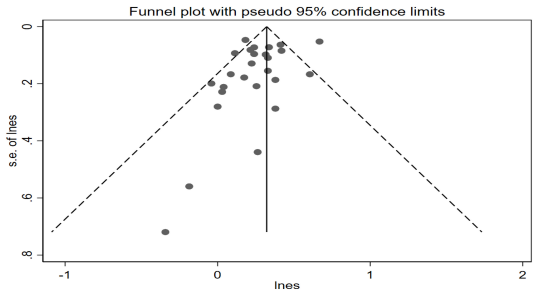


FIGURE 5
Funnel plot of risk of thyroid cancer in diabetes.

inflammatory and oxidative stress caused by metabolic abnormalities. The intracellular anti-oxidant capacity reduced by prolonged inflammatory responses may increase the risk of carcinogenesis of susceptible cells (49). Increased oxidative stress determines thyroid cell inflammatory effects through Toll-like receptor (TLR) activation. Increased TLR expression, activation, and signaling adaptor molecules were found in peripheral blood mononuclear cells from patients with autoimmune thyroid disease (AITD), and TLRs may participate in the pathogenesis of AITD. A significant elevation in TLR endogenous ligands was also observed in the serum of AITD group (50). Moreover, accumulating evidence indicates the association between overactivation of TLR signaling and thyroid cancer progression (51, 52). This reveals the molecular mechanism of thyroid carcinogenesis caused by hyperglycemia to a certain extent. Finally, 70% of patients with DM have vitamin D deficiency (53). Inactivation of deiodinase II (DIO2) enzyme by a Vitamin D deficient environment in patients with DM leads to decreased glucose transporter 4 (GLUT4) transcription by skeletal muscle and adipose tissue, thus resulting in insulin resistance and thyroid carcinogenesis (34, 53).

The strengths of this study should be highlighted as follows: the analyses were limited to cohort studies, which could minimize the influence of biases such as recall and selection biases; the large sample size provided powerful statistical power for quantitative analysis of this correlation between DM and thyroid cancer risk, obtaining more robust results than any single study; subgroup analyses by type of DM and sex were performed to explore the associations between various kinds of DM and thyroid cancer risk and further investigate sex-specific thyroid cancer risk in any type of DM.

This meta-analysis also had some potential limitations. Because DM is shown in the vast majority of studies as a no or yes variable, it is not possible to assess the severity of DM and further explore its association with thyroid cancer risk. Moreover, we could not distinguish controlled vs. uncontrolled DM, because information on DM treatment was not available. In some studies, potential confounding factors were not adjusted such as obesity and age. Although they were adjusted, the adjustment models varied across the selected studies, which might affect the validity of the results. TSH levels and/or TNM classification or staging of thyroid cancer were not monitored in the original literatures included in this study, we cannot assess whether the difference of TSH levels exists between patients with DM and non-DM counterparts and also cannot explore the correlation between DM and progression of thyroid cancer. We expect this problem to be solved in the prospective studies in the future. Diabetes status was ascertained by a self-report in some previous studies. Thus, prediabetes and T2D can be misleading and undiagnosed glucose disorders account for 45.8% on a global scale. However, because of wide area coverage (nine countries), the large sample size (greater than 300,000, and a total of 11,091 cases of thyroid cancer), and the recent literatures accounting for the majority, the impact of this problem is minimized. We believe that our conclusion is still valid.

Conclusion

Our results provided evidence that T2D could increase thyroid cancer risk in both sexes, underlining the demand for developing management and prevention strategies to mitigate thyroid cancer risk in any individual with T2D. Patients with GD are also at high risk for thyroid cancer. Further study is warranted to explore the association between T1D and thyroid cancer risk. Thus, given the increased thyroid cancer risk in patients with DM, DM prevention should be encouraged in all individuals, especially in the high-risk group.

Data availability statement

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

Author contributions

HZ and W-WD conceptualized the research. W-WD and D-LZ conducted statistical analysis. Z-HW, C-ZL, and PZ contributed to data interpretation. W-WD wrote the manuscript draft. All authors contributed to the draft revision and approved the final draft of the manuscript.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.971213/full#supplementary-material

References

1. Miranda-Filho A, Lortet-Tieulent J, Bray F, Cao B, Franceschi S, Vaccarella S, et al. Thyroid cancer incidence trends by histology in 25 countries: a population-based study. *Lancet Diabetes Endocrinol* (2021) 9(4):225–34. doi: 10.1016/s2213-8587(21)00027-9

2. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. *CA Cancer J Clin* (2022) 72(1):7–33. doi: 10.3322/caac.21708

3. Wang J, Yu F, Shang Y, Ping Z, Liu L. Thyroid cancer: incidence and mortality trends in China, 2005-2015. *Endocrine* (2020) 68(1):163–73. doi: 10.1007/s12020-020-02207-6

4. Zhang S, Sun K, Zheng R, Zeng H, Wang S, Chen R, et al. Cancer incidence and mortality in China, 2015. *J Natl Cancer Center* (2021) 1(1):2–11. doi: 10.1016/j.jncc.2020.12.001

5. Li M, Brito JP, Vaccarella S. Long-term declines of thyroid cancer mortality: An international age-Period-Cohort analysis. *Thyroid* (2020) 30(6):838–46. doi: 10.1089/thy.2019.0684

6. Haymart MR. Progress and challenges in thyroid cancer management. *Endocr Pract* (2021) 27(12):1260–3. doi: 10.1016/j.eprac.2021.09.006

7. Fiore M, Oliveri Conti G, Caltabiano R, Buffone A, Zuccarello P, Cormaci L, et al. Role of emerging environmental risk factors in thyroid cancer: A brief review. *Int J Environ Res Public Health 16(7)* (2019) 16(7):1185. doi: 10.3390/ijerph16071185

8. Mannathazhathu AS, George PS, Sudhakaran S, Vasudevan D, Krishna Km J, Booth C, et al. Reproductive factors and thyroid cancer risk: Meta-analysis. *Head Neck* (2019) 41(12):4199–208. doi: 10.1002/hed.25945

9. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* (2004) 27 (5):1047–53. doi: 10.2337/diacare.27.5.1047

10. Jia W, Weng J, Zhu D, Ji L, Lu J, Zhou Z, et al. Standards of medical care for type 2 diabetes in China 2019. *Diabetes Metab Res Rev* (2019) 35(6):e3158. doi: 10.1002/dmrr.3158

11. Ma RCW. Epidemiology of diabetes and diabetic complications in China. *Diabetologia* (2018) 61(6):1249–60. doi: 10.1007/s00125-018-4557-7

12. Jian Gang P, Mo L, Lu Y, Runqi L, Xing Z. Diabetes mellitus and the risk of prostate cancer: an update and cumulative meta-analysis. *Endocr Res* (2015) 40 (1):54–61. doi: 10.3109/07435800.2014.934961

13. Gong Y, Wei B, Yu L, Pan W. Type 2 diabetes mellitus and risk of oral cancer and precancerous lesions: a meta-analysis of observational studies. *Oral Oncol* (2015) 51(4):332–40. doi: 10.1016/j.oraloncology.2015.01.003

14. Zhou Y, Zhang X, Gu C, Xia J. Diabetes mellitus is associated with breast cancer: systematic review, meta-analysis, and in silico reproduction. *Panminerva Med* (2015) 57(3):101–8.

15. Song S, Wang B, Zhang X, Hao L, Hu X, Li Z, et al. Long-term diabetes mellitus is associated with an increased risk of pancreatic cancer: A meta-analysis. *PloS One* (2015) 10(7):e0134321. doi: 10.1371/journal.pone.0134321

16. Kitahara CM, Platz EA, Beane Freeman LE, Black A, Hsing AW, Linet MS, et al. Physical activity, diabetes, and thyroid cancer risk: a pooled analysis of five prospective studies. *Cancer Causes Control* (2012) 23(3):463–71. doi: 10.1007/s10552-012-9896-y

17. Yeo Y, Ma SH, Hwang Y, Horn-Ross PL, Hsing A, Lee KE, et al. Diabetes mellitus and risk of thyroid cancer: a meta-analysis. *PloS One* (2014) 9(6):e98135. doi: 10.1371/journal.pone.0098135

18. Li H, Qian J. Association of diabetes mellitus with thyroid cancer risk: A meta-analysis of cohort studies. *Med (Baltimore)* (2017) 96(47):e8230. doi: 10.1097/md.0000000000008230

19. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *Bmj 372 n71* (2021) 372:n71. doi: 10.1136/bmj.n71

20. Taylor KS, Mahtani KR, Aronson JK. Summarising good practice guidelines for data extraction for systematic reviews and meta-analysis. *BMJ Evid Based Med* (2021) 26(3):88–90. doi: 10.1136/bmjebm-2020-111651

21. Linkeviciute-Ulinskiene D, Patasius A, Zabuliene L, Stukas R, Smailyte G. Increased risk of site-specific cancer in people with type 2 diabetes: A national cohort study. *Int J Environ Res Public Health* (2019) 17(1):246. doi: 10.3390/ijerph17010246

22. Pace R, Rahme E, Dasgupta K. Gestational diabetes mellitus and risk of incident primary cancer: A population-based retrospective cohort study. *J Diabetes* (2020) 12(1):87–90. doi: 10.1111/1753-0407.12988

23. Peng YS, Lin JR, Cheng BH, Ho C, Lin YH, Shen CH, et al. Incidence and relative risk for developing cancers in women with gestational diabetes mellitus: a nationwide cohort study in Taiwan. *BMJ Open* (2019) 9(2):e024583. doi: 10.1136/bmjopen-2018-024583

24. Qi J, He P, Yao H, Song R, Ma C, Cao M, et al. Cancer risk among patients with type 2 diabetes: A real-world study in shanghai, China. *J Diabetes* (2019) 11 (11):878–83. doi: 10.1111/1753-0407.12926

25. Saarela K, Tuomilehto J, Sund R, Keskimäki I, Hartikainen S, Pukkala E. Cancer incidence among Finnish people with type 2 diabetes during 1989-2014. *Eur J Epidemiol* (2019) 34(3):259–65. doi: 10.1007/s10654-018-0438-0

26. Fang Y, Zhang X, Xu H, Smith-Warner SA, Xu D, Fang H, et al. Cancer risk in Chinese diabetes patients: a retrospective cohort study based on management data. *Endocr Connect* (2018) 7(12):1415–23. doi: 10.1530/ec-18-0381

27. Han KT, Cho GJ, Kim EH. Evaluation of the association between gestational diabetes mellitus at first pregnancy and cancer within 10 years postpartum using national health insurance data in south Korea. *Int J Environ Res Public Health 15 (12)* (2018) 15(12):2646. doi: 10.3390/ijerph15122646

28. Bejaimal SA, Wu CF, Lowe J, Feig DS, Shah BR, Lipscombe LL. Short-term risk of cancer among women with previous gestational diabetes: a population-based study. *Diabetes Med* (2016) 33(1):39–46. doi: 10.1111/dme.12796

29. Carstensen B, Read SH, Friis S, Sund R, Keskimäki I, Svensson AM, et al. Cancer incidence in persons with type 1 diabetes: a five-country study of 9,000

cancers in type 1 diabetic individuals. *Diabetologia* (2016) 59(5):980–8. doi: 10.1007/s00125-016-3884-9

30. Dankner R, Boffetta P, Balicer RD, Boker LK, Sadeh M, Berlin A, et al. Time-dependent risk of cancer after a diabetes diagnosis in a cohort of 2. *3 Million Adults. Am J Epidemiol* (2016) 183(12):1098–106. doi: 10.1093/aje/kwv290

31. Luo J, Phillips L, Liu S, Wactawski-Wende J, Margolis KL. Diabetes, diabetes treatment, and risk of thyroid cancer. *J Clin Endocrinol Metab* (2016) 101(3):1243–8. doi: 10.1210/jc.2015-3901

32. Xu HL, Fang H, Xu WH, Qin GY, Yan YJ, Yao BD, et al. Cancer incidence in patients with type 2 diabetes mellitus: a population-based cohort study in shanghai. *BMC Cancer* (2015) 15:852. doi: 10.1186/s12885-015-1887-4

33. Lo SF, Chang SN, Muo CH, Chen SY, Liao FY, Dee SW, et al. Modest increase in risk of specific types of cancer types in type 2 diabetes mellitus patients. *Int J Cancer* (2013) 132(1):182–8. doi: 10.1002/ijc.27597

34. Aschebrook-Kilfoy B, Sabra MM, Brenner A, Moore SC, Ron E, Schatzkin A, et al. Diabetes and thyroid cancer risk in the national institutes of health-AARP diet and health study. *Thyroid* (2011) 21(9):957–63. doi: 10.1089/thy.2010.0396

35. Atchison EA, Gridley G, Carreon JD, Leitzmann MF, McGlynn KA. Risk of cancer in a large cohort of U. *S. veterans diabetes. Int J Cancer* (2011) 128(3):635–43. doi: 10.1002/ijc.25362

36. Johnson JA, Bowker SL, Richardson K, Marra CA. Time-varying incidence of cancer after the onset of type 2 diabetes: evidence of potential detection bias. *Diabetologia* (2011) 54(9):2263–71. doi: 10.1007/s00125-011-2242-1

37. Chodick G, Heymann AD, Rosenmann L, Green MS, Flash S, Porath A, et al. Diabetes and risk of incident cancer: a large population-based cohort study in Israel. *Cancer Causes Control* (2010) 21(6):879–87. doi: 10.1007/s10552-010-9515-8

38. Hemminki K, Li X, Sundquist J, Sundquist K. Risk of cancer following hospitalization for type 2 diabetes. *Oncologist* (2010) 15(6):548–55. doi: 10.1634/theoncologist.2009-0300

39. Adami HO, McLaughlin J, Ekbom A, Berne C, Silverman D, Hacker D, et al. Cancer risk in patients with diabetes mellitus. *Cancer Causes Control* (1991) 2 (5):307–14. doi: 10.1007/bf00051670

40. Standl E, Khunti K, Hansen TB, Schnell O. The global epidemics of diabetes in the 21st century: Current situation and perspectives. *Eur J Prev Cardiol* (2019) 26 (2_suppl):7–14. doi: 10.1177/2047487319881021

41. Popova PV, Klyushina AA, Vasilyeva LB, Tkachuk AS, Vasukova EA, Anopova AD, et al. Association of common genetic risk variants with gestational diabetes mellitus and their role in GDM prediction. *Front Endocrinol (Lausanne)* (2021) 12:628582. doi: 10.3389/fendo.2021.628582

42. Hard GC. Recent developments in the investigation of thyroid regulation and thyroid carcinogenesis. *Environ Health Perspect* (1998) 106(8):427–36. doi: 10.1289/ehp.106-1533202

43. Oberman B, Khaku A, Camacho F, Goldenberg D. Relationship between obesity, diabetes and the risk of thyroid cancer. *Am J Otolaryngol* (2015) 36(4):535–41. doi: 10.1016/j.amjoto.2015.02.015

44. Gristina V, Cupri MG, Torchio M, Mezzogori C, Cacciabue L, Danova M. Diabetes and cancer: A critical appraisal of the pathogenetic and therapeutic links. *BioMed Rep* (2015) 3(2):131–6. doi: 10.3892/br.2014.399

45. Belardi V, Gallagher EJ, Novosyadlyy R, LeRoith D. Insulin and IGFs in obesity-related breast cancer. *J Mammary Gland Biol Neoplasia* (2013) 18(3-4):277–89. doi: 10.1007/s10911-013-9303-7

46. Tamez-Pérez HE, Martínez E, Quintanilla-Flores DL, Tamez-Peña AL, Gutiérrez-Hermosillo H, Díaz de León-González E. The rate of primary hypothyroidism in diabetic patients is greater than in the non-diabetic population. an observational study. *Med Clin (Barc)* (2012) 138(11):475–7. doi: 10.1016/j.medcli.2011.08.009

47. Haymart MR, Repplinger DJ, Leverson GE, Elson DF, Sippel RS, Jaume JC, et al. Higher serum thyroid stimulating hormone level in thyroid nodule patients is associated with greater risks of differentiated thyroid cancer and advanced tumor stage. *J Clin Endocrinol Metab* (2008) 93(3):809–14. doi: 10.1210/jc.2007-2215

48. Zafón C. TSH-suppressive treatment in differentiated thyroid cancer. *A dogma under review. Endocrinol Nutr* (2012) 59(2):125–30. doi: 10.1016/j.endonu.2011.10.002

49. Vigneri P, Frasca F, Sciacca L, Pandini G, Vigneri R. Diabetes and cancer. *Endocr Relat Cancer* (2009) 16(4):1103–23. doi: 10.1677/erc-09-0087

50. Peng S, Li C, Wang X. Et al: Increased toll-like receptors activity and TLR ligands in patients with autoimmune thyroid diseases. *Front Immunol 7:578* (2016) 7:578. doi: 10.3389/fimmu.2016.00578

51. McCall KD, Harii N, Lewis CJ. Et al: High basal levels of functional toll-like receptor 3 (TLR3) and noncanonical Wnt5a are expressed in papillary thyroid cancer and are coordinately decreased by phenylmethimazole together with cell proliferation and migration. *Endocrinology* (2007) 148(9):4226–37. doi: 10.1210/en.2007-0459

52. Peyret V, Nazar M, Martín M. Et al: Functional toll-like receptor 4 overexpression in papillary thyroid cancer by MAPK/ERK-induced ETS1 transcriptional activity. *Mol Cancer Res* (2018) 16(5):833–45. doi: 10.1158/1541-7786.MCR-17-0433

53. Shih SR, Chiu WY, Chang TC, Tseng CH. Diabetes and thyroid cancer risk: literature review. *Exp Diabetes Res* (2012) 2012:578285. doi: 10.1155/2012/578285

frontiers | Frontiers in Endocrinology

TYPE Mini Review
PUBLISHED 21 October 2022
DOI 10.3389/fendo.2022.995503

OPEN ACCESS

EDITED BY
Catherine Fiona Sinclair,
Icahn School of Medicine at Mount Sinai, United States

REVIEWED BY
Maria Morel Espinosa,
Centers for Disease Control and Prevention (CDC), United States

*CORRESPONDENCE
Caroline Serrano-Nascimento
caroline.serrano@unifesp.br
Maria Tereza Nunes
mtnunes@icb.usp.br

SPECIALTY SECTION
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 15 July 2022
ACCEPTED 05 October 2022
PUBLISHED 21 October 2022

CITATION
Serrano-Nascimento C and Nunes MT (2022) Perchlorate, nitrate, and thiocyanate: Environmental relevant NIS-inhibitors pollutants and their impact on thyroid function and human health.
Front. Endocrinol. 13:995503.
doi: 10.3389/fendo.2022.995503

Perchlorate, nitrate, and thiocyanate: Environmental relevant NIS-inhibitors pollutants and their impact on thyroid function and human health

Caroline Serrano-Nascimento[1,2]* and Maria Tereza Nunes[3]*

[1]Instituto de Ciências Ambientais, Químicas e Farmacêuticas (ICAQF), Universidade Federal de São Paulo (UNIFESP), Sao Paulo, Brazil, [2]Laboratório de Endocrinologia Molecular e Translacional (LEMT), Universidade Federal de São Paulo, Sao Paulo, Brazil, [3]Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil

Thyroid disruptors are found in food, atmosphere, soil, and water. These contaminants interfere with the thyroid function through the impairment of thyroid hormone synthesis, plasma transport, peripheral metabolism, transport into the target cells, and thyroid hormone action. It is well known that iodide uptake mediated by the sodium-iodide symporter (NIS) is the first limiting step involved in thyroid hormones production. Therefore, it has been described that several thyroid disruptors interfere with the thyroid function through the regulation of NIS expression and/or activity. Perchlorate, nitrate, and thiocyanate competitively inhibit the NIS-mediated iodide uptake. These contaminants are mainly found in food, water and in the smoke of cigarettes. Although the impact of the human exposure to these anions is highly controversial, some studies indicated their deleterious effects in the thyroid function, especially in individuals living in iodine deficient areas. Considering the critical role of thyroid function and the production of thyroid hormones for growth, metabolism, and development, this review summarizes the impact of the exposure to these NIS-inhibitors on thyroid function and their consequences for human health.

KEYWORDS

NIS, perchlorate, nitrate, thiocyanate, thyroid

Introduction

Several chemicals widely present in the environment affect important biological functions through the disruption of the endocrine system. One classical example of these disruptive phenomena was the discovery of intersex fish in English rivers, which was shown to be related to contaminants that interfere with estrogens action (1). Since then, the knowledge in this field has significantly progressed, and many studies have been published about the endocrine disruptors and their impact to human and other animals' health (2). These important studies are now influencing worldwide regulation and public health policies to mitigate the potential deleterious impacts of the endocrine disruptors in the health and survival of different species.

Although there is a massive progress in the description of new molecules with endocrine disrupting properties, one great challenge in this area is to evaluate the consequences of endocrine disruptors mixtures (3) In fact, the exposure of humans and other animals to complex mixtures of endocrine disruptors complicates the determination of safe levels of exposure (4). In addition, many studies demonstrated that the continuous exposure to low doses of the endocrine disruptors affect several physiological functions (5) Thus, there are several aspects that need to be considered to define the real impact of the exposure to these contaminants on the human health.

It is well known that thyroid hormones play crucial roles in the control of metabolism, normal development, growth, and differentiation processes (6). Therefore, great attention has been given to the thyroid disruptors. Indeed, it has been reported that these disruptors affect several steps of thyroid physiology, like thyroid hormones synthesis, transport, action, and peripheral metabolism (7).

This mini review is centered on anions classified as environmental contaminants that affect thyroid hormone synthesis, particularly those that interfere with the activity of sodium iodide symporter (NIS), which mediates the iodide uptake, the first and limiting step for the thyroid hormone synthesis (8)NIS is expressed at the basolateral membrane of the thyroid follicular cells, as well as in non-thyroidal tissues, like mammary, salivary, and lacrimal glands, stomach, and small intestine (9)In summary, the data about the impact of perchlorate, nitrate and thiocyanate exposure on thyroid function and human health will be addressed herein.

Perchlorate

Perchlorate ($ClO4^-$) is a strong oxidizing anion used in rockets fuels, explosives, fireworks, and missile fuels. Perchlorate is also naturally formed in the atmosphere and is accumulated in arid climate regions (10). In fact, human manufacturing of perchlorate-containing products and the naturally formed perchlorate result in a large occurrence of this compound in the environment, as demonstrated by its presence in irrigation water and soil. Perchlorate is also present in some fertilizers, increasing its accumulation in fruits and vegetables (11) Therefore, human exposure to perchlorate mainly occurs from contaminated food and drinking water.

Perchlorate is a potent inhibitor of iodide uptake mediated by NIS in the thyrocytes, impairing the first step of thyroid hormone synthesis (12). This inhibitory effect has been recognized since the 50's, when perchlorate was commonly used as a therapeutic drug for treating hyperthyroidism (13). Moreover, since this anion displaces iodide from the thyroid, it was used in the "perchlorate challenge" test, for the detection of thyroid iodine organification defects (14).

Several studies contributed to characterize the disruptive effects of perchlorate on thyroid function and to reinforce the importance of the regulation of perchlorate levels in the environment. Perchlorate and iodide are anions with similar charge and size. However, it is worth noting that NIS has a higher selectivity for perchlorate than for iodide. In agreement, *in vitro* studies have shown that iodide transport is essentially abolished in thyrocytes exposed to 10 μM of perchlorate, without alterations in the expression of NIS (12) for many years, it was suggested that perchlorate was a potent inhibitor of NIS-mediated iodide uptake without being transported into the thyrocytes (15). However, elegant studies demonstrated that this anion is actively transported by NIS in an electroneutral stoichiometry (16, 17). Besides the potent inhibition of NIS activity, it has been described that perchlorate also suppresses the thyroglobulin and thyroperoxidase gene expression, which was associated with the impairment of the thyroid hormone synthesis induced by this contaminant (18).

The deleterious effect of perchlorate exposure were previously demonstrated in species that depend on thyroid hormone action to drive their metamorphosis processes, as amphibians and fishes. In accordance, several abnormalities in the development, reproduction, and survival were described in perchlorate-exposed animals (19, 20). In contrast, the exposure to perchlorate has not altered the metamorphosis or the thyroid histopathology of common frogs (21).

In humans, the effects of perchlorate on thyroid function are still controversial. Several studies reported that perchlorate exposure was not associated with alterations in TSH or T4 serum levels in humans (22–24). However, other studies have shown significant alterations in the function of the pituitary-thyroid axis in humans co-exposed to perchlorate and other NIS-inhibitory anions, such as nitrate and/or thiocyanate, especially, but not exclusively, in iodine deficient areas (25, 26). A recent study has also indicated that humans co-exposed to perchlorate, nitrate and thiocyanate presented an increased

central thyroid hormone sensitivity, which seem to be more precise than the single parameters, as TSH or T4 serum levels, to evaluate the homeostasis of the pituitary-thyroid axis (27).

It has been suggested that the disruptive actions of perchlorate on thyroid function are more critical during specific windows of susceptibility, as the pregnancy. Even so, the consequences of maternal exposure to perchlorate in the thyroid function are still controversial (28–31). In fact, the different conclusions about the deleterious effects of perchlorate exposure during this critical developmental period are related to the different ranges of exposure in different human populations as well as to the period of the evaluation in each study. Therefore, more studies are needed to further clarify this issue. Conversely, a study focused on pregnant women with borderline thyroid function living in iodine deficient areas demonstrated that perchlorate exposure impaired the offspring cognitive development. This impairment was not reversed by maternal levothyroxine therapy, suggesting that the fetal thyroid function is more susceptible to the perchlorate-induced disruption (32). In agreement, the offspring rats of pregnant rats exposed to perchlorate presented several alterations in the synaptic function during adulthood (33).

An elegant study from the group of Dr. Nancy Carrasco demonstrated that perchlorate is transported to maternal milk through the activity of NIS that is expressed in the mammary glands (17). Consequently, besides the reduction of iodide transferred to the milk, the newborns could be exposed to high levels of this potent NIS inhibitor, which could potentially impair the central nervous system development, since it is highly dependent on thyroid hormone action (34, 35).

Furthermore, studies have suggested a positive association between perchlorate exposure and the risk to develop papillary thyroid cancer (36, 37). As the incidence of thyroid cancer is increasing worldwide, the contribution of the thyroid disruptors, such as the perchlorate, which has a potent disruptive action on NIS activity and on thyroid function should be addressed.

Finally, in rodents, the chronic exposure to ammonium perchlorate through drinking water altered the serum levels of thyroid hormones and TSH serum levels and the morphology of the thyroid gland (38) Our studies reinforced these data and described some of perchlorate-induced molecular mechanisms involved in the disruption of the hypothalamus-pituitary-thyroid axis. Indeed, the animals exposed to perchlorate presented primary hypothyroidism, as shown by the decreased serum T4 and T3 levels, and increased serum TSH concentration. Additionally, the exposure to perchlorate induced alterations in the expression of genes/proteins involved in the thyroid hormone synthesis and increased several markers of inflammation in the exposed animals (39).

Interestingly, it has been shown that TSH increases the NIS-mediated perchlorate transport into the thyroid cells (16). Furthermore, it has been previously described that perchlorate *per se* induces a unique pattern of gene expression alterations in the thyroid gland, that is completely different from the one induced by iodine deficiency (40) Even though, the molecular mechanisms involved in the regulation of thyroid gene expression need to be further clarified.

Thus, although some studies indicate the potential deleterious effects of perchlorate exposure on thyroid function, there are many controversial results, especially in epidemiologic studies. This reinforces the necessity of more studies to clarify the period as well as the doses of exposure to perchlorate that are potentially more harmful to the health of humans and other animals.

Nitrate

Nitrate (NO3-) is a naturally occurring anion in the environment since it is part of the nitrogen cycle. The plants obtain nitrogen, an essential component for the synthesis of plant proteins, through the absorption of nitrate from the soil and the groundwater (41). Therefore, humans are mainly exposed to nitrate through the consumption of green leafy vegetables, roots, oilseeds, grains, tubers, and nuts. Moreover, nitrate is commonly found in agricultural fertilizers and in preservative additives for cured meats. Accordingly, the presence of nitrate in the environment is greater than the one observed for thiocyanate or perchlorate.

The nitrate levels in the drinking water sources and food have significantly increased in recent decades due to the exacerbated use of nitrogen fertilizers. Alarmingly, the effective nitrate removal from water sources depends on complex and highly costly processes, which are rarely performed (42). In agreement, studies suggest that nitrate concentration in food and water sources will highly increase in the future, due to the increased use of nitrogen fertilizers and the intensification of agricultural activities to support human population growth (43). It is worth noting that the maximum contaminant level of nitrate in the drinking water was defined by the U.S. Environmental Protection Agency (EPA) and the World Health Organization (WHO) as 10 mg/L for nitrate-nitrogen, which is equivalent to 45 mg/L as nitrate (44). Nevertheless, it has been demonstrated that some regions of the world present higher concentrations of nitrate in the water, which greatly exceeds the levels considered safe for human exposure (45).

Interestingly, some types of cancer, as gastric and colorectal cancers, were previously associated with the exposure to nitrate at levels that are considered safe by the regulatory agencies (46, 47). The deleterious effects of nitrate have been related to its conversion into other nitrogen-containing compounds in the body (48). Thus, it is well known that nitrate is converted to nitrite, which can subsequently react with amines and amides in the gastrointestinal tract to form N-nitroso compounds (NOCs), a class of known carcinogenic and cytotoxic substances (49).

Additionally, high levels of nitrate consumption were associated with an increased risk for reproductive problems. Both nitrate and nitrites are precursors of nitric oxide (NO), a lipophilic molecule with several physiological roles. However, excessive production of NO was associated with several pathophysiological events, as reproductive system dysfunctions and impaired production of sexual steroids (50, 51). Other studies indicated that the exposure to high levels of nitrate during pregnancy is a risk factor for spontaneous abortion, fetal death, prematurity, intrauterine growth restriction, low birth weight, congenital malformations, and neonatal death (52). Therefore, besides its carcinogenic potential, the deleterious effects of nitrate on the endocrine system have received increasing attention in the recent years (53). In addition to the nitrate-induced damage to other endocrine glands, some studies suggest that nitrate exposure impairs the thyroid function in humans and other animals.

Nitrate competitively inhibits NIS-mediated iodide uptake with a much lower potency than the one induced by perchlorate (12). Nevertheless, the concentration of nitrate detected in human and environmental samples were much higher than those described for perchlorate. This fact could potentially contribute to the harmful effects induced by nitrate in the body.

Although the *in vitro* assays clearly demonstrated the inhibitory effects of nitrate on NIS function, suggesting an impairment of the thyroid function, the effects of nitrate exposure on pituitary-thyroid axis in humans are still controversial. Indeed, nitrate exposure was associated with increased risk of developing thyroid disorders, especially in susceptible individuals as pregnant women, newborns, and children, as well as in women with urinary iodine levels ≥ 100 µg/L (25, 54–56). Furthermore, chronic exposure to high levels of nitrate through public water supplies was associated with increased risk of developing thyroid cancer (57, 58). However, other studies have not detected any alteration in TSH and/or T4 serum levels in nitrate-exposed humans (31, 59).

Although the inhibition of thyroid function by nitrate is reported in the literature, the molecular mechanisms involved in this phenomenon are not completely elucidated. Indeed, the chronic exposure of male rats to high levels of nitrate increased thyroid weight, induced morphological alterations in the thyroid follicles and altered thyroid hormone production (60, 61). In accordance, a goitrogenic effect was also observed in female rats chronically exposed to nitrate through drinking water. However, no alterations in thyroid hormone or TSH serum levels were observed, which were associated with increased expression of genes involved in the synthesis of thyroid hormones (62).

As discussed before, nitrate exposure increases the production of NO in different tissues, which promotes post-translational modifications, such as nitrosylation of cysteine residues and nitration of tyrosine residues that change the stability, location, and activity of several proteins (63, 64). In this sense, in thyrocytes, it has been shown that the excessive production of NO decreases the expression of transcriptional factors, such as *Foxe1*, inhibits NIS-mediated iodide uptake, and interferes with the signaling pathway triggered by TSH, which potentially inhibits the expression of different genes involved in the biosynthesis of thyroid hormones (65–68). Therefore, although some aspects related to the nitrate-induced impairment of the thyroid function have been described, future studies are needed to unravel the molecular mechanisms involved in the direct effects of this anion on the thyroid, as well as the potential deleterious effects of nitrate on the development of the thyroid gland.

Thiocyanate

Thiocyanate (SCN-) is vastly found in food that contain thioglycosides – such as cassava, bamboo shoots, sweet potatoes, brussels sprouts, cauliflower, corn broccoli, apricots, and almonds. The inhalation of cigarette smoke in another importance source of human thiocyanate contamination since it contains cyanide, which is converted into thiocyanate in the body. Thiocyanate is highly soluble in water and previous data demonstrated a relevant contamination of the groundwater with this anion (69). It is worth noting that the half-life of thiocyanate is approximately 6 days, much longer than the few hours half-lives presented by perchlorate and nitrate.

Thiocyanate has a goitrogenic action since it inhibits NIS-mediated iodide uptake (12). Electrophysiological studies indicated that thiocyanate is transported by NIS into the thyrocytes with a similar stoichiometry of I^- (15, 70). Similar to nitrate, the potency of the thiocyanate-mediated inhibition of NIS activity is lower than the one exerted by perchlorate (12). Nevertheless, epidemiologic studies reported higher levels of thiocyanate in comparison to perchlorate in the serum of the US population (25) In addition, studies demonstrated that besides the inhibitory effect on NIS activity, thiocyanate impairs the iodine organification catalyzed by the thyroperoxide (71, 72).

Thiocyanate is also transported to the milk of breastfeeding mothers who smoke cigarettes. Alarmingly, increased levels of thiocyanate in the maternal milk were correlated with decreased iodide content in the milk. As expected, this condition was associated with higher risk of developing thyroid dysfunctions in the newborns, due to the direct exposure of these individuals to a potent NIS-inhibitor and to decreased levels of the main substrate for the thyroid hormones synthesis (73). Furthermore, epidemiological studies indicated that thiocyanate exposure was associated with the inhibition of thyroid hormone production and the development of thyroid

autoimmunity (74, 75). However, although previous studies indicated that the co-exposure to perchlorate and thiocyanate is potentially deleterious to the thyroid function in adults and in susceptible individuals, as pregnant women, fetus, and newborns, the effects of thiocyanate *per se* in the thyroid are still controversial in humans (27, 30, 31, 76)

Finally, the molecular mechanisms involved in the thiocyanate regulation of thyroid function are not completely understood. Studies using primary thyroid cells cultures exposed to plant extracts rich in thiocyanate demonstrated an increased production of reactive oxygen species, induced cell injury and DNA damage, decreased gene expression and activity of proteins involved in the synthesis of thyroid hormones (77, 78). However, the direct effects of thiocyanate *per se* in the thyrocytes have never been reported.

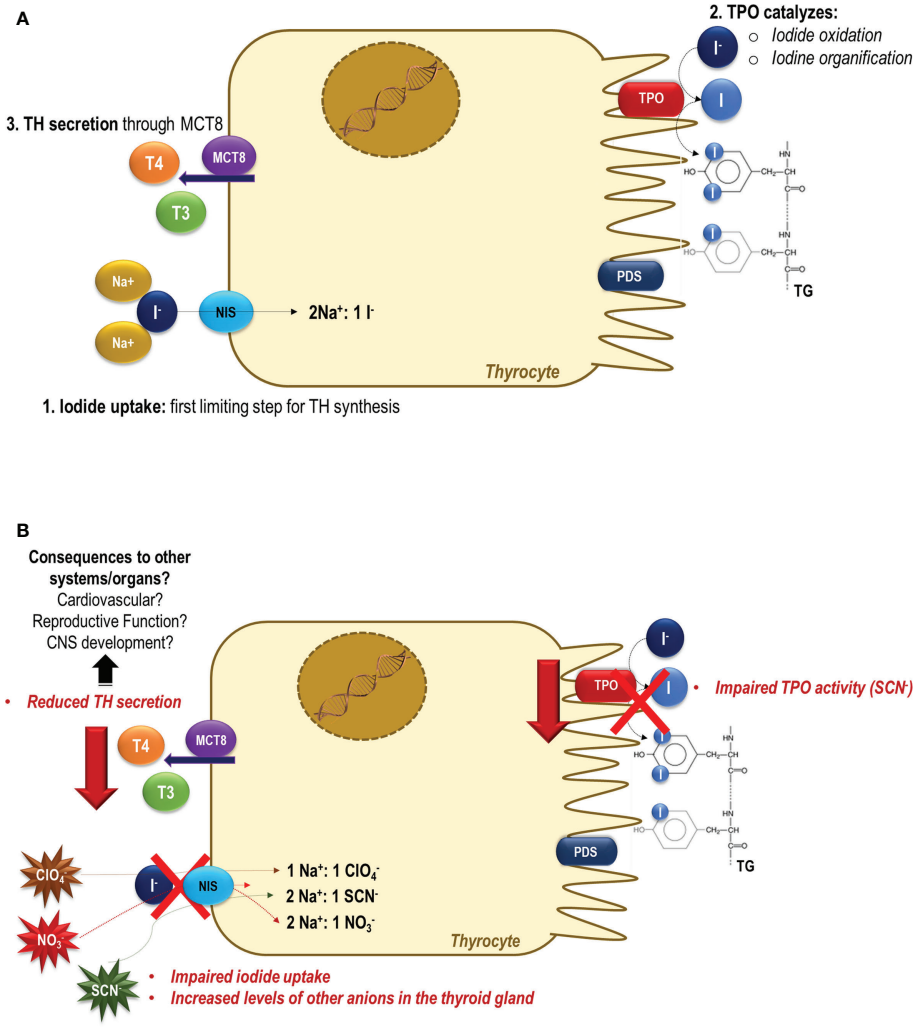


FIGURE 1
Thyroid dysfunction induced by perchlorate, nitrate and/or thiocyanate exposure. **(A)** In a normal condition, the sodium-iodide symporter (NIS) mediates the iodide uptake, the first limiting step for thyroid hormone synthesis. Then, iodide is transported across the apical membrane, and it is oxidized and organified into tyrosyl residues of thyroglobulin (TG) through the activity of thyroid peroxidase (TPO). Under the TSH stimulus, thyroid hormones (TH) are secreted and exert their effects in several tissues/organs, controlling the metabolism, growth, and development (79). **(B)** In the presence of perchlorate, nitrate and/or thiocyanate, NIS-mediated iodide uptake is impaired, and these molecules are actively transported into the thyroid cells (12). The consequences of increased levels of these anions in the intracellular thyrocytes medium are still unclear. It has been reported that besides the inhibition of NIS activity, the SCN- also impairs the organification of iodine catalyzed by TPO activity. The impairment of the activity of these two key proteins involved in the thyroid hormone synthesis could contribute to the reduced production and secretion of thyroid hormones to the blood circulation. The negative consequences of the reduction of the thyroid hormone serum levels are widely described in the literature, but are especially alarming during critical periods of the development, as during the pregnancy and lactation periods.

Current gaps and future perspectives

Several studies have been carried out in recent years and their results have clarified many aspects of the deleterious effects promoted by the exposure of animals to perchlorate, nitrate, and thiocyanate (Figure 1). Nevertheless, there are several aspects and molecular mechanisms that need to be clarified. Indeed, especially the epidemiological data are still controversial, and the impact of the exposure to these NIS-inhibitors on human health are not conclusive. The controversial results may be related to the different methodologies that were used to determine these contaminants in each study, as well as the period of the exposure that was evaluated. Moreover, these anions have a short biological half-life, which could impair these associative analyzes. Additionally, there are no data about the long-term and programming-induced effects of these contaminants, especially during the windows of susceptibility, such as pregnancy and lactation. In general, the harmful effects of these contaminants were observed in iodine-deficient populations and were related to the induction of iodine deficiency. This concern is irrefutable, however there are scarce data about the direct effects of these contaminants on the thyroid gland. It is important to highlight that NIS is expressed in several other tissues as mammary glands, placenta, intestine, kidney, gonads. Therefore, future studies are needed to clarify the effects of these anions in other systems/organs besides the hypothalamus-pituitary-thyroid axis. Finally, it is important to reinforce that the disruption of thyroid function goes beyond the impairment of the synthesis of thyroid hormones, since these hormones affect virtually all organs/systems of the body.

Author contributions

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

Funding

CS-N is supported by a grant from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP: 16/18517-8). MN was supported by grants from FAPESP (13/05629-4) and CNPq (310473/2021-7).

Conflict of interest

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

Publisher's note

References

1. Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Sumpter JP, et al. Estrogenic activity in five united kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ Toxicol Chem* (1997) 16(3):534–42. doi: 10.1002/etc.5620160320

2. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, et al. EDC-2: The endocrine society's second scientific statement on endocrine-disrupting chemicals. *Endocrine Rev* (2015) 36(6):E1-E150. doi: 10.1210/er.2015-1010

3. Casals-Casas C, Desvergne B. Endocrine disruptors: From endocrine to metabolic disruption. *Annu Rev Physiol* (2011) 73:135–62. doi: 10.1146/annurev-physiol-012110-142200

4. Kortenkamp A. Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. *Curr Opin Pharmacol* (2014) 19:105–11. doi: 10.1016/j.coph.2014.08.006

5. Sumpter JP, Johnson AC. Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. *Environ Sci Technol* (2005) 39(12):4321–32. doi: 10.1021/es048504a

6. Mullur R, Liu YY, Brent GA. Thyroid hormone regulation of metabolism. *Physiol Rev* (2014) 94(2):355–82. doi: 10.1152/physrev.00030.2013

7. Duntas LH. Chemical contamination and the thyroid. *Endocrine* (2015) 48 (1):53–64. doi: 10.1007/s12020-014-0442-4

8. Dai G, Levy O, Carrasco N. Cloning and characterization of the thyroid iodide transporter. *Nature* (1996) 379(6564):458–60. doi: 10.1038/379458a0

9. 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

10. Leung AM, Pearce EN, Braverman LE. Environmental perchlorate exposure: potential adverse thyroid effects. *Curr Opin endocrinology diabetes Obes* (2014) 21 (5):372-6. doi: 10.1097/MED.0000000000000090

11. Susarla S, Collette TW, Garrison AW, Wolfe NL, Mccutcheon SC. Perchlorate identification in fertilizers. *Environ Sci Technol* (1999) 33(19):3469–72. doi: 10.1021/es990577k

12. Tonacchera M, Pinchera A, Dimida A, Ferrarini E, Agretti P, Vitti P, et al. Relative potencies and additivity of perchlorate, thiocyanate, nitrate, and iodide on the inhibition of radioactive iodide uptake by the human sodium iodide symporter. *Thyroid* (2004) 14(12):1012–9. doi: 10.1089/thy.2004.14.1012

13. Godley AF, Stanbury JB. Preliminary experience in the treatment of hyperthyroidism with potassium perchlorate. *J Clin Endocrinol Metab* (1954) 14 (1):70–78. doi: 10.1210/jcem-14-1-70

14. Takeuchi K, Suzuki H, Horiuchi Y, Mashimo K. Significance of iodide-perchlorate discharge test for detection of iodine organification defect of the thyroid. *J Clin Endocrinol Metab* (1970) 31(2):144–6. doi: 10.1210/jcem-31-2-144

15. Eskandari S, Loo DDF, Dai G, Levy O, Wright EM, Carrasco N. Thyroid Na +/I- symporter. mechanism, stoichiometry, and specificity. *J Biol Chem* (1997) 272 (43):27230–8. doi: 10.1074/jbc.272.43.27230

16. Tran N, Valentín-Blasini L, Blount BC, McCuistion CG, Fenton MS, Gin E, et al. Thyroid-stimulating hormone increases active transport of perchlorate into thyroid cells. *Am J Physiol Endocrinol Metab* (2008) 294(4):E802-6. doi: 10.1152/ajpendo.00013.2008

17. Dohán O, Portulano C, Basquin C, Reyna-Neyra A, Amzel LM, Carrasco N. The Na+/I- symporter (NIS) mediates electroneutral active transport of the environmental pollutant perchlorate. *Proc Natl Acad Sci U S A*. (2007) 104 (51):20250-5. doi: 10.1073/pnas.0707207104

18. Wu F, Zhou X, Zhang R, Pan M, Peng KL. The effects of ammonium perchlorate on thyroid homeostasis and thyroid-specific gene expression in rat. *Environ Toxicol* (2012) 27(8):445-52. doi: 10.1002/tox.20655

19. Weltje L, Simpson P, Gross M, Crane M, Wheeler JR. Comparative acute and chronic sensitivity of fish and amphibians: A critical review of data. *Environ Toxicol Chem* (2013) 32(5):984-94. doi: 10.1002/etc.2149

20. Bernhardt RR, von Hippel FA, O'Hara TM. Chronic perchlorate exposure causes morphological abnormalities in developing stickleback. *Environ Toxicol Chem* (2011) 30(6):1468-78. doi: 10.1002/etc.521

21. Aviles A, Hulgard K, Green JW, Duus A, Holbech B, Morthorst JE. Effects of sodium perchlorate and 6-propylthiouracil on metamorphosis and thyroid gland histopathology in the European common frog (Rana temporaria). *Aquat Toxicology.* (2022) 244:106094. doi: 10.1016/j.aquatox.2022.106094

22. Lawrence JE, Lamm SH, Pino S, Richman K, Braverman LE. The effect of short-term low-dose perchlorate on various aspects of thyroid function. *Thyroid* (2000) 10(8):659–63. doi: 10.1089/10507250050137734

23. Lawrence J, Lamm S, Braverman LE. Low dose perchlorate (3 mg daily) and thyroid function. *Thyroid* (2001) 11(3):295. doi: 10.1089/105072501750159796

24. Yue B, Ning S, Miao H, Fang C, Li J, Zhang L, et al. Human exposure to a mixture of endocrine disruptors and serum levels of thyroid hormones: A cross-sectional study. *J Environ Sci (China).* (2023) 125:641-649. doi: 10.1016/j.jes.2022.01.017

25. Blount BC, Pirkle JL, Osterloh JD, Valentin-Blasini L, Caldwell KL. Urinary perchlorate and thyroid hormone levels in adolescent and adult men and women living in the united states. *Environ Health Perspect* (2006) 114(12):1865-71. doi: 10.1289/ehp.9466

26. Steinmaus C, Miller MD, Cushing L, Blount BC, Smith AH. Combined effects of perchlorate, thiocyanate, and iodine on thyroid function in the national health and nutrition examination survey 2007-08. *Environ Res* (2013) 123:17-24. doi: 10.1016/j.envres.2013.01.005

27. King L, Huang Y, Li T, Wang Q, Li W, Shan Z, et al. Associations of urinary perchlorate, nitrate and thiocyanate with central sensitivity to thyroid hormones: A US population-based cross-sectional study. *Environ Int* (2022) 164:107249. doi: 10.1016/j.envint.2022.107249

28. Pearce EN, Spencer CA, Mestman JH, Lee RH, Bergoglio LM, Mereshian P, et al. Effect of environmental perchlorate on thyroid function in pregnant women from córdoba, Argentina, and Los Angeles, California. *Endocrine Practice.* (2011) 17(3):412-7. doi: 10.4158/EP10293.OR

29. Steinmaus C, Pearl M, Kharrazi M, Blount BC, Miller MD, Pearce EN, et al. Thyroid hormones and moderate exposure to perchlorate during pregnancy in women in southern California. *Environ Health Perspect* (2016) 124(6):861-7. doi: 10.1289/ehp.1409614

30. Knight BA, Shields BM, He X, Pearce EN, Braverman LE, Sturley R, et al. Effect of perchlorate and thiocyanate exposure on thyroid function of pregnant women from south-West England: A cohort study. *Thyroid Res* (2018) 11(1):9. doi: 10.1186/s13044-018-0053-x

31. Horton MK, Blount BC, Valentin-Blasini L, Wapner R, Whyatt R, Gennings C, et al. CO-occurring exposure to perchlorate, nitrate and thiocyanate alters thyroid function in healthy pregnant women. *Environ Res* (2015) 143(Pt A):1-9. doi: 10.1016/j.envres.2015.09.013

32. Taylor PN, Okosieme OE, Murphy R, Hales C, Chiusano E, Maina A, et al. Maternal perchlorate levels in women with borderline thyroid function during pregnancy and the cognitive development of their offspring: Data from the controlled antenatal thyroid study. *J Clin Endocrinol Metab* (2014) 99(11):4291-8. doi: 10.1210/jc.2014-1901

33. Gilbert ME, Sui L. Developmental exposure to perchlorate alters synaptic transmission in hippocampus of the adult rat. *Environ Health Perspect* (2008) 116 (6):752-60. doi: 10.1289/ehp.11089

34. Kirk AB, Martinelango PK, Tian K, Dutta A, Smith EE, Dasgupta PK. Perchlorate and iodide in dairy and breast milk. *Environ Sci Technol* (2005) 39 (7):2011-7. doi: 10.1021/es048118t

35. Santisteban P, Bernal J. Thyroid development and effect on the nervous system. *Rev Endocrine Metab Disord* (2005) 6(3):217-28. doi: 10.1007/s11154-005-3053-9

36. Zhang L, Fang C, Liu L, Liu X, Fan S, Li J, et al. A case-control study of urinary levels of iodine, perchlorate and thiocyanate and risk of papillary thyroid cancer. *Environ Int* (2018) 120:388-93. doi: 10.1016/j.envint.2018.08.024

37. Wang H, Jiang Y, Song J, Liang H, Liu Y, Huang J, et al. The risk of perchlorate and iodine on the incidence of thyroid tumors and nodular goiter: A case-control study in southeastern China. *Environ Health* (2022) 21(1):4. doi: 10.1186/s12940-021-00818-8

38. Siglin JC, Mattie DR, Dodd DE, Hildebrandt PK, Baker WH. A 90-day drinking water toxicity study in rats of the environmental contaminant ammonium perchlorate. *Toxicological Sci* (2000) 57(1):61-74. doi: 10.1093/toxsci/57.1.61

39. Serrano-Nascimento C, Calil-Silveira J, Dalbosco R, Zorn TT, Nunes MT. Evaluation of hypothalamus-pituitary-thyroid axis function by chronic perchlorate exposure in male rats. *Environ Toxicol* (2018) 33(2):209-19. doi: 10.1002/tox.22509

40. McDougal JN, Jones KL, Fatuyi B, Gray KJ, Blount BC, Valentín-Blasini L, et al. The effects of perchlorate on thyroidal gene expression are different from the effects of iodide deficiency. *J Toxicol Environ Health - Part A: Curr Issues.* (2011) 74 (14):917-26. doi: 10.1080/15287394.2011.573740

41. Crawford NM. Nitrate: Nutrient and signal for plant growth. *Plant Cell* (1995) 7(7):859-68. doi: 10.2307/3870042

42. Ward MH, deKok TM, Levallois P, Brender J, Gulis G, Nolan BT, et al. Workgroup report: Drinking-water nitrate and health - recent findings and research needs. *Environ Health Perspect* (2005) 113(11):1607-14. doi: 10.1289/ehp.8043

43. Jenkinson DS. The impact of humans on the nitrogen cycle, with focus on temperate arable agriculture. *Plant Soil.* (2001) 228(1):3-15. doi: 10.1023/A:1004870606003

44. WHO. *Nitrate and nitrite in drinking water: background document for development of WHO guidelines for drinking water quality*. Geneva: World Health Organization (2016).

45. Jedrychowski W, Maugeri U, Bianchi I. Environmental pollution in central and eastern European countries: A basis for cancer epidemiology. *Rev Environ Health* (1997) 12(1):1-23. doi: 10.1515/REVEH.1997.12.1.1

46. Espejo-Herrera N, Gràcia-Lavedan E, Boldo E, Aragonés N, Pérez-Gómez B, Pollán M, et al. Colorectal cancer risk and nitrate exposure through drinking water and diet. *Int J Cancer.* (2016) 139(2):334-46. doi: 10.1002/ijc.30083

47. Picetti R, Deeney M, Pastorino S, Miller MR, Shah A, Leon DA, et al. Nitrate and nitrite contamination in drinking water and cancer risk: A systematic review with meta-analysis. *Environ Res* (2022) 210:112988. doi: 10.1016/j.envres.2022.112988

48. Bartsch H, Ohshima H, Pignatelli B, Calmels S. Endogenously formed n-nitroso compounds and nitrosating agents in human cancer etiology. *Pharmacogenetics* (1992) 2(6):272-7. doi: 10.1097/00008571-199212000-00005

49. van Breda SG, Mathijs K, Sági-Kiss V, Kuhnle GG, van der Veer B, Jones RR, et al. Impact of high drinking water nitrate levels on the endogenous formation of apparent n-nitroso compounds in combination with meat intake in healthy volunteers. *Environ Health* (2019) 18(1):87. doi: 10.1186/s12940-019-0525-z

50. van Voorhis BJ, Dunn MS, Snyder GD, Weiner CP. Nitric oxide: An autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* (1994) 135(5):1799-806. doi: 10.1210/endo.135.5.7525252

51. del Punta K, Charreau EH, Pignataro OP. Nitric oxide inhibits leydig cell steroidogenesis. *Endocrinology* (1996) 137(12):5377-43.doi: 10.1210/endo.137.12.8940355

52. Ward MH, Jones RR, Brender JD, de Kok TM, Weyer PJ, Nolan BT, et al. Drinking water nitrate and human health: An updated review. *Int J Environ Res Public Health* (2018) 15(7):1557. doi: 10.3390/ijerph15071557

53. Guillette LJ, Edwards TM. Is nitrate an ecologically relevant endocrine disruptor in vertebrates? *Integr Comp Biol* (2005) 45(1):19–27. doi: 10.1093/icb/45.1.19

54. Tajtáková M, Semanová Z, Tomková Z, Szökeová E, Majoroš J, Rádiková Ž, et al. Increased thyroid volume and frequency of thyroid disorders signs in schoolchildren from nitrate polluted area. *Chemosphere* (2006) 62(4):559-64. doi: 10.1016/j.chemosphere.2005.06.030

55. van Maanen JMS, van Dijk A, Mulder K, de Baets MH, Menheere PCA, van der Heide D, et al. Consumption of drinking water with high nitrate levels causes hypertrophy of the thyroid. *Toxicol Lett* (1994) 72(1–3):365-74. doi: 10.1016/0378-4274(94)90050-7

56. Cao Y, Blount BC, Valentin-Blasini L, Bernbaum JC, Phillips TM, Rogan WJ. Goitrogenic anions, thyroid-stimulating hormone, and thyroid hormone in infants. *Environ Health Perspect* (2010) 118(9):1332-7. doi: 10.1289/ehp.0901736

57. Ward MH, Kilfoy BA, Weyer PJ, Anderson KE, Folsom AR, Cerhan JR. Nitrate intake and the risk of thyroid cancer and thyroid disease. *Epidemiology* (2010) 21(3):389-95. doi: 10.1097/EDE.0b013e3181d6201d

58. Said Abasse K, Essien EE, Abbas M, Yu X, Xie W, Jinfang S, et al. Association between dietary nitrate, nitrite intake, and site-specific cancer risk: A systematic review and meta-analysis. *Nutrients* (2022) 14(3):666. doi: 10.3390/nu14030666

59. Bruce GM, Corey LM, Mandel JH, Pleus RC. Urinary nitrate, thiocyanate, and perchlorate and serum thyroid endpoints based on NHANES 2001 to 2002. *J Occup Environ Med* (2013) 55(1):52-8. doi: 10.1097/JOM.0b013e31826bb774

60. Eskiocak S, Dundar C, Basoglu T, Altaner S. The effects of taking chronic nitrate by drinking water on thyroid functions and morphology. *Clin Exp Med* (2005) 5(2):66-71. doi: 10.1007/s10238-005-0068-1

61. Zaki A, Ait Chaoui A, Talibi A, Derouiche AF, Aboussaouira T, Zarrouck K, et al. Impact of nitrate intake in drinking water on the thyroid gland activity in male rat. *Toxicol Lett* (2004) 147(1):27-33. doi: 10.1016/j.toxlet.2003.10.010

62. Ríos-Sánchez E, González-Zamora A, Gonsebatt Bonaparte ME, Meza Mata E, González-Delgado MF, Zámago Amaro A, et al. Regulation of the tpo, tg, Duox2, pds, and Mct8 genes involved in the synthesis of thyroid hormones after subchronic exposure to sodium nitrate in female wistar rats. *Environ Toxicol* (2021) 36(12):2380–91. doi: 10.1002/tox.23351

63. Broillet MC. S-nitrosylation of proteins. *Cell Mol Life Sci* (1999) 55(8-9):1036–42. doi: 10.1007/s000180050354

64. Ju YJ, Lee HW, Choi JW, Choi MS. The role of protein s-nitrosylation in protein misfolding-associated diseases. *Life* (2021) 11(7):705. doi: 10.3390/life11070705

65. Fozzatti L, Vélez ML, Lucero AM, Nicola JP, Mascanfroni ID, Macció DR, et al. Endogenous thyrocyte-produced nitric oxide inhibits iodide uptake and thyroid-specific gene expression in FRTL-5 thyroid cells. *J Endocrinology.* (2007) 192(3):627-37. doi: 10.1677/joe.1.06967

66. Bazzara LG, Vélez ML, Costamagna ME, Cabanillas AM, Fozzatti L, Lucero AM, et al. Nitric oxide/cGMP signaling inhibits TSH-stimulated iodide uptake and expression of thyroid peroxidase and thyroglobulin mRNA in FRTL-5 thyroid cells. *Thyroid* (2007) 17(8):717-27. doi: 10.1089/thy.2007.0086

67. Nicola JP, Peyret V, Nazar M, Romero JM, Lucero AM, del Mar Montesinos M, et al. S-nitrosylation of NF-κB p65 inhibits TSH-induced Na+/I- symporter expression. *Endocrinol (United States).* (2015) 156(12):4741–54. doi: 10.1210/en.2015-1192

68. Montesinos M del M, Nicola JP, Nazar M, Peyret V, Lucero AM, Pellizas CG, et al. Nitric oxide-repressed forkhead factor FoxE1 expression is involved in the inhibition of TSH-induced thyroid peroxidase levels. *Mol Cell Endocrinol* (2016) 420:105-15. doi: 10.1016/j.mce.2015.11.020.

69. Spurr LP, Watts MP, Gan HM, Moreau JW. Biodegradation of thiocyanate by a native groundwater microbial consortium. *PeerJ* (2019) 2019(3):e6498. doi: 10.7717/peerj.6498

70. Yoshida A, Sasaki N, Mori A, Taniguchi S, Ueta Y, Hattori K, et al. Differences in the electrophysiological response to I- and the inhibitory anions SCN- and ClO-4, studied in FRTL-5 cells. *Biochim Biophys Acta Biomembr* (1998) 1414(1–2):231-7. doi: 10.1016/s0005-2736(98)00169-2

71. Willemin ME, Lumen A. Thiocyanate: A review and evaluation of the kinetics and the modes of action for thyroid hormone perturbations. *Crit Rev Toxicol* (2017) 47(7):537–63. doi: 10.1080/10408444.2017.1281590

72. Virion A, Deme D, Pommier J, Nunez J. Opposite effects of thiocyanate on tyrosine iodination and thyroid hormone synthesis. *Eur J Biochem* (1980) 112(1):1-7. doi: 10.1111/j.1432-1033.1980.tb04979.x

73. Laurberg P, Nøhr SB, Pedersen KM, Fuglsang E. Iodine nutrition in breast-fed infants is impaired by maternal smoking. *J Clin Endocrinol Metab* (2004) 89 (1):181–7. doi: 10.1210/jc.2003-030829

74. Singh LH, Chandra AK, Yumnam SD, Sarkar D, Manglem RK, Dhabali T, et al. Thiocyanate in excess develops goiter followed by auto immune thyroid diseases even after effective salt iodization in a rural community of north east India. *Ecotoxicol Environ Saf.* (2021) 208:111711. doi: 10.1016/j.ecoenv.2020.111711

75. Felker P, Bunch R, Leung AM. Concentrations of thiocyanate and goitrin in human plasma, their precursor concentrations in brassica vegetables, and associated potential risk for hypothyroidism. *Nutr Rev* (2016) 74(4):248-58. doi: 10.1093/nutrit/nuv110

76. Bivolarska A, Gatseva P, Nikolova J, Argirova M, Atanasova V. Effect of thiocyanate on iodine status of pregnant women. *Biol Trace Elem Res* (2016) 172 (1):101-7. doi: 10.1007/s12011-015-0583-1

77. Sarkar D, Chandra AK, Chakraborty A, Ghosh S, Chattopadhyay S, Singh LH, et al. Effects of bamboo shoots (Bambusa balcooa) on thyroid hormone synthesizing regulatory elements at cellular and molecular levels in thyrocytes. *J Ethnopharmacol* (2020) 250:112463. doi: 10.1016/j.jep.2019.112463

78. Sarkar D, Chandra AK, Chattopadyay S, Biswas M, Das S, Singh LH, et al. Possible mechanism of bamboo shoots (Bambusa balcooa) induced thyroid disruption – an *in vitro* study. *Hum Exp Toxicol* (2021) 40(3):483-96. doi: 10.1177/0960327120958037

79. Carvalho DP, Dupuy C. Thyroid hormone biosynthesis and release. *Mol Cell Endocrinol* (2017) 458:6–15. doi: 10.1016/j.mce.2017.01.038

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 13 January 2023
DOI 10.3389/fendo.2022.995491

OPEN ACCESS

EDITED BY
Maaike Van Gerwen,
Icahn School of Medicine at Mount Sinai, United States

REVIEWED BY
Sakina Mhaouty-Kodja,
Centre National de la Recherche Scientifique (CNRS), France
Lin Ye,
Jilin University, China
Jing-Cai Liu,
Nanjing Agricultural University, China

*CORRESPONDENCE
Caroline Serrano-Nascimento
caroline.serrano@unifesp.br

SPECIALTY SECTION
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 15 July 2022
ACCEPTED 07 December 2022
PUBLISHED 13 January 2023

CITATION
Sousa-Vidal ÉK, Henrique G, Silva REC and Serrano-Nascimento C (2023) Intrauterine exposure to di(2-ethylhexyl) phthalate (DEHP) disrupts the function of the hypothalamus-pituitary-thyroid axis of the F1 rats during adult life. *Front. Endocrinol.* 13:995491. doi: 10.3389/fendo.2022.995491

Intrauterine exposure to di(2-ethylhexyl) phthalate (DEHP) disrupts the function of the hypothalamus-pituitary-thyroid axis of the F1 rats during adult life

Érica Kássia Sousa-Vidal[1], Guilherme Henrique[2], Renata Elen Costa da Silva[2] and Caroline Serrano-Nascimento[1,2,3]*

[1]Faculdade Israelita de Ciências da Saúde Albert Einstein, Hospital Israelita Albert Einstein, São Paulo, Brazil, [2]Laboratório de Endocrinologia Molecular e Translacional (LEMT), Escola Paulista de Medicina (EPM), Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil, [3]Instituto de Ciências Ambientais, Químicas e Farmacêuticas (ICAQF), Departamento de Ciências Biológicas, Universidade Federal de São Paulo (UNIFESP), Diadema, Brazil

Introduction: DEHP is an endocrine disruptor widely used in the production of malleable plastics. DEHP exposure was associated with altered hypothalamic-pituitary-thyroid (HPT) axis function. Although previous studies reported deleterious effects of DEHP exposure during the intrauterine period, few studies have evaluated the direct effects triggered by this endocrine disruptor on the offspring animals' thyroid function. This study aimed to investigate the impact of intrauterine exposure to DEHP on the HPT axis function programming of the offspring animals during adulthood.

Methods: Pregnant Wistar rats were orally treated with corn oil or corn oil supplemented with DEHP (0.48 or 4.8 mg/kg/day) throughout the gestational period. The offspring rats were euthanized on the 90th postnatal day. Hypothalamus, pituitary, thyroid, and liver were collected to analyze gene expression and protein content through qPCR and Western Blot. Blood was collected to determine TSH and thyroid hormone levels through fluorometric or chemiluminescence immunoassays.

Results: In the adult F1 female rats, the highest dose of DEHP decreased TSH serum levels. In the thyroid, DEHP reduced the gene expression and/or protein content of NIS, TSHR, TG, TPO, MCT8, NKX2.1, PAX8, and FOXE1. These data are consistent with the reduction in T4 serum levels of the F1 DEHP-exposed female rats. In the liver, DEHP exposure increased the mRNA expression of *Dio1* and *Ttr*, while the highest dose of DEHP reduced the mRNA expression of *Ugt1a1* and *Ugt1a6*. Conversely, in the F1 male adult rats, TSHB expression and TSH serum levels were increased in DEHP-exposed animals. In the thyroid,

except for the reduced protein content of TSHR, none of the evaluated genes/proteins were altered by DEHP. TH serum levels were not changed in the DEHP-exposed F1 male rats compared to the control group. Additionally, there were no significant alterations in the expression of hepatic enzymes in these animals.

Discussion/Conclusions: Our results demonstrated, for the first time, that intrauterine exposure to DEHP disrupts the HPT axis function in male and female offspring rats and strongly suggest that DEHP exposure increases the susceptibility of the offspring animals to develop thyroid dysfunctions during adulthood.

KEYWORDS

phthlates, DEHP, endocrine disruptors, hypothalamus-pituitary-thyroid axis, intrauterine period, DOHaD

1 Introduction

Phthalates are plasticizers used in producing polyvinyl chloride plastics and several manufactured goods, such as toys, food packaging, and medical devices. Phthalates are not covalently bound as plasticizes, and thus can easily leach into the water, food, and environment (1, 2).

Di(2-ethylhexyl) phthalate (DEHP) is the most common member of the class of phthalates, and several studies indicated that humans are highly exposed to DEHP (3, 4). Indeed, DEHP exposure was considered a potential risk to human health (5–7). The estimated daily intake of DEHP for adult humans was 0.5-30 µg/kg/day, although some studies have reported higher levels of exposure to this phthalate (8–10). Once in the body, DEHP is metabolized to mono(2-ethylhexyl) phthalate (MEHP), a highly toxic compound (11).

DEHP and MEHP are compounds that interfere with the endocrine function in humans and other animals (12). It is worth noting that the thyroid hormones control metabolic processes essential for normal development and growth (13–15). Moreover, the thyroid hormones are transferred through the placenta to the fetal compartment during the intrauterine period, and the thyroid hormones' actions are crucial to the development of the central nervous system (16–18).

Although it has been suggested in several epidemiological studies, the negative association between thyroid hormone serum levels and DEHP exposure is still controversial in humans (19–22). On the contrary, the disruptive effect of DEHP exposure on the thyroid function of the most susceptible individuals, such as newborns, children, and pregnant women, was extensively reported in the literature (23–25).

In animal models, the direct and chronic exposure to high doses of DEHP (250 to 750 mg/kg/day) was related to increased serum levels of TRH and TSH, decreased thyroid hormones serum concentration, altered expression of genes/proteins involved in the thyroid hormone synthesis, altered peripheral metabolism and impaired plasma transport of thyroid hormones to the target tissues (26–28).

It is well known that the intrauterine period is essential for programming health or disease during adulthood (29–31). Nevertheless, although it has been described that *in-utero* exposure to DEHP was related to neurobehavioral and neurodevelopment impairments due to alterations in thyroid homeostasis (32, 33), limited studies investigated the effects of DEHP early-life exposure directly in the thyroid function of the offspring animals. Therefore, this study aimed to evaluate the impact of intrauterine exposure to DEHP in the programming of the HPT axis of the adult F1 rat offspring.

2 Material and methods

2.1 Animals and experimental protocol

Virgin female and male Wistar rats were obtained from the Animal Breeding Centre at the Institute of Biomedical Sciences, University of Sao Paulo. The animals were maintained in polysulfone rat cages in the Experimental and Training Center of the Hospital Israelita Albert Einstein at constant temperature (23 ± 1°C), 12:12-h light-dark cycle schedule, and fed with rat chow (NUVLAB, CR1; Nuvital, Brazil) and water (polysulfone bottles) *ad libitum*. The female and male rats were mated, and the presence of spermatozoa in the vaginal smear was defined as the first day of gestation. Thereafter, each pregnant rat was randomly assigned to one of 3 treatment groups: control, 0.48 mg/kg/day (DEHP 0.48), or 4.8 mg/kg/day (DEHP 4.8). Four pregnant rats were used in each experimental group. Each

pregnant rat had one litter of 10-12 animals per group. The number of offspring animals did not vary among the groups. DEHP was dissolved in corn oil (Figure 1). The treatment doses were based on the NOAEL dose of DEHP, which was defined as 4.8 mg/kg/day (34, 35).

From the first day of gestation until the birth of the pups, pregnant dams were orally dosed once a day with corn oil (vehicle control) or the doses mentioned above of DEHP by placing a pipette tip containing the dosing solution into the mouth (Figure 1). This method of drug administration was chosen to mimic oral exposure in humans (35). The volume of each dosing mixture in corn oil was adjusted daily based on changes in the dam's weight.

The DEHP treatment was interrupted after the birth of the offspring rats. After weaning, the rats were separated by sex and kept on standard chow and filtered water until the 90th postnatal day (PND90). Then, the animals were weighed, anesthetized, and euthanized by decapitation. The euthanasia was performed in the morning between 8h00 and 11h00AM. The female offspring were in the metestrus and diestrus phases of the estrous cycle on the day of the euthanasia. Blood, hypothalamus, pituitary, thyroid, and liver were collected, stored at -80°C, and processed as described below.

The experimental protocol was approved by the Ethics Committee on Animal Experimentation of the Hospital Israelita Albert Einstein (CEUA 3326/2018), following the ethical principles in animal research adopted by the National Council for the Control of Animal Experimentation.

2.2 Evaluation of gene expression

Hypothalamus, pituitary, thyroid, and liver were homogenized in TRIzol® Reagent (Invitrogen Life Technologies Carlsbad, CA, USA), using Polytron® equipment (KINEMATIC), following the manufacturer's recommendations (36). In addition, gene expression of the hypothalamus and pituitary (*Tshb, Cga, Trh*), thyroid transcription factors (*Nkx2.1, Pax8, Foxe1*), thyroid differentiation markers (*Slc5a5, Tshr, Tpo, Tg, Mct8*), deiodinases (*Dio1*, *Dio3*), hepatic enzymes (*Ttr, Ugt1a1, Ugt1a6*) was evaluated through RT-qPCR assays. The relative mRNA expression was calculated according to the $2^{-\Delta\Delta Ct}$ method using *Rpl19* mRNA expression as the internal control. Primer sequences are described in Supplementary Table 1.

2.3 Evaluation of protein content

Hypothalamus, pituitary, and thyroid were homogenized in RIPA lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl, 1% Nonidet P-40; 0.5% sodium deoxycholate; 1 mM EDTA and 0.1% SDS) supplemented with protease inhibitors (Protease Inhibitor Tablets, Thermo Scientific Pierce™), using Polytron® equipment (KINEMATIC). Western Blotting was performed as previously described (36). Briefly, nitrocellulose membranes were blocked with 3% BSA solution and incubated with specific primary and secondary antibodies, described in Supplementary Table 2. Blots were developed using the enhanced chemiluminescence (ECL) kit (Bio-Rad). Densitometric analyses were performed using Image J. 1.4 software (National Institutes of Health). Ponceau staining was used for thyroid total protein normalization, as previously described (37).

2.4 Determination of TSH, T3, and T4 serum levels

T_4 and T_3 rat serum concentrations were determined by a chemiluminescent immunoassay (Roche). TSH serum levels were

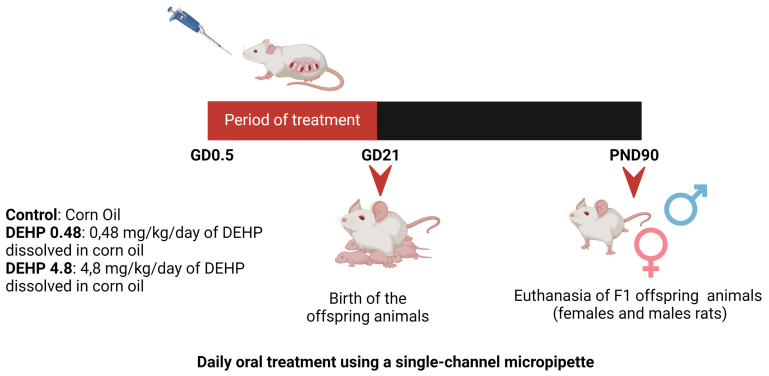


FIGURE 1
Schematic representation of the experimental protocol. Pregnant Wistar rats were randomly divided into 3 experimental groups: Control, DEHP 0.48 mg/kg/day and DEHP 4.8 mg/kg/day. Pregnant rats orally received corn oil (control) or corn oil supplemented with DEHP (0.48 or 4.8 mg/kg/day) throughout pregnancy (GD0.5 – GD21). After birth, F1 offspring male and female rats were maintained with normal rat chow and filtered water. The offspring animals were anesthetized and euthanized in the PND90. Created with BioRender.com.

determined by a fluorometric immunoassay, as previously described (37).

2.5 Statistical analysis

All data are reported as means ± SEM. The number of animals used in the study is indicated in the legends of the figures. Statistical analysis was performed using the GraphPad Prism Software – Version: 6.0. Data was subjected to a normality test (Kolmogorov-Smirnov) and then to unpaired One-Way ANOVA followed by Student-Newman-Keuls post hoc test and/or Dunnet's multiple comparisons test *post hoc* test. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Intrauterine exposure to DEHP alters TSH and thyroid hormones serum levels in the F1 female rats

As presented in Table 1, the exposure of F1 female rats *in utero* to DEHP did not alter body weight but altered the serum levels of the TSH and thyroid hormones during adulthood. Indeed, the female rats exposed to the highest dose of DEHP treatment during the intrauterine period presented lower serum TSH levels than the control group. Moreover, *in-utero* exposure to DEHP reduced T4 serum levels in the F1 female rats. Interestingly, there was a significant increase in the T3 total serum levels in the animals exposed to the lowest dose of DEHP during the intrauterine period.

3.2 Intrauterine exposure to DEHP alters the gene expression and protein content of the F1 female rats' hypothalamus and pituitary

As demonstrated in Figure 2, the intrauterine exposure to DEHP has not altered TRH's gene and protein expression in the hypothalamus of the F1 female rats during adulthood. Interestingly, exposure to the lowest dose of DEHP significantly increased the gene expression and protein content of the alpha and beta subunits of TSH in the pituitary of the F1 female rats. However, there were no significant alterations in these subunits' gene/protein content in the animals exposed to the highest dose of DEHP (Figure 3).

3.3 Intrauterine exposure to DEHP reduces the gene expression and protein content of the F1 female rats' thyroid gland

Intrauterine exposure to both doses of DEHP has also reduced the expression of genes/proteins involved in the synthesis and secretion of thyroid hormones (Figure 4). Indeed, there was a significant decrease in the gene expression of *Foxe1* and *Nkx2.1* transcription factors in both treatment doses, while the *Pax8* gene expression was reduced only in the animals exposed to the highest dose of DEHP. In addition, both doses of DEHP exposure reduced the thyroid expression of *Slc5a5, Tpo, Tshr*, and *Mct8* mRNAs compared to the control animals (Figure 4A). In agreement, the *in-utero* DEHP-exposed F1 female rats presented a significant reduction in the content of NIS, TPO, and TSHR, critical proteins involved in synthesizing thyroid hormones (Figure 4B).

3.4 Intrauterine exposure to DEHP alters the gene expression of the F1 female rats' liver

Finally, the gene expression of type I deiodinase (*Dio1*) was significantly increased in the liver of female rats exposed to both doses of DEHP. There was no significant alteration in the expression of type 3 deiodinase (*Dio3*). Moreover, intrauterine exposure to the highest dose of DEHP reduced the mRNA expression of *Ugt1a* and *Ugt1a6*, glucuronosyltransferases involved in the depuration of thyroid hormones. The mRNA expression of the transthyretin (*Ttr*), an essential plasma protein

TABLE 1 Body weight, thyroid hormones and TSH serum levels in adult F1 female rats that were exposed or not to DEHP during intrauterine period.

	Control	DEHP 0.48 mg/kg/day	DEHP 4.8 mg/kg/day*
Body weight	221 ± 4,2	216 ± 6,9	227 ± 4,9
T_3 (ng/dL)	1,20 ± 0,05	1,36 ± 0,02**	1,19 ± 0,02
T_4 (µg/dL)	5,90 ± 0,38	5,01 ± 0,13@	4,14 ± 0,18***
TSH (ng/mL)	10,63 ± 1,0	10,72 ± 2,1	5,05± 0,8*

Results are expressed by means ± SEM, n = 5-7 per group. @P = 0.06, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. Control.

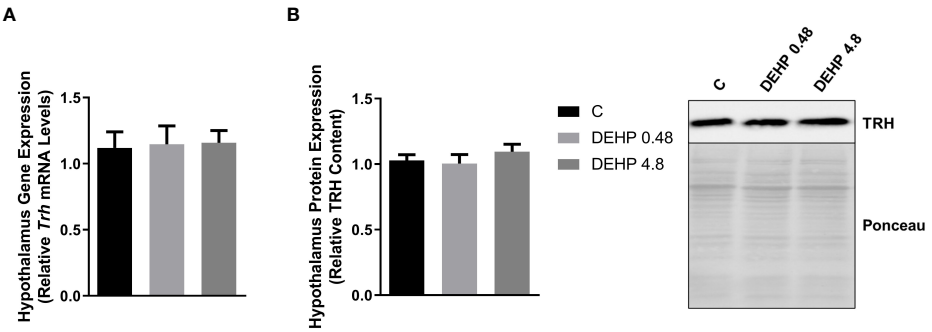


FIGURE 2
Impact of DEHP exposure during the intrauterine period on TRH expression in the hypothalamus of adult F1 female rats. **(A)** Gene expression (*Trh*) was evaluated by Real-Time PCR and normalized by the expression of the constitutive gene *Rpl19*. **(B)** The protein content of thyrotropin-releasing hormone (TRH) was evaluated by Western Blot and normalized by Western Blot and normalized by Ponceau staining of the membranes. Representative western blots are shown in the right panel. Results are expressed as means ± SEM as fold change or in arbitrary units (AU). Values are expressed as mean ± SEM, in arbitrary units, n = 8-9 animals. P > 0.05 (One-Way ANOVA).

transporter of thyroid hormones, was increased in the liver of DEHP-exposed adult F1 female rats (Figure 5).

3.5 Intrauterine exposure to DEHP alters TSH serum levels but doesn't change thyroid hormones serum levels in the F1 male rats

In contrast to the results obtained in the F1 female rats, adult F1 male rats have not presented alterations in the thyroid hormones serum levels. However, both studied doses significantly elevated the TSH serum levels in the DEHP-exposed animals. Notably, the offspring's body weight did not vary in the DEHP-exposed animals (Table 2).

3.6 Intrauterine exposure to DEHP alters the gene expression and protein content of the F1 male rats' hypothalamus and pituitary

Interestingly, the *in-utero* exposure to the lowest dose of DEHP increased the mRNA expression of *Trh* in the hypothalamus of adult F1 male rats, even though there were no significant alterations in the TRH protein content in these animals (Figure 6). Furthermore, as demonstrated in Figure 7, DEHP exposure has not altered the alpha subunit of TSH expression in the pituitary of the F1 male rats. In contrast, the animals exposed to the lowest dose of DEHP during the intrauterine period presented increased mRNA expression and protein content of the beta subunit of TSH in the pituitary.

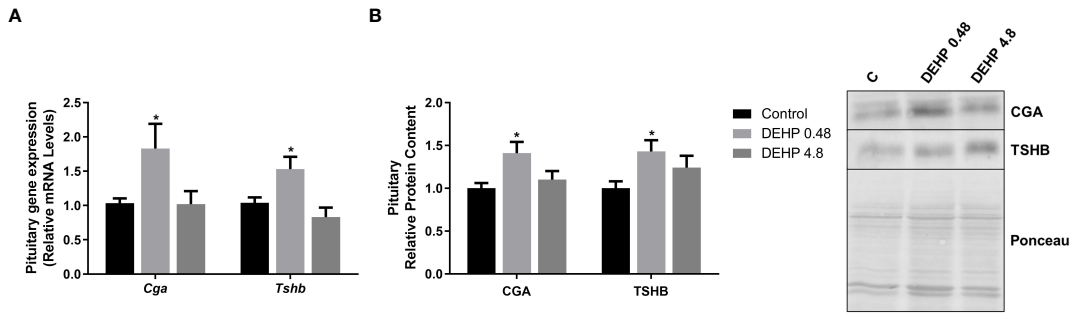


FIGURE 3
Impact of DEHP exposure during intrauterine period in the expression of alpha and beta subunits of TSH in the pituitary of adult F1 female rats. **(A)** Gene expression (*Cga, Tshb*) was evaluated by Real-Time PCR and normalized by the expression of the constitutive gene *Rpl19*. **(B)** The protein content of CGA and TSHB was assessed by Western Blot and normalized by Ponceau staining of the membranes. Representative western blots are shown in the right panel. Values are expressed as mean ± SEM in arbitrary units, n=10-14 animals. * p < 0.05 vs. Control (One-Way ANOVA).

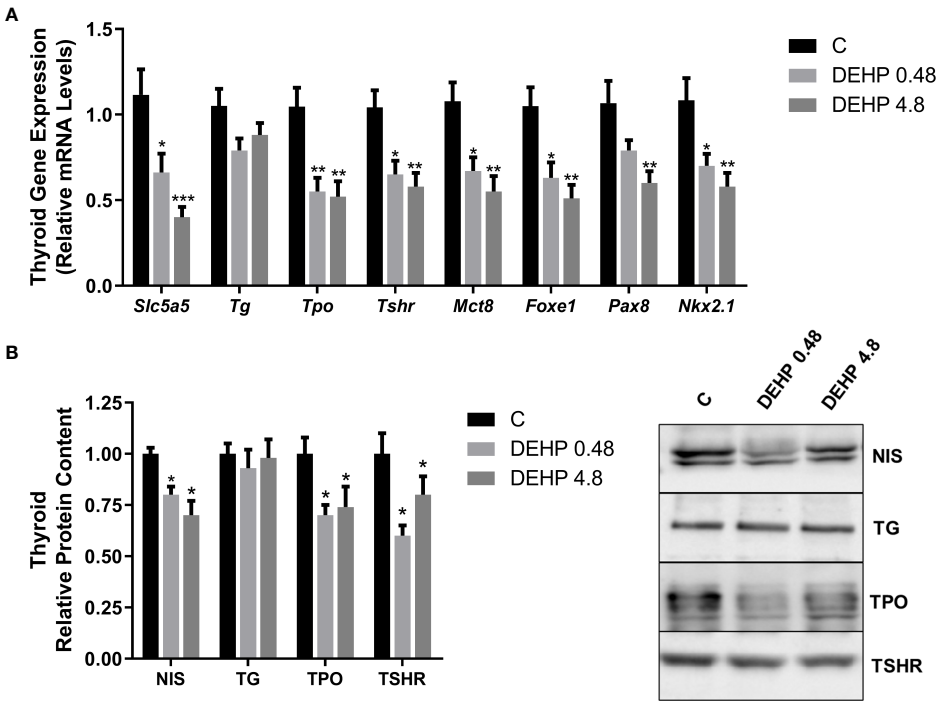


FIGURE 4
Impact of DEHP exposure during the intrauterine period on thyroid gene and protein expression in adult F1 female rats. **(A)** Gene expression (*Slc5a5, Tg, Tpo, Tshr, Mct8, Foxe1, Pax8, Nkx2.1*) was evaluated by Real-Time PCR and normalized by the expression of the constitutive gene *Rpl19*. **(B)** The protein content of NIS, TG, TPO, and TSHR was evaluated by Western Blot and normalized by Ponceau staining of the membranes (Supplementary Figure 1). Representative western blots are shown in the right panel. Values are expressed as mean ± SEM, in arbitrary units, n=8-10. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Control. (One-Way ANOVA).

3.7 Intrauterine exposure to DEHP affects the gene expression and protein content of the F1 male rats' thyroid gland

Interestingly, the exposure of F1 male rats to DEHP *in utero* has not significantly altered the mRNA expression of *Slc5a5, Tg, Tpo, Tshr, Mct8, Foxe1, Pax8*, and *Nkx2.1* (Figure 8A). However, DEHP-exposed animals presented a significant decrease in the TSRH protein content in the thyroid (Figure 8B).

3.8 Intrauterine exposure to DEHP doesn't alter the gene expression of the F1 male rats' liver

The mRNA expression of *Dio1*, *Dio3, Ugt1a*, *Ugt1a6*, and *Ttr* were not altered in the liver of adult F1 DEHP-exposed male rats compared to the control group (Figure 9).

In summary, Figure 10 presents a schematic representation of the deleterious effects of intrauterine

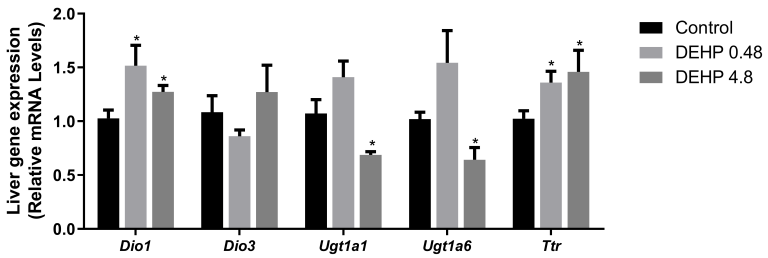


FIGURE 5
Impact of DEHP exposure during intrauterine period in the liver gene expression in adult F1 female rats. Gene expression (*Dio1, Dio3, Ugt1a, Ugt1a6, Ttr*) was evaluated by Real-Time PCR and was normalized by the expression of the constitutive gene *Rpl19*. Values are expressed as mean ± SEM in arbitrary units, n=7-10 animals. * $p < 0.05$, vs. Control (One-Way ANOVA).

TABLE 2 Body weight, thyroid hormones and TSH serum levels in adult F1 male rats that were exposed or not to DEHP during intrauterine period.

	Control	DEHP 0.48 mg/kg/day	DEHP 4.8 mg/kg/day
Body Weight	381 ± 9,4	365 ± 10,1	398 ± 7,7
T_3 (ng/dL)	1,15 ± 0,02	1,18 ± 0,03	1,19 ± 0,02
T_4 (µg/dL)	7,20 ± 0,36	7,20 ± 0,31	6,31 ± 0,44
***TSH (ng/mL)**	7,71 ± 0,8	16,08 ± 5,2*	15,14 ± 2,2*

Results are expressed by means ± SEM, n = 5-12 per group. *P < 0.05 vs. Control.

exposure to DEHP in the HPT axis function of F1 offspring during adulthood.

4 Discussion

The data presented in this study demonstrated that intrauterine exposure to DEHP disrupts the HPT axis of the offspring rats during adulthood. Our results also suggest that the male and female offspring present different sensitivity to DEHP exposure.

The disruptive effects of DEHP exposure in the HPT axis were previously reported in the literature (38–40). However, although these studies were very elucidative about the deleterious effects of DEHP on thyroid function, the studied doses were much higher than those used herein. Moreover, most of the previous studies were performed in male rats. It is well known that the prevalence of thyroid diseases is higher in women than in men (41). For that reason, we aimed to evaluate the consequences of DEHP exposure in both genders. Therefore, our study was the first to describe the effects of intrauterine exposure to DEHP in the programming of the HPT axis both in male and female offspring.

The adverse effects triggered by intrauterine exposure to DEHP were previously reported in the reproductive function and neurobehavioral of the offspring (32, 42). However, the impact of this exposure directly on thyroid function is poorly described (33, 43). Therefore, this is a limitation of the present study since it is difficult to compare the obtained results with those presented in the literature.

In the hypothalamus of the adult F1 female rats, there was no alteration in the gene/protein expression of TRH. Contrary to these results, the male offspring rats presented increased *Trh* mRNA expression in the lowest treatment dose without significant alterations in the TRH protein content. Sun et al. also observed increased TRH content in the hypothalamus of male rats directly exposed to high doses of DEHP (50 to 500 mg/kg/day) (39).

It is well described that thyroid hormones exert a negative feedback loop in the hypothalamus and pituitary (44). Therefore, our data suggest an impairment in the HPT axis function both in the male and female offspring since there was an increased expression of *Trh* in the hypothalamus of the male rats even in the absence of thyroid hormones alterations, and the *Trh* expression was not upregulated in the females with reduced T4 levels.

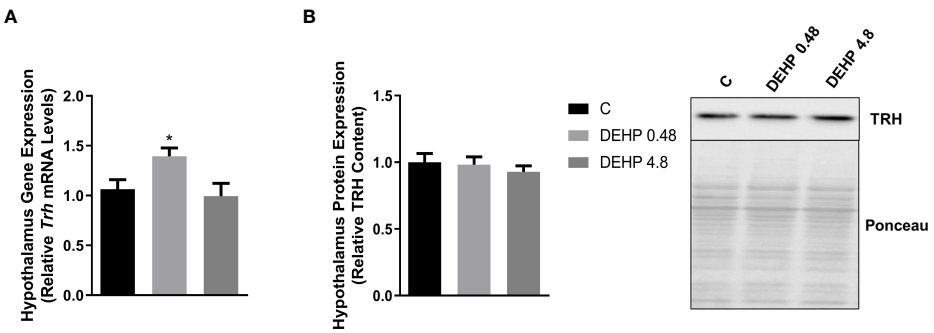


FIGURE 6
Impact of DEHP exposure during intrauterine period in the TRH expression in the hypothalamus of adult F1 male rats. **(A)** Gene expression (*Trh*) was evaluated by Real-Time PCR and normalized by the expression of the constitutive gene *Rpl19*. **(B)** The protein content of thyrotropin-releasing hormone (TRH) was evaluated by Western Blot and normalized by Ponceau staining of the membranes. Representative western blots are shown in the right panel. Values are expressed as mean ± SEM, in arbitrary units, n = 8-10 animals. * p < 0.05 vs. Control (One-Way ANOVA).

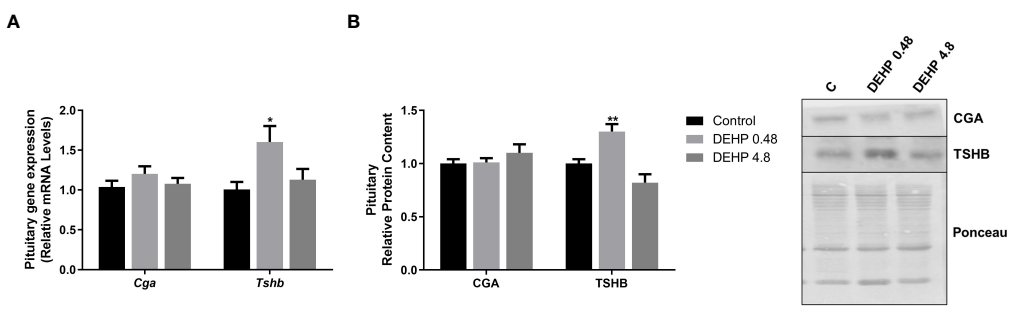


FIGURE 7
Impact of DEHP exposure during intrauterine period in the expression of alpha and beta subunits of TSH in the pituitary of adult F1 male rats. **(A)** Gene expression (*Cga, Tshb*) was evaluated by Real-Time PCR and normalized by the expression of the constitutive gene *Rpl19*. **(B)** The protein content of CGA and TSHB was evaluated by Western Blot and normalized by Ponceau staining of the membranes.. Representative western blots are shown in the right panel. Values are expressed as mean ± SEM, in arbitrary units, n= 13-14 animals. * $p < 0.05$, ** $p < 0.01$ vs. Control (One-Way ANOVA).

In the pituitary, the adult F1 female rats presented increased gene/protein expression of CGA, especially in the lowest dose of DEHP treatment. It is well known that the glycoprotein hormones, such as TSH, FSH, and LH, are composed of a common alpha-subunit and a specific beta-subunit, which confers biological specificity to these hormones. (45). Therefore, the increased expression of CGA in the female offspring rats' pituitary suggests that the synthesis and secretion of FSH and LH are potentially altered in the DEHP-exposed animals. These results follow previous data about the

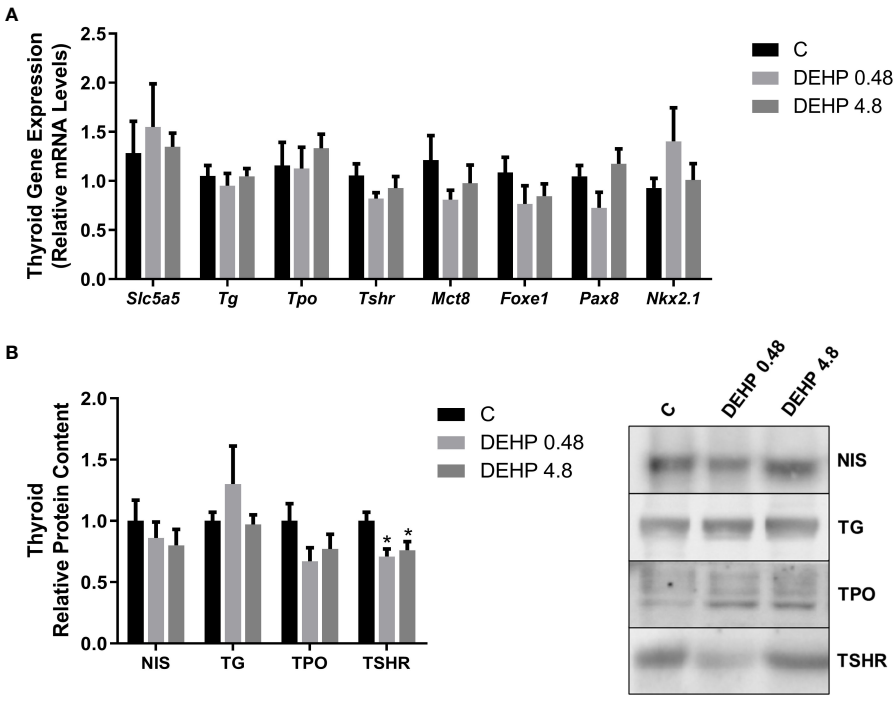


FIGURE 8
Impact of DEHP exposure during the intrauterine period on thyroid gene and protein expression in the adult F1 male rats. **(A)** Gene expression (*Slc5a5, Tg, Tpo, Tshr, Mct8, Foxe1, Pax8, Nkx2.1*) was evaluated by Real-Time PCR and normalized by the expression of the constitutive gene *Rpl19*. **(B)** The protein content of NIS, NIS, TG, TPO, and TSHR was evaluated by Western Blot and normalized by Ponceau staining of the membranes (Supplementary Figure 2). Representative western blots are shown in the right panel. Values are expressed as mean ± SEM, in arbitrary units, n=8-10. * $p < 0.05$ vs. Control. (One-Way ANOVA).

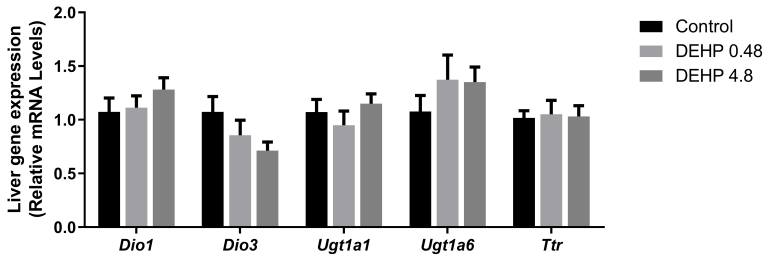


FIGURE 9
Impact of DEHP exposure during intrauterine period in the liver gene expression in adult F1 male rats. Gene expression (*Dio1, Dio3, Ugt1a, Ugt1a6, Ttr*) was evaluated by Real-Time PCR and was normalized by the expression of the constitutive gene *Rpl19*. Values are expressed as mean ± SEM in arbitrary units, n=7-10 animals. $p > 0.05$ (One-Way ANOVA).

disruptive effects of DEHP exposure, including during the intrauterine period, in the hypothalamus-pituitary-ovary axis function and the reproductive outcomes in female rats (35, 42). Interestingly, the exposure to DEHP during the intrauterine period decreased TSH levels only in the F1 female offspring exposed to the highest dose of treatment

The exposure to DEPH increased the content of the TSHB in the adult F1 male rats. This result was consistent with the increased TSH serum levels that were observed in the DEHP-exposed animals. Even though the effects of DEHP exposure on the expression/secretion of TSH are still controversial, Dong et al. demonstrated that the perinatal exposure to DEHP (30 to 750 mg/kg/day) increased the TSH serum levels of the pups at PND 7, PND 14, and PND 21 (43). The results also agree with previous results that demonstrated increased TSH levels in the male offspring rats of rat dams exposed to 600 mg/kg/day of DEHP (33). Moreover, our results are consonant with previous studies carried out in zebrafish that reported a significant increase in the *Tshb* expression MEHP-exposed animals (40 and 200 µg/L) (46). Nevertheless, studies performed with male rats chronically exposed to DEHP (600 mg/kg/day) presented decreased levels of TSH. (40). These discordant results indicate that the exposure period is crucial to determine the DEHP-triggered effects on the TSH expression in the pituitary.

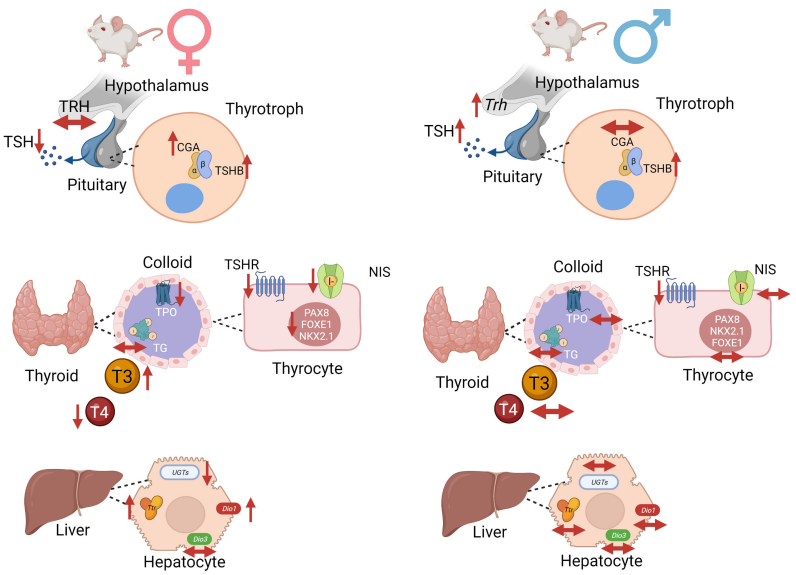


FIGURE 10
Schematic representation of DEHP effects in the HPT axis of adult F1 female and male offspring animals. In summary, intrauterine exposure to DEHP induced hypothyroidism in adult female offspring rats and subclinical hypothyroidism in adult male offspring rats. Created with BioRender.com.

TSH is the primary regulator of thyroid morphology and all the steps involved in thyroid hormone synthesis. TSH triggers its effects through the binding in the TSHR expressed in the basolateral membrane of thyrocytes (47). As described herein, the intrauterine exposure to DEHP triggered different results in the thyroid of the adult F1 female and male offspring rats. In the F1 female rats, there was a significant reduction in the expression of genes/proteins involved in the synthesis and secretion of thyroid hormones in both studied doses of DEHP, which was consistent with the reduced serum levels of T4 in these animals. However, in the males, thyroid gene expression was not altered by DEHP exposure, although TSHR expression was reduced in these animals. Moreover, the thyroid hormone levels were not changed in the adult F1 males, although TSH levels were significantly increased in both studied doses.

The disruptive effect of DEHP on TSH, TSH signaling pathway, and thyroid hormone levels have been previously reported, especially in male rats. Indeed, it has been demonstrated that the exposure of rats to high doses of DEHP (50 and 500 mg/kg/day) for two weeks reduced T3 and T4 levels and the *Tshr* mRNA expression in the thyroid gland (48). Rats chronically and directly exposed to DEHP (250, 500, or 750 mg/kg/day) presented reduced levels of thyroid hormones or increased levels of TSH (26, 27, 49).

Indeed, thyroid transcription factors are regulated by TSH and are critical to maintaining thyroid function and controlling the expression of thyroid differentiation genes (50, 51). Thus, the significant reduction of *Pax8*, *Nkx2.1*, and *Foxe1* mRNA in the thyroid of F1 female rats could justify the decrease in the expression of the other thyroid genes. Additionally, the reduced TSH serum levels observed in the females exposed to the highest dose of DEHP could also contribute to the reduced thyroid transcriptional activity. Therefore, in the males, the absence of thyroid gene expression regulation, even with high serum circulating levels of TSH, suggests that the thyroid gland is hypofunctional in these animals. This hypothesis is strengthened by the reduced expression of TSHR in the thyroid of DEHP-exposed animals.

The impact of DEHP exposure on thyroid gene expression and function is still inconclusive. In fact, previous studies demonstrated that chronic exposure to high doses of DEHP (150, 300, 600 mg/kg/day) induced a stimulatory effect on *Slc5a5, Tpo, Tshr*, and *Tg* mRNA expression in rats (40). In agreement, rats perinatally exposed to DEHP also presented increased expression of genes/proteins involved in the biosynthesis of thyroid hormones (43). On the other hand, Kim et al. demonstrated reduced expression of TSHR in male rats exposed to DEHP (50 and 500mg/kg/day) for 14 days (48). These results are in accordance with previous studies that demonstrated reduced expression of NIS and TPO in rats chronically treated with high doses of DEHP (500 and 750 mg/kg/day) (27). Although this is the first study to evaluate the impact of *in utero* DEHP exposure in the thyroid gene/protein expression of the offspring during adulthood, previous studies have clearly demonstrated impairment in the thyroid hormones secretion and action in DEHP-exposed animals during critical windows of susceptibility (32, 33, 52). These data reinforce that gender, the treatment dose, and the period of exposure are crucial to determining the effects of DEHP on the HPT axis.

Although the female rats presented decreased expression of genes/proteins involved in thyroid hormone production, T3 levels were increased in the lowest treatment dose. Previous data indicated increased T3 serum levels and decreased T4 levels in animals exposed to DEHP (300 mg of DEHP/kg/day) (40, 53).

Thyroid hormone serum levels depend on the thyroid secretion rate and the expression/activity of deiodinases in the peripheral tissues (54). Therefore, the increased expression of *Dio1* mRNA in the liver of F1 female rats could contribute to the increased circulating levels of T3. Conversely, there were no significant alterations in the *Dio1* mRNA expression in the liver of adult F1 male rats, suggesting a sexually different response in the liver of DEHP-exposed animals.

The regulation of deiodinases expression by DEHP has already been reported in the literature. Indeed, the exposure of male rats to DEHP (600 mg/kg/day) for six months increased the *Dio1* mRNA expression in the liver (55). Perinatal exposure to DEHP was also related to increased *Dio1* mRNA levels in PND14 and PND21 rats (43). Furthermore, zebrafish exposed to significant environmental concentrations of DEHP presented increased expression of *Dio1* and *Dio 2* transcripts (46).

Transthyretin (*Ttr*) is the main transport protein of thyroid hormones in rats (56). In the present study, *in-utero* exposure to DEHP increased the *Ttr* mRNA expression in the liver of female offspring but not in the male one. In contrast, previous studies have demonstrated a suppressive effect of chronic DEHP exposure in the TTR expression, which was associated with decreased thyroid hormone serum levels (27). Future studies are needed to unravel the sexually dimorphic response of *Ttr* mRNA expression in DEHP-exposed animals and elucidate the molecular mechanisms involved in the differential regulation of *Ttr* mRNA expression in animals exposed during critical periods of development or during adulthood.

Finally, UDP-glucuronyltransferases (UGTs) enzymes catalyze the glucuronidation and peripheral degradation of thyroid hormones (56). The regulation of the expression of these enzymes presented a different response in the DEHP-exposed male and female offspring. Future studies might elucidate the molecular pathways in regulating these crucial enzymes in DEHP-exposed animals.

This study has some limitations. First, although we reported several alterations in the gene/protein expression in the HPT axis glands, we couldn't wholly elucidate the molecular mechanisms involved in this disruption. We have preliminary data suggesting the participation of epigenetic mechanisms in regulating thyroid transcriptional activity, but additional studies will be needed to elucidate their involvement in the disruption of

thyroid gene transcription. Moreover, the morphological analysis of the thyroid gland would contribute to understanding if the disruption of the thyroid function in the offspring animals is related to the impairment of the follicle's structure.

In summary, the data presented herein indicated, for the first time, that intrauterine exposure to DEHP causes long-term damage in the HPT axis function both in the male and female offspring. Furthermore, the results suggest that exposure to DEHP programs the offspring's thyroid function, increasing their susceptibility to developing thyroid dysfunctions during adulthood.

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 animal study was reviewed and approved by Ethics Committee on Animal Experimentation of the Hospital Israelita Albert Einstein (CEUA 3326/2018).

Author contributions

ES-V performed the treatments and experiments, processed the tissues, collected the data, performed the analysis, and wrote the paper. GH processed the tissues, collected the data, and performed the analysis. RS collected the data, performed the analysis. CS-N conceived and designed the analysis, performed the treatments, and wrote the paper. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by a grant from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) to CSN (16/18517-8).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.995491/full#supplementary-material

References

1. Bergé A, Cladière M, Gasperi J, Coursimault A, Tassin B, Moilleron R. Meta-analysis of environmental contamination by phthalates. *Environ Sci pollut Res Int* (2013) 20:8057–76. doi: 10.1007/s11356-013-1982-5

2. Hongjun Y, Wenjun X, Qing L, Jingtao L, Hongwen Y, Zhaohua L. Distribution of phthalate esters in topsoil: A case study in the yellow river delta, China. *Environ Monit Assess* (2013) 185:8489–500. doi: 10.1007/s10661-013-3190-7

3. Dickson-Spillmann M, Siegrist M, Keller C, Wormuth M. Phthalate exposure through food and consumers' risk perception of chemicals in food. *Risk Anal* (2009) 29:1170–81. doi: 10.1111/j.1539-6924.2009.01233.x

4. Rowdhwal SSS, Chen J. Toxic effects of di-2-ethylhexyl phthalate: an overview. *BioMed Res Int* (2018) 22(2018):1750368. doi: 10.1155/2018/1750368

5. Trnka B, Polan M, Zigmont VA. Exposure to di-2-ethylhexyl phthalate (DEHP) and infertility in women, NHANES 2013-2016. *Reprod Toxicol* (2021) 103:46–50. doi: 10.1016/j.reprotox.2021.05.010

6. Zou Q-Y, Hong S-L, Kang H-Y, Ke X, Wang X-Q, Li J, et al. Effect of di-(2-ethylhexyl) phthalate (DEHP) on allergic rhinitis. *Sci Rep* (2020) 10:14625. doi: 10.1038/s41598-020-71517-6

7. Crobeddu B, Ferraris E, Kolasa E, Plante I. Di(2-ethylhexyl) phthalate (DEHP) increases proliferation of epithelial breast cancer cells through progesterone receptor dysregulation. *Environ Res* (2019) 173:165–73. doi: 10.1016/j.envres.2019.03.037

8. Clark KE, David RM, Guinn R, Kramarz KW, Lampi MA, Staples CA. Modeling human exposure to phthalate esters: A comparison of indirect and biomonitoring estimation methods. *Hum Ecol Risk Assess* (2011) 17:923–65. doi: 10.1080/10807039.2011.588157

9. Serrano SE, Braun J, Trasande L, Dills R, Sathyanarayana S. Phthalates and diet: a review of the food monitoring and epidemiology data. *Environ Health* (2014) 13:1–14. doi: 10.1186/1476-069X-13-43

10. Huang C-F, Wang I-J. Changes in urinary phthalate metabolite levels before and after the phthalate contamination event and identification of exposure sources in a cohort of Taiwanese children. *Int J Environ Res Public Health* (2017) 14:935. doi: 10.3390/ijerph14080935

11. Erkekoglu P, Kocer-Gumusel B. Genotoxicity of phthalates. *Toxicol Mech Methods* (2014) 24:616–26. doi: 10.3109/15376516.2014.960987

12. Martinez-Arguelles DB, Campioli E, Culty M, Zirkin BR, Papadopoulos V. Fetal origin of endocrine dysfunction in the adult: The phthalate model. *J Steroid Biochem Mol Biol* (2013) 137:5–17. doi: 10.1016/j.jsbmb.2013.01.007

13. Mantzouratou P, Lavecchia AM, Xinaris C. Thyroid hormone signalling in human evolution and disease: A novel hypothesis. *J Clin Med* (2022) 11:43. doi: 10.3390/jcm11010043

14. Gereben B, Zavacki AM, Ribich S, Kim BW, Huang SA, Simonides WS, et al. Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr Rev* (2008) 29:838–98. doi: 10.1210/er.2008-0019

15. Mullur R, Liu Y-Y, Brent GA. Thyroid hormone regulation of metabolism. *Physiol Rev* (2014) 94:355–82. doi: 10.1152/physrev.00030.2013

16. Zuñiga LFF, Muñoz YS, Pustovrh MC. Thyroid hormones: Metabolism and transportation in the fetoplacental unit. *Mol Reprod Dev* (2022) 89:526–39. doi: 10.1002/mrd.23647

17. Patel J, Landers K, Li H, Mortimer RH, Richard K. Thyroid hormones and fetal neurological development. *J Endocrinol* (2011) 209:1–8. doi: 10.1530/JOE-10-0444

18. Bernal J. Thyroid hormone regulated genes in cerebral cortex development. *J Endocrinol* (2017) 232:R83–97. doi: 10.1530/JOE-16-0424

19. Tsai H-J, Wu C-F, Tsai Y-C, Huang P-C, Chen M-L, Wang S-L, et al. Intake of phthalate-tainted foods and serum thyroid hormones in Taiwanese children and adolescents. *Sci Rep* (2016) 6:30589. doi: 10.1038/srep30589

20. Zhao Y, Song X, Ding S, Qi W, Zhang Y, Xu Q, et al. The associations of urinary DEHP metabolite levels, serum thyroid hormones, and thyroid-related genes among the adolescent students from China: a cross-sectional study. *Environ Sci pollut Res* (2022) 29:19081–97. doi: 10.1007/s11356-021-16909-5

21. Huang P-C, Waits A, Chen H-C, Chang W-T, Jaakkola JJK, Huang H-B. Mediating role of oxidative/nitrosative stress biomarkers in the associations between phthalate exposure and thyroid function in Taiwanese adults. *Environ Int* (2020) 140:105751. doi: 10.1016/j.envint.2020.105751

22. Derakhshan A, Shu H, Broeren MAC, Lindh CH, Peeters RP, Kortenkamp A, et al. Association of phthalate exposure with thyroid function during pregnancy. *Environ Int* (2021) 157:106795. doi: 10.1016/j.envint.2021.106795

23. Huang P-C, Tsai C-H, Liang W-Y, Li S-S, Huang H-B, Kuo P-L. Early phthalates exposure in pregnant women is associated with alteration of thyroid hormones. *PloS One* (2016) 11:e0159398. doi: 10.1371/journal.pone.0159398

24. Huang P-C, Chang W-H, Wu M-T, Chen M-L, Wang I-J, Shih S-F, et al. Characterization of phthalate exposure in relation to serum thyroid and growth hormones, and estimated daily intake levels in children exposed to phthalate-tainted products: A longitudinal cohort study. *Environ pollut* (2020) 264:114648. doi: 10.1016/j.envpol.2020.114648

25. Yao H, Han Y, Gao H, Huang K, Ge X, Xu Y, et al. Maternal phthalate exposure during the first trimester and serum thyroid hormones in pregnant women and their newborns. *Chemosphere* (2016) 157:42–8. doi: 10.1016/j.chemosphere.2016.05.023

26. Ye H, Ha M, Yang M, Yue P, Xie Z, Liu C. Di2-ethylhexyl phthalate disrupts thyroid hormone homeostasis through activating the Ras/Akt/TRHr pathway and inducing hepatic enzymes. *Sci Rep* (2017) 7:40153. doi: 10.1038/srep40153

27. Liu C, Zhao L, Wei L, Li L. DEHP reduces thyroid hormones *via* interacting with hormone synthesis-related proteins, deiodinases, transthyretin, receptors, and hepatic enzymes in rats. *Environ Sci pollut Res* (2015) 22:12711–9. doi: 10.1007/s11356-015-4567-7

28. Kim S, Park G-Y, Yoo YJ, Jeong JS, Nam KT, Jee S-H, et al. Di-2-ethylhexylphthalate promotes thyroid cell proliferation and DNA damage through activating thyrotropin-receptor-mediated pathways *in vitro* and *in vivo*. *Food Chem Toxicol* (2019) 124:265–72. doi: 10.1016/j.fct.2018.12.010

29. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of *in utero* and early-life conditions on adult health and disease. *New Engl J Med* (2008) 359:61–73. doi: 10.1056/NEJMra0708473

30. Hocher B. More than genes: The advanced fetal programming hypothesis. *J Reprod Immunol* (2014) 104–105:8–11. doi: 10.1016/j.jri.2014.03.001

31. Bianco-Miotto T, Craig JM, Gasser YP, van Dijk SJ, Ozanne SE. Epigenetics and DOHaD: from basics to birth and beyond. *J Dev Orig Health Dis* (2017) 8:513–9. doi: 10.1017/S2040174417000733

32. Lv J, Li Y, Chen J, Li R, Bao C, Ding Z, et al. Maternal exposure to bis(2-ethylhexyl) phthalate during the thyroid hormone-dependent stage induces persistent emotional and cognitive impairment in middle-aged offspring mice. *Food Chem Toxicol* (2022) 163:112967. doi: 10.1016/j.fct.2022.112967

33. Zhang Y, Zhang W, Fu X, Zhou F, Yu H, Na X. Transcriptomics and metabonomics analyses of maternal DEHP exposure on male offspring. *Environ Sci pollut Res* (2018) 25:26322–9. doi: 10.1007/s11356-018-2596-8

34. Lyche JL, Gutleb AC, Bergman Å, Eriksen GS, Murk AJ, Ropstad E, et al. Reproductive and developmental toxicity of phthalates. *J Toxicol Environ Health Part B* (2009) 12:225–49. doi: 10.1080/10937400903094091

35. Blystone CR, Kissling GE, Bishop JB, Chapin RE, Wolfe GW, Foster PMD. Determination of the di-(2-ethylhexyl) phthalate NOAEL for reproductive development in the rat: Importance of the retention of extra animals to adulthood. *Toxicological Sci* (2010) 116:640–6. doi: 10.1093/toxsci/kfq147

36. Serrano-Nascimento C, Calil-Silveira J, Dalbosco R, Zorn TT, Nunes MT. Evaluation of hypothalamus-pituitary-thyroid axis function by chronic perchlorate exposure in male rats. *Environ Toxicol* (2018) 33:209–19. doi: 10.1002/tox.22509

37. Sander H, Wallace S, Plouse R, Tiwari S, Gomes AV. Ponceau s waste: Ponceau s staining for total protein normalization. *Anal Biochem* (2019) 575:44–53. doi: 10.1016/j.ab.2019.03.010

38. Xu Q, Zhao T, Ri H, Ye J, Zhao W, Zhang Y, et al. Di(2-ethylhexyl) phthalate induced thyroid toxicity *via* endoplasmic reticulum stress: *In vivo* and *in vitro* study. *Environ Toxicol* (2022) 37:2924–36. doi: 10.1002/tox.23648

39. Sun D, Zhou L, Wang S, Liu T, Zhu J, Jia Y, et al. Effect of di-(2-ethylhexyl) phthalate on the hypothalamus-pituitary-thyroid axis in adolescent rat. *Endocr J* (2022) 69:EJ17–0272r. doi: 10.1507/endocrj.EJ17-0272r

40. Wu H, Zhang W, Zhang Y, Kang Z, Miao X, Na X. Novel insights into di-(2-ethylhexyl)phthalate activation: Implications for the hypothalamus-pituitary-thyroid axis. *Mol Med Rep* (2021) 23:290. doi: 10.3892/mmr.2021.11930

41. Okosieme OE, Khan I, Taylor PN. Preconception management of thyroid dysfunction. *Clin Endocrinol (Oxf)* (2018) 89:269–79. doi: 10.1111/cen.13731

42. Niermann S, Rattan S, Brehm E, Flaws JA. Prenatal exposure to di-(2-ethylhexyl) phthalate (DEHP) affects reproductive outcomes in female mice. *Reprod Toxicol* (2015) 53:23–32. doi: 10.1016/j.reprotox.2015.02.013

43. Dong J, Cong Z, You M, Fu Y, Wang Y, Wang Y, et al. Effects of perinatal di (2-ethylhexyl) phthalate exposure on thyroid function in rat offspring. *Environ Toxicol Pharmacol* (2019) 67:53–60. doi: 10.1016/j.etap.2019.01.012

44. Chiamolera MI, Wondisford FE. Minireview: Thyrotropin-releasing hormone and the thyroid hormone feedback mechanism. *Endocrinology* (2009) 150:1091–6. doi: 10.1210/en.2008-1795

45. Pierce JG. Eli Lilly lecture: The subunits of pituitary thyrotropin–their relationship to other glycoprotein hormones. *Endocrinology* (1971) 89:1331–44. doi: 10.1210/endo-89-6-1331

46. Zhai W, Huang Z, Chen L, Feng C, Li B, Li T. Thyroid endocrine disruption in zebrafish larvae after exposure to mono-(2-ethylhexyl) phthalate (MEHP). *PloS One* (2014) 9:e92465. doi: 10.1371/journal.pone.0092465

47. Vassart G, Dumont JE. The thyrotropin receptor and the regulation of thyrocyte function and growth. *Endocr Rev* (1992) 13:596–611. doi: 10.1210/edrv-13-3-596

48. Kim MJ, Kim HH, Song YS, Kim O-H, Choi K, Kim S, et al. DEHP down-regulates tshr gene expression in rat thyroid tissues and FRTL-5 rat thyrocytes: A potential mechanism of thyroid disruption. *Endocrinol Metab* (2021) 36:447. doi: 10.3803/EnM.2020.920

49. Zhang P, Guan X, Yang M, Zeng L, Liu C. Roles and potential mechanisms of selenium in countering thyrotoxicity of DEHP. *Sci Total Environ* (2018) 619–620:732–9. doi: 10.1016/j.scitotenv.2017.11.169

50. Fernandez LP, Lopez-Marquez A, Santisteban P. Thyroid transcription factors in development, differentiation and disease. *Nat Rev Endocrinol* (2015) 11:29–42. doi: 10.1038/nrendo.2014.186

51. Kambe F, Nomura Y, Okamoto T, Seo H. Redox regulation of thyroid-transcription factors, pax-8 and TTF-1, is involved in their increased DNA-binding activities by thyrotropin in rat thyroid FRTL-5 cells. *Mol Endocrinol* (1996) 10:801–12. doi: 10.1210/mend.10.7.8813721

52. Zhao L-L, Shen R, Sun C-C, Zhang S-Y, Wang B, Sun M-F, et al. Prenatal di-(2-ethylhexyl) phthalate maternal exposure impairs the spatial memory of adult mouse offspring in a phase- and gender-dependent manner. *Environ Sci pollut Res* (2020) 27:28267–75. doi: 10.1007/s11356-020-09181-6

53. Sherif NAE-H, El-Banna A, Abdel-Moneim RA, Sobh ZK, Balah MIF. The possible thyroid disruptive effect of di-(2-ethyl hexyl) phthalate and the potential protective role of selenium and curcumin nanoparticles: A toxicological and histological study. *Toxicol Res (Camb)* (2022) 11:108–21. doi: 10.1093/toxres/tfab122

54. Bianco AC, da Conceição RR. The deiodinase trio and thyroid hormone signaling. *Thyroid Hormone Nucl Receptor* (2018), 1801:67–83. doi: 10.1007/978-1-4939-7902-8_8

55. Dong X, Dong J, Zhao Y, Guo J, Wang Z, Liu M, et al. Effects of long-term *in vivo* exposure to di-2-ethylhexylphthalate on thyroid hormones and the TSH/TSHR signaling pathways in wistar rats. *Int J Environ Res Public Health* (2017) 14:44. doi: 10.3390/ijerph14010044

56. van der Spek AH, Fliers E, Boelen A. The classic pathways of thyroid hormone metabolism. *Mol Cell Endocrinol* (2017) 458:29–38. doi: 10.1016/j.mce.2017.01.025

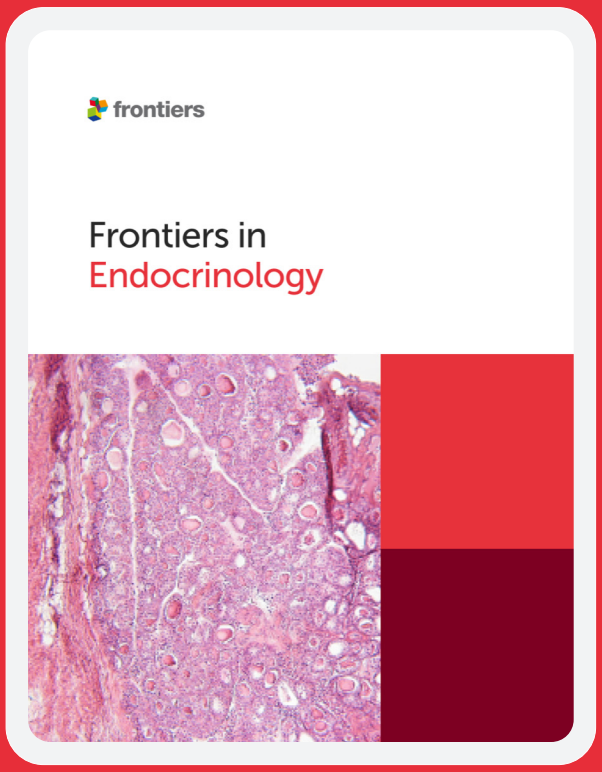
Frontiers in Endocrinology

Explores the endocrine system to find new therapies for key health issues

The second most-cited endocrinology and metabolism journal, which advances our understanding of the endocrine system. It uncovers new therapies for prevalent health issues such as obesity, diabetes, reproduction, and aging.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact