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Thyroid at the Crossroads of Public Health Challenges: From the SARS-CoV-2 Pandemic to Endocrine Disrupting Chemicals

Sofia Macedo Mireles

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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THYROID AT THE CROSSROADS OF PUBLIC HEALTH CHALLENGES: FROM THE SARS-COV-2 PANDEMIC TO ENDOCRINE DISRUPTING CHEMICALS

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Afiliação - Instituto de Patologia e Imunologia Molecular da Universidade do Porto (IPATIMUP), Instituto de Investigação e Inovação em Saúde (i3S)

Ao meu filho,

“São as nossas escolhas que revelam o que realmente somos, muito mais do que as nossas capacidades."

J.K. Rowling

Host Institutions

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PUBLICATIONS

This thesis integrates seven articles, five of them already published, and of those, three are first authorship studies. The first original study is regarding the SARS-CoV-2 infection in the thyroid of COVID-19 deceased patients and the expression of the molecules involved in the viral infection: ACE2, TMPRSS2, and Furin (**Article I**); a review paper (**Article II**) was written, following the rationale of the latter, aiming to understand the COVID-19 systemic impact, emphasizing potential thyroid involvement in long-term complications. The third paper was an original article (**Article III**) and described the expression of the three molecules involved in SARS-CoV-2 infection (ACE2, TMPRSS2, and Furin) but aimed to understand their putative role on thyroid tumourigenesis, using a series of patients with benign or malignant thyroid neoplasms. The fourth paper (**Article IV**), one systematic review, addressed the state of the art on endocrine-disrupting chemicals exposure and its impact on endocrine neoplasia. Lastly, on the scope of the previous work, our group also conducted an original study (**Article V**), regarding the effects of the endocrine-disrupting chemical, Tributyltin, on the thyroid of male rats. This thesis also includes results of two articles in preparation, one addressing a $BRAF^{V600E}$ thyroid-targeted transgenic zebrafish model (**Article VI,** in preparation) and preliminary results of Bisphenol A exposure to the same transgenic model (**Article VII,** in preparation).

Article I

Macedo S, Pestana A, Santos L, Neves C, Guimarães S, Duarte-Neto A, Dolhnikoff M, Saldiva P, Alves G, Oliveira R, Cabanes D, Carneiro F, Sobrinho-Simões M, Soares P. Detection of SARS-CoV-2 infection in thyroid follicular cells from a COVID-19 autopsy series. Eur Thyroid J. 2022 Jul 19;11(4): e220074. https://doi.org/10.1530/ETJ-22-0074

Article II

Boaventura P, **Macedo S**, Ribeiro F, Jaconiano S, Soares P. Post-COVID-19 Condition: Where Are We Now? *Life*. 2022; 12(4):517. https://doi.org/10.3390/life12040517

Article III

Azevedo MT*, Macedo S*, Canberk S, Cardoso L, Gaspar TB, Pestana A, Batista R, Sobrinho-Simões M, Soares P. Significance of Furin Expression in Thyroid Neoplastic

Transformation. Cancers (Basel). 2023 Aug 1;15(15):3909. https://doi.org/10.3390/cancers15153909 *authors contributed equally to this work

Article IV

Macedo S*, Teixeira E*, Gaspar TB*, Boaventura P, Soares MA, Miranda-Alves L, Soares P. Endocrine-disrupting chemicals and endocrine neoplasia: A forty-year systematic review. Environ Res. 2023 Feb 1; 218:114869. https://doi.org/10.1016/j.envres.2022.114869 *authors contributed equally to this work

Article V

Rodrigues-Pereira P, **Macedo S**, Gaspar TB, Canberk S, Selmi-Ruby S, Máximo V, Soares P, Miranda-Alves L. Relevant dose of the environmental contaminant, tributyltin, promotes histomorphological changes in the thyroid gland of male rats. Mol Cell Endocrinol. 2020 Feb 15; 502:110677. https://doi.org/10.1016/j.mce.2019.110677

Article VI - *In preparation*

Macedo S, Gianetti K, Ferreira T, Almeida A.L, Sobrinho-Simões M, Soares P, and Ferreira M.G**.** TP53 is required for transformation of $BRAF^{V600E}$-expressing thyroid cells in zebrafish.

Article VII - *In preparation*

Macedo S, Caridade, L, and Soares P**.** VII. Effect of the endocrine disruptor, Bisphenol A, in zebrafish embryos with thyroid cells targeted with BRAFV600E-expression.

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TABLE OF CONTENTS

PUBLICATIONS ..... **X**

ACKNOWLEDGEMENTS/ AGRADECIMENTOS ..... **XII**

TABLE OF CONTENTS ..... **XVI**

FIGURES INDEX ..... **XX**

ABBREVIATIONS ..... **XXII**

ABSTRACT ..... **XXVIII**

RESUMO ..... **XXX**

CHAPTER I | General Introduction **1**

1. The thyroid gland 1
   1.1. Thyroid physiology 1
   1.2. Thyroid disorders 3
      1.2.1. Hypothyroidism/ Hyperthyroidism 3
      1.2.2. Subacute thyroiditis 3
      1.2.3. Auto-immune thyroid disease 4
      1.2.4. Benign lesions of the thyroid 4
         1.2.4.1. Thyroid follicular nodular disease 4
         1.2.4.2. Follicular thyroid adenoma 5
      1.2.5. Malignant lesions of the thyroid 5
         1.2.5.1. Papillary thyroid Carcinoma 6
         1.2.5.2. Follicular thyroid Carcinoma 6
         1.2.5.3. Oncocytic thyroid carcinoma 7
         1.2.5.4. Poorly differentiated thyroid carcinoma 7
         1.2.5.5. Anaplastic thyroid carcinoma 7
         1.2.5.6. Medullary thyroid carcinoma 8
         1.2.5.7. Molecular genetics of thyroid cancer 8
   1.3. The risk factors underlying thyroid disease 9
      1.3.1. Demographic factors 9

1.3.2. Ionizing Radiation 9
1.3.3. Iodine levels....10
1.3.4. Viruses....10
1.3.4.1. Viruses and thyroid cancer....11
1.3.4.2. Sars-cov-2 virus and thyroid....12
1.3.5. Endocrine-disrupting chemicals 16
1.3.5.1. Thyroid gland endocrine disruptors 17
1.3.5.2. EDCs and thyroid cancer incidence 21
2. Zebrafish as a model for thyroid disease 22
2.1. Zebrafish animal model 22
2.2. Zebrafish thyroid development.... 22
2.3. Zebrafish as a cancer model system 23
3. Thyroid at the crossroads of public health 24
CHAPTER II | Research Aims 27
CHAPTER III | Materials and Methods 29
1. Patients' collection and clinicopathological data 29
2. Molecular techniques 29
3. Morphometric studies 29
4. Ecotoxicological studies 29
5. Systematic Review 30
6. Statistical analysis 30
CHAPTER IV | Results 31
I.Detection of SARS-CoV-2 infection in thyroid follicular cells from a COVID-19 autopsy series 31
II.Post-COVID-19 Condition: Where Are We Now? 45
III.Significance of Furin Expression in Thyroid Neoplastic Transformation.... 61
IV.Endocrine-disrupting chemicals and endocrine neoplasia: A forty-year systematic review 87

V.Relevant dose of environmental contaminant, tributyltin, promotes histomorphological changes in the thyroid gland of male rats 135

VI.TP53 is required for transformation of $BRAF^{V600E}$-expressing thyroid cells in zebrafish……149

VII.Effect of the endocrine disruptor, Bisphenol A, in zebrafish embryos with thyroid cells targeted with $BRAF^{V600E}$-expression 173

CHAPTER V | General discussion 187

CHAPTER VI | Conclusion 191

REFERENCES 193

FIGURES INDEX

Figure 1 -The Hypothalamic-Pituitary-Thyroid axis and the regulation of hormonal balance. 2

Figure 2 - SARS-CoV-2 entry in the host cell and the involvement of ACE2, TMPRSS2, and Furin proteins in the viral infection.....13

Figure 3 – (A) Schematic representation of the construct used to generate thyroid-expressing human BRAF^{V600E} zebrafish. Briefly, a thyroid-specific thyroglobulin promoter (tg promoter) was placed upstream of the human BRAF^{V600E} coding sequence, followed by a polyA with a downstream GFP fusion with a heart marker (cmlc2), used for positive confirmation of successful transgenesis; (B) Representative fluorescence stereoscopic images of transgenic zebrafish thyroid follicles expressing mCherry fluorescence in lower magnification (24x) and (B1) higher magnification (120x). 161

Figure 4 – Representative thyroid histology sections of control and tg-BRAF^{V600E} animals at different timepoints. 163

Figure 5 – Morphometric quantification of thyroid hyperplasia of tg-BRAF^{V600E} (n=8) and control fish (n=11); (A) Mean thyroid area ($p<0.0001$); (B) Mean follicles number ($p<0.001$) and (C) Mean follicle size ($p<0.0001$), at 3 mpf. 164

Figure 6 - Representative thyroid histology sections of control and tg-BRAF^{V600E} animals at 12 mpf. Note the enlarged follicles, resembling goiter, in the tg-BRAF^{V600E} animals. 164

Figure 7 - Body Mass Index (BMI) measured from (A) controls (n=22) and tg- BRAF^{V600E} (n=52) at 3 mpf; and (B) controls (n=25) and tgBRAF^{V600E} (n=28) at 12 mpf. Unpaired T-test comparison (**** - $p<0.0001$) and (*** - $p<0.001$), respectively. 165

Figure 8 - mRNA expression of tg ($p>0.05$), pax8 ($p>0.05$) and slc5a5 ($p<0.05$) genes in thyroid of tg-BRAFV600E (n=16) in comparison with controls (n=5) at 3 mpf. 166

Figure 9 - Protein expression of ERK and AKT proteins in thyroid tissue of tg-BRAFV600E (n=19) and control (n=17) at 3 mpf. No significant differences were observed ($p>0.05$). 167

Figure 10 – Representative thyroid histology sections of control (tp53^{M214K}) and tg-BRAFV600E tp53^{M214K} animals at different timepoints.....168

Figure 11 - Morphometric quantification of thyroid follicles of tg-BRAFV600E tp53^{M214K} (n=6) and control tp53^{M214K} fish (n=3); (A) Mean thyroid area; (B) Mean follicles number and (C) Mean follicle size, at 3 mpf. No statistically significant differences were detected (p>0.05). 169

Figure 12 - Body Mass Index (BMI) measured from (A) tp53^{M214K} (n=26) and tg-mCherry BRAF^{V600E} tp53^{M214K} (n=34) at 3 mpf; and (B) tp53^{M214K} (n=25) and tgBRAF^{V600E} (n=28) at 12 mpf. Unpaired T-test comparison (p>0.05). 169

Figure 13 - mRNA expression of tg (p<0.05), pax8 (p>0.05) and slc5a5 (p>0.05) genes in thyroid of tg-BRAF^{V600E} tp53^{M214K} (n=5) in comparison with tp53^{M214K} controls (n=5) at 3 mpf. 170

Figure 14 - Protein expression of ERK and AKT proteins in thyroid tissue of tg-BRAF^{V600E} (n=20) and tp53^{M214K}control fish (n=17). No significant differences were observed (p>0.05 171

Figure 15 - Preliminary concentration assay of WT zebrafish exposed to BPA for a 7-day period. (A) Percentage of hatched embryos (p<0.05); (B) Heart rate at 72hpf (p<0.05); (C) Percentage of abnormal behaviour (p<0.05); (D) Percentage of pericardial edema (p<0.05); (E) Percentage of tail abnormalities (p<0.05); (F) Mortality rate (p>0.05). 184

Figure 16 - BPA 21-day exposure to transgenic zebrafish larvae (tg-BRAF^{V600E}) in comparison with control larvae (tg-mCherry). mRNA quantification of (A) tg, (B) pax8, (C) tsh, and (D) slc5a5. No statistically significant differences were detected (p>0.05). 185

ABBREVIATIONS

ACE Angiotensin converting enzyme

ACE2 Angiotensin converting enzyme 2

AITD Autoimmune thyroid disorders

AKT V-Akt murine thymoma viral oncogene homolog

ALK Anaplastic lymphoma receptor tyrosine kinase

AMP Adenosine Monophosphate

Ang 1-7 Angiotensin 1-7

Ang 1-9 Angiotensin 1-9

Ang I Angiotensin I

Ang II Angiotensin II

ARAF A-Raf proto-oncogene, serine/threonine kinase

ARNm Ácido ribonucleico mensageiro

ATA American thyroid association

ATC Anaplastic thyroid carcinoma

BAC Bacterial artificial chromosome

BDE-47 2,2,4,4-tetrabromodiphenyl ether

BMI Body Mass Index

BMR basal metabolic rate

bp Base pairs

BPA Bisphenol A

BPF Bisphenol F

BPS Bisphenol S

BRAF B-type Raf kinase

C cell Parafollicular cell

cDNA Complementary deoxyribonucleic acid

cmcl2 Cardiac myosin light chain

COVID-19 Coronavirus disease 2019

CRAF C-Raf proto-oncogene, serine/threonine kinase

Cre Cre recombinase

CT Threshold cycle

DE Disruptor endócrino

Dio1 Deiodinase 1

Dio2 Deiodinase 2

DNA deoxyribonucleic acid

dpf Days *post* fertilization

dpi Days *post* injection

E Envelope protein

EBV Epstein–Barr virus

EDC Endocrine disrupting chemical

EDTA (Ethylenedinitrilo)tetraacetic acid

EGFP Enhanced green fluorescent protein

ERK Extracellular regulated kinase

FCT Fundação para a ciência e tecnologia

FFPE Formalin-fixed paraffin-embedded

FNA Fine needle aspiration

FND Thyroid follicular nodular disease

FR flame retardant

FTA Follicular thyroid adenomas

FTC Follicular thyroid carcinoma

GD Graves Disease

Globocan Global Cancer Observatory

H&E Hematoxylin and eosin staining

HCV Hepatitis C virus

HEXXH Histidine- Glutamine-X-X-Histidine

HFV Human Foamy Virus

HIV Human Immunodeficiency Virus

HM Heavy metal

HPA The Human Protein Atlas

HPF High power fields

hpf Hours *post* fertilization

hpi Hours *post* injection

HPT Hypothalamic-pituitary-thyroid axis

HT Hashimoto's Thyroiditis

HTLV-1 Human T-cell leukemia virus type 1

IARC International agency for research on cancer

IGC Instituto gulbenkian de ciência

IgG Immunoglobulin G

IHC Immunohistochemistry

LOF Loss of function

M Membrane protein

mAb Monoclonal antibody

MAPK Mitogen-activated protein kinases

mCh Monomeric mCherry

MCS Multicloning site

ME Middle entry clone

MEHP Mono-(2-ethylhexyl) phthalate

MEK Mitogen-activated protein kinase kinase

mpf Months *post* fertilization

mRNA Messenger ribonucleic acid

MS222 Tricaine Methanesulfonate

MTC Medullary thyroid carcinoma

NIS Sodium-iodide symporter

NIS Sodium-Iodine Symporter

nkx2.1a thyroid transcription factor-1

nnhex haematopoietically expressed homeobox

NRAS Neuroblastoma RAS Viral (v-ras) Oncogene Homolog

NTC Non template control

NTRK1 Neurotrophic Tyrosine Kinase, Receptor, Type 1

OTC Oncocytic thyroid carcinoma

pA PolyA

PAX2.1 Paired box gene 2.1

pax2a paired box gene 2a

PAX8 Paired box 8

PBDE polybrominated diphenyl ether

PCB Polychlorinated biphenyl

PCR Polymerase chain reaction

PDTC Poorly differentiated thyroid carcinoma

PFC Perfluorinated compound

PFOA Perfluorooctanoic acid

PFOS Perfluorooctanesulfonic acid (),s

PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase

PI3K Phosphoinositide 3-kinase gene

PPAR Peroxisome proliferator-activated receptor

PPARG Peroxisome proliferator-activated receptor gamma

PTC Papillary thyroid carcinoma

RAAS Renin-angiotensin-aldosterone system

RAS Rat Sarcoma viral oncogene homolog

RBD Receptor Binding Domain

RET Rearranged during Transfection

RIPA Radioimmunoprecipitation Assay Buffer

RNA Ribonucleic acid

RPM Revolutions Per Minute

RT-PCR Real-time polymerase chain reaction

S Spike protein

SARS-CoV-2 severe acute respiratory syndrome coronavirus 2

SAT Subacute thyroiditis

SDS PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS Sodium dodecyl sulfate

slc5a5 Solute carrier family 5 (sodium/iodide cotransporter) subunit alpha

SV40 Simian Virus 40

T2A Thosea asigna virus 2A

T3 Triiodothyronine

T4 Thyroxine

TAE Tris base, acetic acid and EDTA solution

TBBPA Tetrabromobisphenol A

TBBPS Tetrabromobisphenol S

TBT Tributyltin

TC Thyroid Cancer

TERT Telomerase reverse transcriptase

tg Thyroglobulin

TH Thyroid Hormone

Thr Threonine

TMPRSS2 Transmembrane serine protease 2

TPO Thyroperoxidase

TRAb Thyroid stimulating hormone receptor antibody

TRH Thyrotropin-releasing hormone

TSH Thyroid stimulating hormone

TSHr Thyroid stimulating hormone receptor

UGT1ab glucuronosyltransferase 1

WHO World health organization

WISH Whole-mount *in situ* hybridization

WT Wild-type

ABSTRACT

The thyroid gland, a crucial component of the endocrine system, plays a critical role in regulating various physiological processes by secreting hormones that influence embryonic development, metabolism, and overall body function. A multitude of environmental threats pose risks to public health, including pollution, infectious agents like the SARS-CoV-2 virus, and the introduction of harmful substances such as Endocrine-Disrupting Chemicals (EDCs) into the ecosystems. These threats, in turn, play a role in various issues, and so it is of great importance to recognize and comprehend them.

This thesis investigated the thyroid gland as a key focal point to address two of these emerging challenges: the impact of the SARS-CoV-2 virus on thyroid follicular cells and the persistent threat posed by the EDCs in thyroid function.

We have proven that thyroid follicular cells can be directly infected by the SARS-CoV-2 virus, by detecting the presence of viral particles in thyroid tissues of a series of COVID-19 deceased patients. In the context of COVID-19, characterized by systemic involvement and sometimes chronic complications, we reviewed the risk factors contributing to the patient's prolonged symptoms (post-COVID-19 condition), and the potential of thyroid involvement in this condition. We also examined the molecules involved in the mechanisms of the viral infection: ACE2, TMPRSS2 and Furin in a series of benign and malignant thyroid neoplasms and we observed that overexpression of Furin mRNA, one of the key molecules for this process, revealed to be correlated with poor prognostic factors for thyroid tumours, such as lymph node metastization.

EDCs are substances known to interfere with the endocrine system's normal functioning, and the thyroid is particularly vulnerable to their effects. We conducted a comprehensive review spanning four decades of studies, which underscored thyroid's susceptibility to EDCs, linking their exposure to endocrine neoplasia. We have also demonstrated the harmful effects of a well-known EDC, Tributyltin, in the thyroid gland of male rats.

To confront the challenges of studying EDCs, a transgenic zebrafish model expressing $BRAF^{V600E}$ mutation, specifically in thyroid cells was developed. This model exhibits abnormal thyroid morphogenesis and hyperplasia which allowed us to have a unique platform for preliminary assessments of EDCs' potential role in the early onset of thyroid disease and the promotion of cancer within a genetically predisposed context.

This research places its findings within the landscape of public health and hopefully provides foundation for strategies that mitigate the impact of infectious diseases as the

SARS-CoV-2 on thyroid health and address the complex relationship between EDCs and thyroid neoplasia, integrating efforts to safeguard thyroid health and human well-being.

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RESUMO

A glândula tiroideia, uma componente crucial do sistema endócrino, desempenha um papel crítico na regulação de vários processos fisiológicos, secretando hormonas que influenciam o desenvolvimento embrionário, o metabolismo e em geral homeostasia do organismo. Uma multitude de ameaças ambientais, como a poluição, agentes infeciosos como o vírus SARS-CoV-2 e a introdução de substâncias nocivas para os ecossistemas, como os Disruptores Endócrinos (DEs), apresentam um risco para a saúde pública. Essas ameaças, por sua vez, desempenham um papel ao nível da saúde pública, sendo, por isso, de grande importância reconhecê-las e compreendê-las.

Esta tese investigou a glândula tiroideia como um órgão chave desafiado por dois destes reptos emergentes: o impacto do vírus SARS-CoV-2 nas células foliculares da tiróide e a ameaça persistente colocada pelos DEs na função tiroideia.

Nós comprovamos que as células foliculares da tiroide podem ser diretamente infetadas pelos vírus SARS-CoV-2, ao detetarmos a presença de partículas virais em tecidos de tiroide de uma série de pacientes que faleceram no decurso de infeção por COVID-19. No contexto da COVID-19, que é caracterizada por ter um envolvimento sistémico e por vezes complicações crónicas, nós fizemos uma revisão dos fatores de risco que podem contribuir para os sintomas prolongados destes pacientes (condição pós-COVID-19) e o potencial envolvimento da tiroide nesta condição. Examinamos também as moléculas envolvidas nos mecanismos da infeção viral: ACE2, TMPRSS2, e Furina, numa série de neoplasias benignas e malignas de tiroide e observamos que a sobrexpressão do ARNm da Furina, uma molécula chave para este processo, revelou estar correlacionada com fatores de pior prognóstico em tumores da tiroide, como metastização dos linfonodos.

Os DEs são substâncias que se sabe que interferem no funcionamento normal do sistema endócrino, e a tiróide é particularmente suscetível aos efeitos destes compostos. Realizamos uma revisão sistemática que abrangeu quatro décadas de estudos, onde se destacou a susceptibilidade da tiróide a DEs, estabelecendo uma correlação entre a exposição a essas substâncias e a neoplasia endócrina. Demonstramos também os efeitos nocivos de um DE bem conhecido, o Tributilestanho, na tiroide de ratos macho. Para confrontar os desafios de estudar os DEs, foi desenvolvido um modelo de peixe-zebra transgénico, que expressa a mutação $BRAF^{V600E}$ especificamente nas células da tiroide. Este modelo exibia uma morfologia anormal da tiroide e hiperplasia, o que nos permitiu ter uma plataforma única para a avaliações preliminares do potencial dos DEs de induzir

precocemente doença da tiroide e promover o desenvolvimento de cancro, num contexto de predisposição genética.

Este trabalho insere as suas descobertas no panorama da saúde pública, esperando que se forneça uma base para estratégias de forma a amenizar o impacto de doenças infecciosas como o SARS-CoV-2, na saúde da tiróide, e abordar a relação complexa entre os DEs e a neoplasia da tiróide, realizando esforços de forma a proteger a saúde da tiróide e o bem-estar humano.

CHAPTER I | GENERAL INTRODUCTION

1. The thyroid gland

The thyroid gland stands as a central organ within the network of the endocrine system, assuming an essential role in the coordination of diverse physiological processes throughout the body. It is the largest endocrine gland, located in the neck, just below the Adam's apple with a butterfly-shaped structure consisting of two lobes that are connected by a thin tissue, the isthmus (Armstrong et al., 2023).

The thyroid parenchyma is primarily composed of two distinct endocrine cell types: follicular cells and parafollicular cells (Khan & Farhana, 2023). Follicular cells are responsible for synthesizing and releasing thyroid hormones, while parafollicular cells, or C cells, produce calcitonin, involved in calcium regulation (Khan & Farhana, 2023). This thesis will be centred in thyroid follicular cells and associated diseases.

The thyroid gland is composed of small spherical structures, the follicles, that predominantly synthesize two key hormones: thyroxine (T4) and triiodothyronine (T3). The hormone T4 represents roughly 90% of the total hormone produced by the gland, initially in an inactive state and that later undergoes conversion into the active form, T3, within various tissues of the body (Armstrong et al., 2023). These hormones assume a critical role in overseeing the body's metabolism, energy generation, and growth processes (Armstrong et al., 2023; Nilsson & Fagman, 2017).

1.1. Thyroid physiology

The physiology of the gland is a finely tuned system. The release of thyroid hormones is managed by the hypothalamus-pituitary-thyroid (HPT) axis. The hypothalamus initiates this process by secreting thyrotropin-releasing hormone (TRH), prompting the pituitary gland to release thyroid-stimulating hormone (TSH) (Figure 1). Subsequently, TSH acts as the catalyst, inducing the thyroid gland to synthesize and release the vital hormones T3 and T4 (Feldt-Rasmussen et al., 2021) (Figure 1).

The HPT axis employs a negative feedback mechanism to support hormonal balance. Elevated thyroid hormone levels suppress the release of TRH and TSH, thereby diminishing additional hormone production. Conversely, decreased thyroid hormone levels prompt the secretion of TRH and TSH, fostering an increase in hormone production to restore balance (Feldt-Rasmussen et al., 2021; Gavrila & Hollenberg, 2019) (Figure 1).

They play a crucial role in the development and function of the nervous system. T4 and T3, are crucial for fetal neural development, shaping the central nervous system and preventing congenital hypothyroidism-related impairments. Throughout life, these hormones maintain neural functions, impacting myelination, synaptic transmission, and brain metabolism. Imbalances can lead to cognitive and mood changes, highlighting the vital role of thyroid function in neurological well-being (Feldt-Rasmussen et al., 2021; Gavrila & Hollenberg, 2019).

Thyroid hormones are essential for maintaining the basal metabolic rate (BMR), which determines the body's baseline energy expenditure at rest; influencing how the body uses energy. T3, the more active form, directly influences cellular respiration and the utilization of nutrients, such as carbohydrates and fats, for energy. In essence, thyroid hormones act as metabolic regulators, ensuring a proper balance in energy metabolism throughout the body's tissues. An adequate level of thyroid hormones is crucial for maintaining overall metabolic efficiency, body temperature, and energy homeostasis. Imbalances, such as hypothyroidism or hyperthyroidism, can lead to disruptions in BMR, resulting in symptoms like weight gain or loss, fatigue, and alterations in energy utilization (Jacobsen et al., 2006; M. J. Kim et al., 2018).

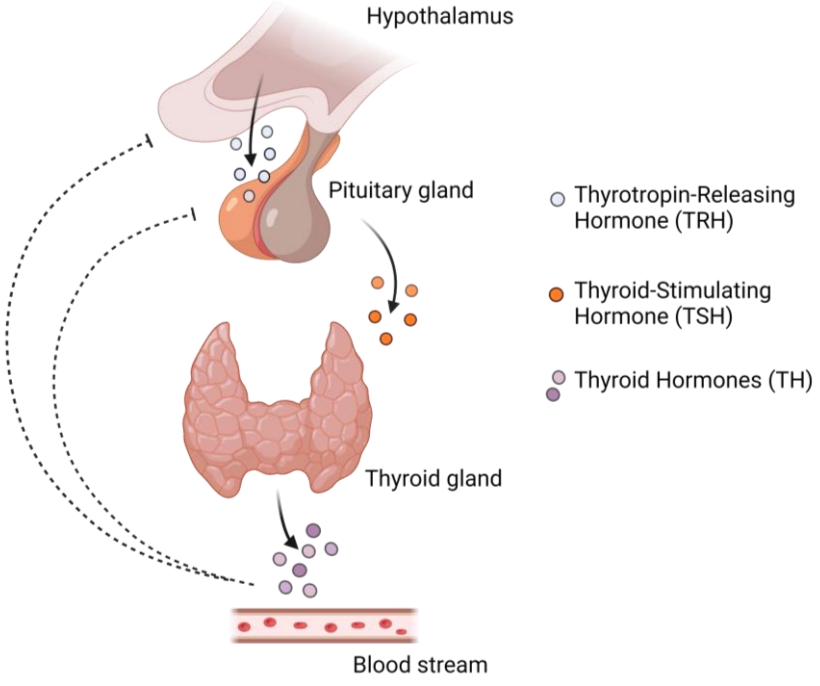

Figure 1 -The Hypothalamic-Pituitary-Thyroid axis and the regulation of hormonal balance.

1.2. Thyroid disorders

Disorders of the thyroid gland encompass a spectrum of conditions that disrupt the normal functioning of the thyroid and can present inflammatory (mainly autoimmune) or neoplastic characteristics. Thyroid autoimmune disorders, prevalent worldwide, can result from either in overactivity or underactivity of the thyroid gland. These conditions may lead to the enlargement of the thyroid gland, causing direct symptoms and discomfort in affected individuals (Moini et al., 2020).

1.2.1. Hypothyroidism/ Hyperthyroidism

Hypothyroidism is characterized by insufficient production of thyroid hormones (T3 and T4), and manifests with symptoms such as fatigue, weight gain, cold intolerance, dry skin, and muscle weakness. Common causes of this disease include autoimmune thyroiditis (Hashimoto's disease), iodine deficiency, certain medications, and surgical removal of the thyroid. Timely identification and appropriate management, often involving thyroid hormone replacement therapy, are crucial for alleviating symptoms and restoring hormonal balance in individuals with hypothyroidism (Chaker et al., 2017).

Hyperthyroidism, on the other hand, results from the excessive production of thyroid hormones, leading to symptoms such as weight loss, rapid heartbeat, anxiety, heat intolerance, and tremors. Common causes include Graves' disease, an autoimmune disorder, toxic nodular goiter, and thyroiditis. Effective management of hyperthyroidism often involves medications to regulate hormone levels, radioactive iodine therapy, or, in some cases, surgical intervention. Early diagnosis and targeted treatment are essential to alleviate symptoms and restore thyroid function to a balanced state (De Leo et al., 2016).

1.2.2. Subacute Thyroiditis

Subacute Thyroiditis (SAT), also known as De Quervain thyroiditis or granulomatous thyroiditis is a self-limited, inflammatory condition affecting the thyroid gland. It typically follows a viral infection, such as a respiratory infection, associated with painful symptoms and various systemic symptoms (Ray et al., 2022). The inflammation results in the enlargement of the thyroid gland, the release of thyroid hormones stored, leading to a phase of temporary hyperthyroidism. As the inflammation progresses, the damaged thyroid tissue may release an excess of thyroid hormones into the bloodstream. Simultaneously, the insufficient production of new thyroid hormones can result in a temporary phase of

hypothyroidism (Ray et al., 2022). Unlike chronic autoimmune thyroiditis, subacute thyroiditis is often a self-limiting condition. However, during the recovery phase, the thyroid gland may become underactive, resulting in hypothyroidism (Ray et al., 2022).

1.2.3. Auto-immune thyroid disease

Auto-immune thyroid diseases (AITD) are conditions in which the immune system mistakenly targets and attacks the thyroid gland, leading to dysfunction and potential long-term health issues. Among the diverse autoimmune thyroid diseases, two of the most prevalent are Hashimoto's thyroiditis and Graves' disease.

Hashimoto's Thyroiditis (HT) is a chronic autoimmune condition marked by inflammation of the thyroid gland, leading to the gradual destruction of thyroid tissue. The immune system produces antibodies that erroneously target and damage the thyroid follicular cells, resulting in a reduction in thyroid hormone production. This autoimmune attack manifests with typical symptoms of hypothyroidism, as mentioned above (Antonelli et al., 2015; Ferrari et al., 2020).

Graves' Disease (GD), on the other hand, represents an autoimmune disorder characterized by an overactive thyroid, a condition known as hyperthyroidism. In this scenario, specific antibodies, thyroid-stimulating immunoglobulins (TSI), like TSH receptor antibody (TRAb) stimulate the thyroid gland to produce excessive thyroid hormones. Common symptoms of Graves' disease encompass weight loss, rapid heartbeat, anxiety, heat intolerance, and Graves' ophthalmopathy, marked by bulging eyes. Diagnosis involves blood tests to measure thyroid hormones, TSI, and thyroid function. Treatment options vary and may include anti-thyroid medications, radioactive iodine therapy, or, in certain cases, surgical interventions to remove part (or all) of the thyroid. Each condition requires tailored management strategies to restore thyroid function and alleviate associated symptoms (Antonelli et al., 2015; Ferrari et al., 2020).

1.2.4. Benign lesions of the thyroid

1.2.4.1. Thyroid follicular nodular disease

Thyroid follicular nodular disease (FND), previously designated as multinodular goiter, is identified by an enlarged thyroid encompassing multiple nodules, which can range in size and composition (Baloch et al., 2022). While common, most thyroid nodules are non-cancerous (benign), often discovered incidentally during imaging studies or physical examinations. Although most nodules are asymptomatic, larger ones or those causing

compression may lead to difficulty swallowing or breathing. Diagnosis typically involves imaging studies and fine needle aspiration (FNA) biopsy to determine whether a nodule is benign or cancerous. The presence of these nodules can impact thyroid function, potentially giving rise to either hyperthyroidism or hypothyroidism since the nodules may not respond adequately to TSH stimulation due to autonomous functioning (Can & Rehman, 2020; Mittal et al., 2020). This disruption in the feedback loop can result in imbalances in thyroid hormone levels, leading to conditions such as hypothyroidism or hyperthyroidism, depending on the nodules' specific characteristics and impact on thyroid function (Can & Rehman, 2020).

1.2.4.2. Follicular thyroid adenoma

Follicular thyroid adenomas (FTAs) represent the most frequent benign neoplasms originating from follicular cells (McHenry & Phitayakorn, 2011; Norris & Farci, 2023). These entities are characterized by well-differentiated follicular structures and are encapsulated. FTAs do not invade surrounding tissues or blood vessels, and their cellular features closely resemble normal thyroid follicular cells (McHenry & Phitayakorn, 2011; Norris & Farci, 2023). While non-cancerous, follicular thyroid adenomas can sometimes present challenges in diagnosis, as their histological characteristics may overlap with those of the malignant counterpart, follicular thyroid carcinomas (FTCs). FNA biopsies and thorough histopathological examinations are crucial for accurate diagnosis and differentiation between benign and malignant follicular thyroid lesions. Most of the patients are asymptomatic and the majority of the nodules are incidentally discovered by ultrasound or palpation (McHenry & Phitayakorn, 2011; Norris & Farci, 2023). Hence, surgical removal is usually not recommended, unless they are symptomatic adenomas or when diagnostic uncertainty persists.

1.2.5. Malignant lesions of the thyroid

Thyroid cancer is the most frequent neoplasm in the endocrine system (Raue & Frank-Raue, 2016). This malignancy is characterized by having a wide variety of histotypes, with a very heterogeneous frequency among them (Pizzato et al., 2022). The most common histotypes include papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), both well differentiated, originating from follicular cells. Anaplastic thyroid carcinoma (ATC) is a rare, and extremely aggressive and undifferentiated subtype of thyroid cancer,

characterized by rapid growth, invasion of surrounding tissues, and a high chance of metastasis. Finally, the poorly differentiated thyroid carcinoma (PDTC) is also a rare and aggressive subtype of thyroid cancer that exhibits biological characteristics intermediate between well-differentiated thyroid cancers, such as papillary and follicular carcinomas, and undifferentiated anaplastic thyroid carcinomas (Garcia-Rostan & Sobrinho-Simões, 2011).

In this thesis, we address the classification of thyroid tumours, acknowledging the latest update from the revised classification by the WHO Classification of Tumours Editorial Board (Baloch et al., 2022). However, in order to align with the temporal context of the publications, our results and discussions will refer to the previous classification.

1.2.5.1. Papillary Thyroid Carcinoma

Papillary Thyroid Carcinoma (PTC) is the predominant histotype, accounting for more than 80-85% of all thyroid malignancies (Limaiem et al., 2019; LiVolsi, 2011). Histologically, the nuclear features, although not diagnostic, are characteristic of this subtype with clear or empty nuclei appearance that results from the finely dispersed chromatin (orphan Annie-eye nuclei appearance) (LiVolsi, 2011). The classic papillary thyroid carcinoma histotype (cPTC) is identified by well-formed papillae, but several subtypes of PTC can present distinct morphologies, wherein tumour cells exhibit distinct nuclear characteristics (Baloch et al., 2022). Macroscopically, PTCs can be unifocal or multifocal entities, exhibiting variable sizes, with an average diameter of 2-3 centimeters (LiVolsi, 2011). This histotype is characterized by an indolent behavior, and exhibits good prognosis, with up to 25-year survival rates (LiVolsi, 2011).

1.2.5.2. Follicular Thyroid Carcinoma

Follicular thyroid carcinoma (FTC) represents 10-15% of all thyroid cancers. It is a malignant epithelial tumour with follicular cell differentiation that lacks the nuclear features of papillary thyroid carcinoma (Sobrinho-Simoes et al., 2011). FTC are frequently isolated tumours, encapsulated, with minimal capsular invasion, or, not so frequent, may be highly invasive and present extensive infiltration of the capsule and/or vascular invasion. Microscopically, these tumours present small/medium-sized follicles. Solid or rarely trabecular growth patterns can also be present (Schmid & Farid, 2006; Sobrinho-Simoes et al., 2011). Although presenting in general, a prognosis similar to PTC, it also depends on

the degree of the tumour invasion; minimally invasive tumours present much better prognosis than widely invasive carcinomas (Sobrinho-Simoes et al., 2011).

1.2.5.3. Oncocytic thyroid carcinoma

Oncocytic thyroid carcinoma (OCA) represent about 3% of all thyroid carcinomas and can present a more aggressive behaviour. It is an invasive malignant follicular cell neoplasm, and constituited by at least 75% of oncocytic cells (Baloch et al., 2022). These tumours tend to be associated with vascular invasion and distant metastases, making this variant more aggressive than the other well-differentiated thyroid tumours (Baloch et al., 2022; Goffredo et al., 2013).

1.2.5.4. Poorly differentiated thyroid carcinoma

Poorly Differentiated Thyroid Carcinoma (PDTC) is a rare subtype of thyroid carcinoma, accounting for 1-3% of all thyroid tumours (Ibrahimpasic et al., 2019). The biological behaviour falls somewhere between that of differentiated thyroid carcinomas (PTC and FTC) and anaplastic thyroid carcinomas (ATC) (Bellini et al., 2021). The Turin proposal outlines three criteria for diagnosing PDTC: the presence of a solid/trabecular/insular growth pattern; the absence of nuclear features typical of PTC; and the presence of at least one of the following three features: a mitotic index ≥3 high power fields (HPFs), necrosis, or convoluted nuclei. PDTCs are more frequently detected in older patients, with worse clinical pathological features such as advanced locoregional disease and distant metastases (Ibrahimpasic et al., 2019).

1.2.5.5. Anaplastic thyroid carcinoma

Anaplastic thyroid carcinoma (ATC) is an extremely rare, undifferentiated, and and very aggressive neoplasm (Bible et al., 2021). It represents less than 2% of all cases of thyroid carcinoma but accounts for 20-50% of all deaths for this malignancy (Chintakuntlawar et al., 2019). These lesions can be characterized by the presence of spindle shaped cells with a high mitotic index, infiltrative growth pattern, necrotic areas and vascular invasion (Limaiem et al., 2019). Patients with ATC are older and, locoregional and distant metastases are frequent at the time of presentation (Alobuia et al., 2020; Limaiem et al., 2019). These tumours exhibit resistance to conventional therapies (radioactive iodine), posing a significant challenge in the management of the disease (Chintakuntlawar et al., 2019).

1.2.5.6. Medullary thyroid carcinoma

Medullary thyroid carcinoma (MTC) is a rare neuroendocrine tumour that originates from the thyroid parafollicular cells (Lee et al., 2017). It accounts for 2-4% of all thyroid tumours (Ceolin et al., 2019). This neoplasm in the majority of cases (75%) occurs sporadically but 25% account for the familial syndrome multiple endocrine neoplasia type 2 (MEN2). The first tends to appear at older ages as the latter at younger ages (second and third decade of life) due to its hereditary nature (Kim & Kim, 2021). Patients frequently present an isolated nodule accompanied by an increase in the parathyroid hormone. This lesion is characterized by nests of rounded cells or with ovoid structure with fibrovascular stroma (Master & Burns, 2017). Patient outcomes are typically associated with factors such as tumour stage, patient age, tumour size, and the presence of local or distant metastases at the time of presentation. Nevertheless, the majority of patients diagnosed in the early stages of the disease have a favourable prognosis (Master & Burns, 2017). This neoplasia will not be further addressed in the present thesis.

1.2.5.7. Molecular genetics of thyroid cancer

Thyroid cancer, a heterogeneous disease with various histotypes, exhibits distinct mutations in critical genes through either point mutations or chromosomal rearrangements (Pak et al., 2015).

In PTC, prevalent mutations include the $BRAF^{V600E}$ mutation, occurring in 45-80% of cases (Vuong et al., 2017), and RAS mutations in 15%, with HRAS and NRAS being frequently mutated (Nylén et al., 2020). Additionally, around 10-20% of PTC cases involve RET/PTC rearrangements (Hu et al., 2021), fusions of the RET proto-oncogene with various partner genes, or NTRK fusions (Salvatore et al., 2021). As for FTC, it is often characterized by RAS mutations, occurring in approximately 50% of cases (Giordano, 2018; Macerola et al., 2021), and a subset of cases (up to 40%) exhibits PAX8-PPARγ rearrangements (Nikiforova et al., 2003).

ATC, known for its aggressive nature, is characterized by a high frequency of TP53 mutations, occurring in approximately 80% of cases (Elia et al., 2022; Reddi et al., 2015). BRAF mutations, specifically the V600E mutation, are observed. TERT promoter mutations can also be found in high frequencies (Elia et al., 2022). PDTCs, on the other hand, can

have higher frequencies of RAS and PI3K/AKT gene fusions (RET, ALK, NTRK1, NTRK3) (Xu & Ghossein, 2016; Xu & Ghossein, 2020)

Overall, the molecular characterization of thyroid cancer histotypes is of great importance since the presence of specific mutations are closely related to the tumour morphology and can influence prognosis, treatment strategies, and overall clinical management.

1.3. The risk factors underlying thyroid disease

The thyroid gland's susceptibility to environmental triggers, such as radiation and contaminants, stems from its unique physiological characteristics and vital endocrine functions.

1.3.1. Demographic factors

Sex and age are recognized as significant demographic variables when it comes to exerting influence on the incidence of thyroid disease. Women exhibit a higher susceptibility to thyroid disorders compared to men, namely cancerous nodules (Liu et al., 2017). The risk of thyroid disorders rises with age, particularly affecting older individuals who are more prone to conditions like hypothyroidism and nodular goiter (Moini et al., 2020). For thyroid cancer, the peak of incidence is in the fourth decade of life. Age is also a significant factor in the prognosis of thyroid cancer patients, since the risk of progression is higher in individuals > 55 years (K. Kim et al., 2018). Regular monitoring is crucial for early detection and management.

1.3.2. Ionizing Radiation

Ionizing radiation (IR) manifests as waves or particles with the potential to displace electrons from atoms and consequently with the potential to induce DNA strand breaks and genetic alterations, such as mutations and rearrangements (El-Benhawy et al., 2022). Due to its anatomical location, the thyroid gland is one of the target organs for radiation-related disease (El-Benhawy et al., 2022). Exposure to IR, particularly during childhood or in occupational settings, emerges as a significant risk factor for thyroid cancer, in fact, until recent years, childhood exposure to ionizing radiation was considered the only established risk factor for thyroid cancer (Kitahara & Schneider, 2022). This exposure may result from medical treatments, nuclear accidents, or certain occupations that entail increased radiation exposure. After US atomic bombings in 1945, exposure to radiation impacted severely Japanese children’s thyroids, that showed an increased risk of developing thyroid cancer in

the five decades after atomic radiation exposure (Fiore et al., 2019). After Chernobyl (1986), accident, there was an increased incidence of thyroid cancer among children and adolescents exposed to radioactivity many years after the Chernobyl accident (Fiore et al., 2019). The impact of radiation on the thyroid emphasizes the importance of rigorous safety measures and monitoring, which calls for the need of awareness and preventive strategies in vulnerable populations.

1.3.3. Iodine levels

Iodine levels play a pivotal role in thyroid health, as it is essential for the synthesis of thyroid hormones. Both insufficient and excessive intake of iodine can contribute to thyroid problems. Iodine deficiency can lead to disorders like goiter and hypothyroidism (Kalarani & Veerabathiran, 2022). Conversely, excessive iodine intake, often due to dietary supplements or certain medications, can also disrupt thyroid function and cause long-term hypothyroidism, known as the Wolf-Chaikof effect (Kalarani & Veerabathiran, 2022).

Despite extensive worldwide initiatives to increase iodine intake, it is estimated that one-third of the global population resides in regions with iodine deficiency (Vanderpump, 2019). Iodine deficiency is now emerging as a concern even in industrialized nations, previously considered iodine-sufficient (Vanderpump, 2019). Striking a balance in iodine intake is crucial for maintaining optimal thyroid function and preventing associated disorders. Regular monitoring of iodine levels, particularly in populations at risk as children and pregnant women, is essential for ensuring thyroid health.

1.3.4. Viruses

The association between viral infections and thyroid disorders such as Hashimoto's thyroiditis has been explored, showing an increased incidence of Hashimoto's thyroiditis following viral infections, particularly those affecting the upper respiratory tract (Desailloud & Hober, 2009; Scappaticcio et al., 2021). Viruses are associated with inflammatory-immune responses, that can impact lifelong thyroid function. Thyroid hormones can modulate innate and adaptive immune responses through genomic and nongenomic mechanisms. T4 and T3 hormone concentrations can stimulate the production and release of cytokines, potentially contributing to the "cytokine storm" observed in systemic viral infections (De Vito et al., 2011).

Subacute thyroiditis (SAT) is a condition that involves inflammation of the thyroid gland, and it is often associated with viral infections. The initial descriptions indicated a tendency

to manifest following upper respiratory tract infections or sore throats, explaining the association with viral infections as the underlying cause (Desailloud & Hober, 2009). Desailloud and Hober (2009) conducted a review, highlighting that the literature consistently provides well-described direct evidence of viral presence in the thyroid, which is closely associated with illnesses affecting the organ. Retroviruses such as Human Foamy Virus (HFV) and mump virus (mumps) are described on SAT, other virus such as Human T-cell Leukemia Virus Type 1 (HTLV-1), HFV, Human Immunodeficiency Virus (HIV), and Simian Virus 40 (SV40) were identified in Graves Disease (GD). Additionally, in Hashimoto's Thyroiditis (HT), the presence of HTLV-1, enterovirus, rubella, mump, HSV, Epstein-Barr and parvovirus were documented (Desailloud & Hober, 2009).

This association suggests a potential link between viral infections and the development or exacerbation of thyroid diseases but the interplay between genetic factors, specific viral strains, and the individual's immune response emphasizes the complexity of the relationship between viral infections and the development of thyroid disorders (Desailloud & Hober, 2009; Scappaticcio et al., 2021).

1.3.4.1. Viruses and thyroid cancer

Viruses and biological agents are believed to contribute to 25% of all human cancers globally, with Epstein–Barr virus (EBV) alone accounting for 2% of these cases (Almeida et al., 2019). EBV exhibits a very high worldwide seroprevalence and is classified as a human carcinogen within the herpesvirus family, implicated in various malignancies. It has been extensively studied in thyroid specimens, particularly in cases of autoimmune and neoplastic lesions. The presence of EBV in thyroid cancer (TC) is reported with considerable variation (attributed to differences in population characteristics and the methodologies) (Almeida et al., 2019). In a series of 57 thyroid cancer patients and 18 samples from healthy controls, EBV DNA was identified in 71.9% of the samples, and its presence was linked to a progressive development of thyroid tumours, demonstrating that EBV may have a role in thyroid tumourigenesis (Moghoofei et al., 2019). Despite these investigations, the precise role of EBV in thyroid diseases remains unclear (Almeida et al., 2019) so there is an urgency to understand the tumourigenic mechanisms of EBV in thyroid cancer.

Hepatitis C virus (HCV) infection also has been highlighted to be associated with diseases beyond liver-related conditions, including autoimmune thyroid disorders and as a risk factor for several cancers, including hepatocellular carcinoma, and it has been questioned the implication of HCV in thyroid cancer risk but the results have not been

consistent (Wang et al., 2017). Wang et al (2017) conducted a meta-analysis involving five studies, encompassing 367 cases of thyroid cancer. After adjusting for heterogeneity of the data, the results revealed a significant association, indicating that HCV infection is linked to an increased risk of thyroid cancer (Wang et al., 2017).

The investigation of the relationship between the COVID-19 (caused by the SARS-CoV-2 virus) and thyroid cancer, has been analysed in two different ways; 1) the effect of thyroid cancer on covid-19 risk, or 2) the effect of COVID-19 on predisposition to thyroid cancer (Tsang et al., 2020). As reviewed by Tsang, regarding the first, individuals with small, well-differentiated thyroid tumours and not undergoing active treatment, are deemed less likely to face an increased risk for COVID-19 or adverse outcomes, and the subset of patients actively undergoing therapy, may be immunocompromised, having a higher risk for COVID-19. This population with increased susceptibility to acquire respiratory viruses, faces a multifactorial risk, and are estimated to be ten times more likely to acquire sepsis than those without cancer, often succumbing to non-cancer-related diseases (Tsang et al., 2020). Secondly, to this date, no data seems to connect other coronaviruses to thyroid cancer, but none of them had such high rates of infection worldwide as COVID-19.

1.3.4.2. Sars-cov-2 virus and thyroid

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a coronavirus that emerged in late 2019 in Wuhan, China. It is the virus responsible for the coronavirus disease (COVID-19) pandemic, which has had widespread and profound impacts on global health, economies, and daily life. SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus from the coronaviridae family (genus Betacoronavirus). It shares 79% nucleotide sequence identity with the previous SARS-CoV, accountable for the 2002–2004 SARS outbreak and both belong to the coronaviruses family, which also includes the MERS-CoV virus (Middle East Respiratory Syndrome) (Jackson et al., 2022). Within the architecture of the virus, essential structural components include the nucleocapsid (N), membrane (M), envelope (E), and spike (S) proteins, depicted in Figure 2. Among these, the S protein stands out with its distinctive crown-like morphology and its pivotal role in facilitating the virus's entry into host cells by engaging with the receptor on the cell surface (Jackson et al., 2022).

Comprising two subunits, S1 and S2 (Figure 2), the S protein epitomizes the virus's ability to recognize and bind to host cells, a crucial step in the infection process. S1 contains the receptor-binding domain (RBD) (Xia et al., 2020), which interacts with host cell receptors,

while S2 attaches the protein to the membrane and facilitates membrane fusion, enabling viral entry into the host cell (Jackson et al., 2022). The SARS-CoV-2 virus is primarily known to target the human respiratory tract, but it can be widespread throughout the body. This ability is mainly due to the ubiquitous cellular presence of its main receptor angiotensin-converting enzyme 2 (ACE2) (Lan et al., 2020; Shang et al., 2020). To ensure a highly efficient infection, the SARS-CoV-2 virus utilizes not only the ACE2 receptor but also relies on two main proteases: TMPRSS2 and Furin (Jackson et al., 2022) (Figure 2).

Thyroid is one of the organs with higher expression levels of ACE2 (Wang et al., 2020), making this organ an obvious target for infection. In fact, associations between SARS-CoV-2 infection and thyroid-related issues have emerged. Studies have reported clinical signs of subacute thyroiditis (Brancatella et al., 2020; Rotondi et al., 2021) and thyrotoxicosis (Harris & Al Mushref, 2021; Lania et al., 2020) in COVID-19 patients, suggesting a possible impact on the thyroid gland. The mechanisms and extent of the virus' involvement in the thyroid are not yet fully understood, making it an area of interest for further investigation. Exploring the interaction between SARS-CoV-2 and the thyroid is crucial for a comprehensive understanding of the virus's effects on various organs and systems within the human body.

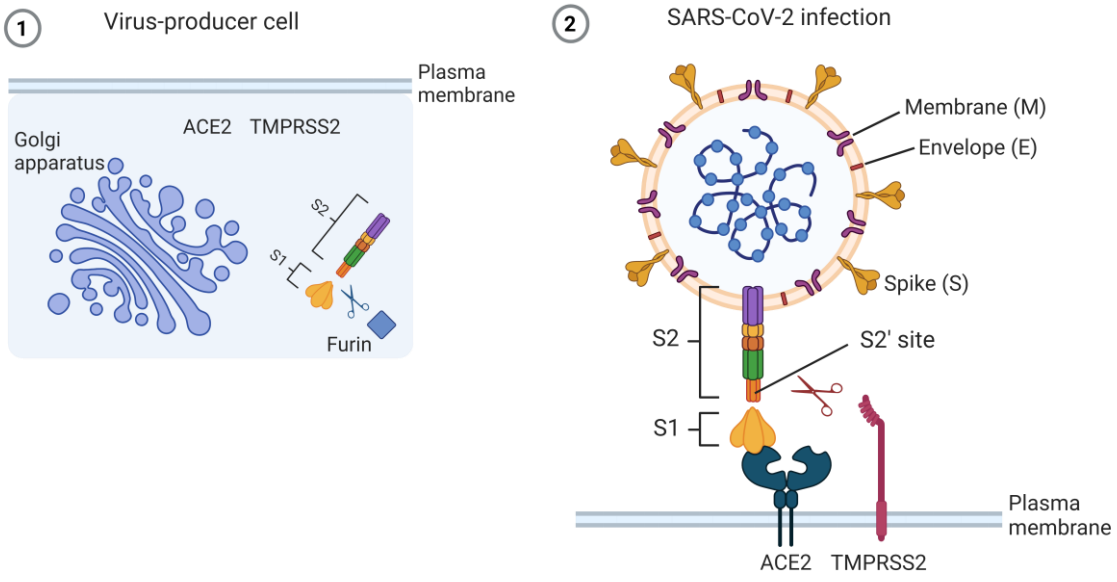

Figure 2 - SARS-CoV-2 entry in the host cell and the involvement of ACE2, TMPRSS2, and Furin proteins in the viral infection.

Angiotensin-convertase enzyme 2 (ACE2)

ACE2 is a zinc metallo-carboxypeptidase discovered in the late 2000s by independent research groups (Donoghue et al., 2000; Tipnis et al., 2000). It encodes an 805-aminoacid protein primarily found in the plasma membrane. Its N-terminal segment faces the extracellular space, encompassing the active catalytic site characterized by the conserved zinc binding motif sequence HEXXH (Histidine-Glutamine-X-X-Histidine), a hallmark in the peptidase family (Kuba et al., 2010; Lubbe et al., 2020). A common ancestral origin between ACE2 and its homolog ACE was proposed due to their 40% identity and 61% similarity (Donoghue et al., 2000).These molecules are fundamental in the renin-angiotensin-aldosterone system (RAAS) and despite the great homology, ACE and ACE-2 exert a counter-regulatory effect on the regulation of this system (Beyerstedt et al., 2021; Tipnis et al., 2000). converts angiotensin I (AngI) into angiotensin 1-9 and angiotensin II (Ang II) into angiotensin 1–7, resulting in low blood pressure through blood vessel dilation. On the other hand, ACE converts angiotensin I (Ang I) into Ang II, which constricts blood vessels and raises blood pressure (Bank et al., 2021; Beyerstedt et al., 2021).

ACE2 is the functional receptor of the SARS-CoV-2 virus (Figure 2), as it was for the previous SARS-CoV virus (Li et al., 2003; Shang et al., 2020). Recent studies reported that SARS-CoV-2 shows a much higher affinity to this receptor in comparison with previous SARS-CoV virus, a feature probably related to the higher spreading of the disease (Ortega et al., 2020).

Transmembrane serine protease 2 (TMPRSS2)

The TMPRSS2 belongs to the type II transmembrane serine proteases (TTPs), that nearly comprises one third of all proteases (Hedstrom, 2002). It is constituted by 492 amino acids and contains four domains: A N-terminal portion localized in the intracellular region, a type II transmembrane domain, linker regions with different protein-protein interaction domains and a C-terminal serine protease domain (Hooper et al., 2001; Wettstein et al., 2022). The proprotein convertases are a family of membrane-associated serine proteases involved in the activation of proproteins, such as prohormones, by removing the blocking portion of the protein (Jackson et al., 2022) but TMPRSS2 protease-specific function in the organism remains uncertain (Chen et al., 2010). TMPRSS2 is highly expressed in the prostate and may play a role in tumour prostate biology through modulation of angiogenesis and tubulogenesis in microvesicular endothelial cells (Aimes et al., 2003; Chen et al., 2010).

In the presence of TMPRSS2, the viral infection process involves the binding of the S1 subunit to the ACE2 receptor, which exposes the S2 subunit. Subsequently, this enables TMPRSS2 to cleave the S2' site at the cell surface (Figure 2). This cleavage step is essential for a successful membrane fusion to occur (Baughn et al., 2020; Jackson et al., 2022).

Furin

Furin is a transmembrane type I, calcium strictly dependent serine endoprotease (Dahms et al., 2016). This protein is ubiquitously expressed and belongs to the evolutionarily ancient family of subtilisin/kexin like proprotein convertases (PCSKs) (Braun & Sauter, 2019). This proteases family is responsible for the cleavage and conversion of immature pro-proteins into their biologically active forms (Braun & Sauter, 2019). This molecule encodes a protein of 794 amino acids and is a single polypeptide constituted by an N-terminal signal peptide (SP), an inhibitory pro-domain, a catalytic endopeptidase domain, a P domain, a cysteine-rich domain (CRD) and a C-terminal cytoplasmic domain (Osman et al., 2022). The pro-domain acts like a chaperone that controls the correct guiding, folding, activation and transport of furin (Thomas, 2002). Furin cleavage is important for the activation of many molecules such as growth factors, receptors, and enzymes (Seidah & Prat, 2012). Its overexpression, at the mRNA level, seems to be related to malignancy in several organs such as breast and ovary (Page et al., 2007), head, and neck tumours (Bassi et al., 2003), and non-small cell lung carcinoma (Schalken et al., 1987).

Unlike other coronaviruses, SARS-CoV-2 requires the cleavage of its S1-S2 subunits within the virus-producing cell (Hoffmann et al., 2020). During virus biosynthesis within infected cells, the S protein undergoes cleavage by the furin protease within the Golgi apparatus, which remains linked throughout virus maturation (Figure 2). In viral infection, the S1 subunit binds to its receptor, while the S2 subunit anchors the S protein to the membrane, facilitating fusion (Jackson et al., 2022).

ACE2, TMPRSS2 and Furin in tumourigenesis

The ACE2, TMPRSS2, and Furin molecules gained prominence during the SARS-CoV-2 pandemic, due to their crucial roles in the virus's infection mechanism. This significance appeared particularly relevant for the thyroid gland, given reports of high expression levels of ACE2, TMPRSS2, and Furin mRNA in this organ (Cao et al., 2021; Li et al., 2020; Wang

et al., 2020; Zhou & Gao, 2021). However, whether these molecules have a role in tumourigenesis is not yet well clarified.

ACE2 role in cancer is still controversial, while ACE2's promotion of angiogenesis could suggest a potential oncogenic role, some studies propose its tumour-suppressor function associated with the inhibition of tumour growth and metastasis (Bao et al., 2020; Chai et al., 2020; Niu et al., 2021; Zhang et al., 2019). TMPRSS2, on the other hand, has been reported to be dysregulated in tumours, being its high expression correlated with promotion of prostate cancer metastization (Lucas et al., 2014) and low expression associated with poor prognosis in breast invasive carcinoma (Xiao et al., 2022). Furin role in tumourigenesis it still not known, but its overexpression has been documented in different types of cancer, such as sarcoma (Jaaks & Bernasconi, 2017), breast (Cheng et al., 1997), ovary (Page et al., 2007) and colorectal cancer (He et al., 2020), and more recently with thyroid cancer (Poyil et al., 2023). While ACE2, TMPRSS2, and Furin molecules have been implicated in various cancers, the precise mechanisms underlying their roles in tumourigenesis, particularly in the thyroid gland, remain largely elusive.

Post-COVID-19 condition

COVID-19, now recognized as a systemic infection with chronic complications, exhibits more extensive and intense post-viral symptoms compared to other conditions (Yong, 2021). Individuals who have had a SARS-CoV-2 infection and subsequently present with symptoms such as profound fatigue, muscle weakness, dyspnea, anxiety, and cognitive impairment, without any alternative medical explanation, may receive a diagnosis of Post-COVID-19 condition, as outlined by the World Health Organization (WHO) (WHO, 2021). The thyroid is a potential target for the virus, but the role of thyroid dysfunction in post-COVID-19 condition remains unclear. Proposed mechanisms include direct viral effects, systemic inflammation, and hypothalamic-pituitary-thyroid axis dysfunction(Campi et al., 2021). Limited studies on post-COVID thyroid status emphasize the need for further investigation.

1.3.5. Endocrine-disrupting chemicals

Endocrine-disrupting chemicals (EDCs) are exogenous substances that interfere with the normal functioning of the endocrine system, including the thyroid gland and its hormone synthesis. These chemicals may disrupt the delicate balance of hormonal regulation, potentially contributing to dysfunction within the thyroid. This interference can occur through

mechanisms that affect hormone production, secretion, and overall thyroid function (Macedo et al., 2023).

EDCs are exogenous substances that are now recognized as significant contributors to tumourigenesis. Recent studies have enlightened the enduring impact of EDCs, with even those long banned continuing to play a role in the increasing incidence of neoplasia. The rise in hormone-related neoplasms observed over the last few decades predisposes to a critical examination of the potential involvement of EDCs in this disease (Pierozan et al., 2020). Notably, approximately 80% of EDCs have been identified as potentially tumourigenic (Bräuner et al., 2021; Rocha et al., 2021; Yoon et al., 2014), underscoring the need to unravel the complex link between EDC exposure and the increasing prevalence of hormone-related cancers (Sung et al., 2021).

The prevalence of thyroid cancer has exhibited a consistent rise over the past few decades and although the use of advanced imaging technology has played a role in detecting smaller tumours earlier or incidentally, the exposure to environmental risk factors, notably EDCs, has been associated with the onset of thyroid cancer (Alsen et al., 2021; Fiore et al., 2019). Among the potential health concerns associated with EDC exposure, thyroid neoplasia stands out as a recent area of investigation.

1.3.5.1. Thyroid Gland Endocrine Disruptors

The thyroid gland is particularly susceptible to endocrine disruption, probably due to the chemical similarity between thyroid hormones and certain EDCs. EDCs, which include various synthetic and natural compounds, can interfere with the normal function of the endocrine system. For the purpose of this work, we will only explore the synthetic EDCs, known for their impact on the thyroid.

Organotins

Organotins refer to a class of organic compounds that contain tin atoms bonded to carbon-containing groups. They are recognized for their broad-spectrum antimicrobial properties, making them effective in preventing the growth of marine organisms on ship hulls. One of the most well-known types of organotins is the tributyltin (TBT), which has historically been used in various applications such as antifouling paints for ships and as stabilizers in plastics. Other examples include triphenyltin and dimethyltin compounds. These compounds have been raising environmental concerns for many years due to their persistence and toxicity.

Despite the regulatory restrictions imposed on organotin compounds since the 1980s, elevated concentrations of these substances persist in coastal regions, particularly in harbors and shipyards, including those along the Brazilian coast (Bertuloso et al., 2015; Podratz et al., 2015). Human contamination by TBT can occur through consumption of contaminated fish or shellfish and water, among others (Rodrigues-Pereira et al., 2020).

EDCs exert their effects across various endocrine axes, encompassing the hypothalamic-pituitary-thyroid (HPT) axis. Recent studies have proved TBT has a thyroid disruptor at environmental relevant concentrations (Andrade et al., 2022; Andrade et al., 2018; Rodrigues-Pereira et al., 2020). Low doses of TBT were able to induce morphological alterations in the thyroid follicles of male rats (Rodrigues-Pereira et al., 2020). TBT also increased TPO expression and reduced antioxidant enzyme activities, leading to oxidative stress, in the thyroid of female rats (Andrade et al., 2018).

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are a group of synthetic organic chemicals composed of biphenyl molecules with varying numbers of chlorine atoms attached. Developed for use in a wide range of industrial applications due to their stability, insulating properties, and resistance to heat, PCBs were widely used in electrical transformers, capacitors, coolants, and other industrial equipment. However, their chemical stability led to persistence in the environment, and PCBs are now recognized as persistent organic pollutants.

The literature shows that PCBs have the ability of interact with thyroid hormone receptors affecting the production of thyroid hormones (Djordjevic et al., 2020). Nevertheless, PCBs are correlated with either increase and decrease of the THs levels, without affecting TSH levels (Zoeller, 2010). On the other hand, animal studies have been more consistent in showing an inverse association between PCBs and the thyroid hormones (Little et al., 2022), inducing in a dose-dependent manner, a decrease in circulating T3 and T4 levels in different models, as reviewed by Little et al., (Little et al., 2022).

Bisphenols

Bisphenols are a group of chemicals characterized by having two phenol functional groups. One of the most widely known bisphenols is bisphenol A (BPA), which has been extensively used in the production of plastics and epoxy resins. These materials are employed in the manufacturing of numerous consumer products, including food and beverage containers, medical devices, and thermal receipt papers.

In the existing literature, research on bisphenol A (BPA) and its analogues has consistently demonstrated an impact on thyroid function, and recently, these compounds where shown to induce the downregulation of key thyroid-related genes in mice, including TSH receptor, TPO (thyroperoxidase), TG (thyroglobulin), and NIS (sodium-iodide symporter). Notably, the analogues TBBPA (tetrabromobisphenol A) and TBBPS (tetrabromobisphenol S) exhibit more pronounced effects when compared to BPA and BPS (Hu et al., 2023). With the aim of eliminating BPA, some common substitutes including bisphenol S (BPS) and bisphenol F (BPF) were applied. Despite efforts to reduce BPA usage, bisphenols, as a group, continue to be of concern in environmental and health research.

Perfluorinated compounds

Perfluorinated compounds (PFCs) represent a class of synthetic chemicals characterized by the presence of strong carbon-fluorine bonds, which make them highly stable and resistant to degradation. These compounds find widespread use in various industrial and consumer applications due to their unique water- and grease-resistant properties. PFCs have been employed in the manufacturing of non-stick coatings, water repellents, firefighting foams, and other products. Most well-known PFCs include perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), but they have been banned. However, their persistence in the environment and bioaccumulation in living organisms, including humans, still raise environmental and health concerns.

Animal studies with some PFAs revealed these chemicals were able to reduce the circulating levels of thyroid hormones in diet-exposed animals, by increasing their metabolic clearance rate (Dong et al., 2016).

Heavy metals

Heavy metals (HM) are metallic elements characterized by their relatively high density, atomic weight, and potential toxicity when present in excessive amounts. They occur naturally in the environment, but human activities such as industrial processes, mining, and agriculture can release them into the environment, leading to potential contamination.

HM exhibit an increased tendency to accumulate in the thyroid, when compared to the accumulation levels observed in other tissues (Vigneri et al., 2017). HM are recognized for their dual capacity as endocrine disruptors of perturbing the regular hormonal system, as it is the case of cadmium (Cd), lead (Pb), mercury (Hg), arsenic (As), and manganese (Mg),

or as inducers of tumourigenesis, exhibiting characteristics of carcinogens, as it is the case for cadmium (Cd), arsenic (As), Nickel (Ni), beryllium (Be), and chromium (Cr) (Fiore et al., 2019; van Gerwen et al., 2022; Vigneri et al., 2017). Moreover, certain heavy metals play a crucial role in influencing the function and maintaining the homeostatic mechanisms of the thyroid gland. Volcanic areas are frequently enriched in thyroid cancer cases (Malandrino et al., 2020; Pellegriti et al., 2009; Vigneri et al., 2017)

In vivo studies with exposure to these compounds have revealed thyroid disfunction. One study of exposure of sexually mature zebrafish to cadmium chloride induced reproductive toxicity, that resulted in malformations in their offspring. They exhibited reduced thyroid hormone (TH) levels, T3 andT4. Furthermore, the study revealed up-regulation of thyroid receptor genes and increased expression of thyroid hormone synthesis and metabolism-related genes, indicating disruptions in the thyroid endocrine system's functional development in the exposed zebrafish offspring (Tian et al., 2020). Other study revealed that Exposure of wild-type (WT) zebrafish embryos to elevated concentrations of copper and zinc resulted in significant disruptions in the thyroid endocrine system, with increased T4 and reduced T3 concentrations in fish exposed to both compounds. Transcriptional changes in genes associated with the hypothalamic-pituitary-thyroid (HPT) axis were observed, highlighting the molecular impact of copper and zinc on thyroid function (Zhong et al., 2022).

Flame retardants

Flame retardants (FR) are chemical compounds specifically designed to slow down or inhibit the ignition and spread of fires. They are commonly incorporated into a wide range of house-hold materials and products, including furniture, electronics, textiles, plastics, and building materials, to enhance fire safety, by delaying the onset of combustion. Flame retardants encompass different classes such as brominated flame retardants (e.g., polybrominated diphenyl ethers or PBDEs), organophosphorus flame retardants, and nitrogen-containing flame retardants.

Studies using zebrafish model, revealed impact in the thyroid gland, once exposed to these compounds. Exposure of zebrafish embryo-larvae to 2,2,4,4-tetrabromodiphenyl ether (BDE-47) and tetrabromobisphenol A (TBBPA) revealed significant impacts on the TH system. TBBPA exposure led to mortality, developmental retardation, and morphological deformities, while BDE-47 induced yolk sac and craniofacial deformities, curved spines, and shorter tails. Both compounds disrupted the TH system by altering gene expression in the

HPT axis, influencing deiodinases, transport proteins, thyroid follicle synthesis proteins, and glucuronidation enzymes. Whole-mount *in situ* hybridization (WISH) analyses showed tissue-specific changes in thyroid receptor and deiodinase enzyme expression, particularly in the brain, liver, pronephric ducts, and craniofacial tissues (Parsons et al., 2019).

Phthalates

Phthalates are a group of synthetic chemicals commonly used as plasticizers to enhance the flexibility, durability, and transparency of various plastic products. These chemicals are characterized by their ability to soften plastics and improve their elasticity, making them prevalent in items such as toys, food packaging, cosmetics, and medical devices. Phthalates are not chemically bound to the plastics they modify, which makes them prone to leaching into the environment and, subsequently, human exposure.

Exposure of zebrafish embryos to different concentrations of mono-(2-ethylhexyl) phthalate (MEHP) led to significant disruptions in the thyroid endocrine system. MEHP exposure resulted in a decrease in whole-body T4 and increase in T3 contents. Upregulation of genes related to thyroid hormone metabolism: Deiodinase 2 (Dio2) and glucuronosyltransferase 1 (UGT1ab) was also observed. Additionally, elevated transcription of genes involved in thyroid development (Nkx2.1 and Pax8), thyroid hormone synthesis (TSHβ, NIS, and TG), and increased Deiodinase 1 (Dio1) expression indicated complex alterations in the hypothalamic-pituitary-thyroid (HPT) axis. However, a significant down-regulation of genes encoding proteins involved in thyroid hormone transport (transthyretin, TTR) was observed after MEHP exposure. Overall, acute exposure to MEHP-induced thyroid endocrine toxicity (Zhai et al., 2014)

1.3.5.2. EDCs and thyroid cancer incidence

EDCs are recognized for their ability to influence thyroid function and are implicated in an elevated risk of specific cancers (Bräuner et al., 2021; Rocha et al., 2021; Yoon et al., 2014). Multiple studies have indicated that exposure to specific EDCs can disrupt thyroid function and is linked to an elevated risk of various adverse health outcomes. These consequences encompass developmental abnormalities, thyroid disorders, and diverse forms of cancer (Alsen et al., 2021). The accumulating evidence underscores the potential impact of EDC exposure on thyroid cancer. Specifically, implicated in this association include certain congeners of flame retardants, polychlorinated biphenyls (PCBs), and

phthalates, along with particular pesticides (Alsen et al., 2021). In our review, phthalates exhibited the highest risk for TC among all EDCs in the study. Pesticides, HM, Bisphenols and Flame retardants exposure also demonstrated an elevated risk of TC (Macedo et al., 2023). Overall, the findings underscore the diverse and impactful associations between specific EDCs and thyroid cancer risk, emphasizing the need for further research in this area.

2. Zebrafish as a model for thyroid disease

2.1. Zebrafish animal model

The zebrafish (*Danio rerio*) is a cypriniform teleost fish that belongs to the lineage of ray-finned fishes (McCluskey & Braasch, 2020). It is a small tropical freshwater fish native of South Asia, well-known for its distinctive blue and silver stripes that extend along its body, the zebrafish has become a prominent model organism in scientific research.

The zebrafish has emerged as a versatile model organism for biomedical research, namely in the oncology field. It possesses numerous advantages such as its genetic similarity to humans, at least 80% of the genes have an orthologue in humans (Howe et al., 2013). Its rapid development, transparency during embryonic stages, and the ability to easily manipulate its genome make zebrafish an excellent model for investigating various aspects of cancer, including tumour initiation, progression, and response to therapies.

The transparency of zebrafish embryos allows the easy visualization of thyroid development through fluorescent markers and is a promising biological tool to investigate the effects of environmental factors on thyroid cancer initiation and progression. Additionally, the zebrafish model allows for high-throughput drug screening, providing a cost-effective platform to identify potential therapeutic agents for cancer treatment. Overall, the zebrafish model stands as a valuable tool in the understanding of cancer biology, including thyroid cancer, and it can be very useful for translational applications in the development of targeted therapies.

2.2. Zebrafish Thyroid Development

Thyroid hormones are essential for all vertebrates, contributing to the normal development of organisms and the precise regulation of metabolism (Maenhaut et al., 2015; Wendl et al., 2002). Despite the anatomical differences, the hypothalamus-pituitary axis is well conserved between mammals and fish (Blanton & Specker, 2007).

In zebrafish, as some other vertebrates, the thyroid follicle cells and C-cells are not localized in the same organ (Alt et al., 2006). The thyroid is not encapsulated by a connective tissue but instead is constituted by several follicles dispersed along the aorta, more precisely, between the first gill arch and the bulbus arteriosus (Alt et al., 2006; Wendl et al., 2002). The follicles can be aggregated in groups of two or more or can also be isolated. They are filled with colloid, and the quantity of colloid is dependent upon the metabolic activity of thyroid cells and the TSH production (Fournie et al., 2005; Wendl et al., 2002). The shape is irregular and each follicle can vary in size (14 µm -140 µm) (Wendl et al., 2002). In teleost, the production of THs (T3 and T4) is similar to humans and involves the uptake of iodine, iodination of thyroglobulin, and subsequent release of hormones into the bloodstream (Wendl et al., 2002).

Zebrafish thyroid expresses key transcription factors for thyroid differentiation such as thyroid transcription factor-1 (nkx2.1a), haematopoietically expressed homeobox (nnhex), paired box gene (pax2a), as early as 24 hours-*post*-fertilization (hpf) (Porazzi et al., 2009; Wendl et al., 2002), followed by paired box gene 8 (pax8) that starts expressing at 28 hpf (Porazzi et al., 2009). For thyroid differentiation, zebrafish later starts to express thyroglobulin (tg) and the sodium iodine symporter, also known as NIS (solute carrier family 5 member 5, slc5a5) at 32 and 40 hpf, respectively (Alt et al., 2006).

The functionality of hormone-producing follicles can be evaluated through T4 immunostaining, and zebrafish express T4 immuno-positive cells by 80 hpf (Porazzi et al., 2009). By 5 days *post*-fertilization, some follicles start to become apparent, usually the cells are round, with a diameter ranging from 5 to 10µm (Alt et al., 2006).

2.3. Zebrafish as a Cancer Model System

The zebrafish emerges as a robust *in vivo* model, particularly useful in the preclinical investigations for cancer predisposition. Zebrafish possess the ability to develop tumours that closely mimic human disease (White et al., 2013). This similarity in tumour characteristics provides researchers the opportunity of studying cancer in a model system that shares key features with human pathology, but in more rapid and less expensive conditions, when compared to the standard mice models.

Interestingly, when studying the genetics of tumours, due to the evolutionary genome duplication event that occurred in fish, these models are able to tolerate more easily some gene mutations when comparing with rodents. This allows the animal to endure longer, facilitating more comprehensive and prolonged studies (Kobar et al., 2021). One of the first

models of cancer to appear in zebrafish was the fish with spontaneous *TP53* gene mutation (*tp53*^{M214K}*).* The mutant animals developed mainly malignant peripheral nerve sheath tumours (PNST) with a tumour incidence of 28% by 16.5 months (Berghmans et al., 2005). Another zebrafish model demonstrated the cooperation between the tumour suppressor gene mutation with the activating mutation in the serine/threonine kinase BRAF (BRAF^{V600E}). These fish expressed the BRAF mutation specifically in the melanocytes (under the control of the *mitfa* promoter). BRAF^{V600E} transgenic models developed nevi, and in a background of loss of *tp53* the animals developed invasive melanoma (Patton et al., 2005).

Specifically in thyroid, a zebrafish model was more recently characterized. The animal expressed BRAF^{V600E} mutation in the thyrocytes, under the expression of the thyroglobulin (tg) promoter and developed invasive thyroid carcinoma (Anelli et al., 2017). Furthermore, once a model is established, zebrafish represents a powerful tool for drug screening in order to identify novel therapies for cancer treatment (Kobar et al., 2021), but also to test putative tumour promotor chemicals, as is the case of EDCs.

3. Thyroid at the crossroads of public health

In 1920, public health was defined as "*the science and art of preventing disease, prolonging life, and promoting health and efficiency through organized community effort*" (Turnock, 2012). It is a multidisciplinary field that focuses on the enhancement of the well-being of entire populations. To achieve that, community efforts must be made in order to prevent diseases and consequently prolong life with quality. Environmental factors may play a crucial role in public health, as they can significantly impact the health of individuals and communities. Environmental threats to public health are numerous, as pollution, infectious agents, such as the SARS-CoV2 virus, and the introduction of harmful substances into the ecosystems such as the EDCs. These threats will consequently contribute to a range of health issues, from infectious diseases, as the COVID-19 pandemic, to more chronic conditions, as auto-immune diseases, or cancer. Understanding these threats are essential components of the public health purpose. The thyroid gland is a key target of the public health challenges. It is an essential organ of the endocrine system, and plays a critical role in embryonic development, regulation of the metabolism, energy balance, and various other physiological processes. The continuous exposure of the thyroid to environmental factors has great impact and can result in development of thyroid disorders and eventually, cancer.

In the complex web of health, the research on this thesis falls within the scope of the *One Health* approach. The World Health Organization (WHO) defines it as a holistic paradigm that recognizes the interconnection of people, animals, plants, and their shared environment, i.e., aims to address health challenges by considering the broader system rather than focusing on the individual element.

CHAPTER II | RESEARCH AIMS

As our global community struggles with challenges such as the COVID-19 pandemic and environmental pollution, along with excessive use of natural resources, environmental imbalances affecting various species and causing widespread health impacts emerged. My research, situated at the nexus of the thyroid, encompasses the SARS-CoV-2 pandemic and the pervasive influence of EDCs which aligns with the *One Health* principles. The thyroid, a fundamental endocrine organ, emerges as a sentinel reflecting the interplay between environmental factors, viral threats, and the balance of the endocrine system.

The focus of this thesis is to comprehensively investigate the multifaceted challenges of the thyroid in different contexts:

Covid and thyroid

1- At the time we were conducting this thesis, SARS-CoV-2 pandemic appeared. The number of studies regarding COVID-19 effects on thyroid of infected patients was increasing but, at the time, there was no demonstration if the thyroid gland could be a target for infection. So, we questioned if SARS-CoV-2 virus could directly infect the thyroid cells, and if so, how was the expression of the molecules involved in the machinery for infection in the thyroid of these patients. Through an exploration of the thyroid profile in COVID-19 deceased patients, our objective was to determine whether the thyroid gland could be directly infected by the virus, aiming to elucidate the origins of thyroid disorders observed in patients with SARS-CoV-2 infection. This investigation aimed to establish a putative link between the viral infection and thyroid-related conditions, such as subacute thyroiditis, observed in patients with COVID-19, contributing to a more comprehensive understanding of the implications of the virus on thyroid health. At this point, literature showed that some patients infected by the virus, exhibited more extensive and intense post-viral symptoms compared to other conditions, and so we wondered if the thyroid being a potential target for the virus, would have a role in post-COVID-19 condition, and so we conducted a review of the literature on the matter.

2- Expanding on our initial study of thyroid infection in COVID-19 deceased patients, and understanding the crucial functions of the molecules ACE2, TMPRSS2 and Furin in facilitating virus entry into host cells, we identified a less explored field for further investigation - whether these molecules were highly expressed or not in the thyroid gland, and if so, we asked if they could they potentially be involved in thyroid tumourigenesis.

EDCs and thyroid

3- To unravel the complex relation between environmental factors and thyroid function, we extended our investigation to the humans' environmental exposure to hazard chemicals, such as the EDCs and its possible implication in the worldwide increase of not only thyroid neoplasia, but also other endocrine neoplasms. Our question was if there was clear evidence in humans, about exposure to EDC and risk to the development of endocrine cancer. For that, we conducted a 40-year systematic review approaching EDCs exposure and endocrine tumourigenic risk in humans for six endocrine organs: thyroid, breast, ovary, uterus, testis and prostate.

4- In collaboration with a Brazilian group, we had the opportunity to study EDCs impact in mammal's thyroid. While TBT is a recognized EDC, many conducted studies employ high doses of exposure, making it challenging to ascertain the relevance of the TBT concentrations found in the environment. We asked if TBT could have an impact in the thyroid at an environmental relevant dose, for a short-period of time. To answer the question, we have conducted an exposure assay with a low dose of TBT for 15 days, using male rats as models.

5- One of the unmet needs in the study of the effects of EDCs in organisms is the scarcity of robust *in vivo* models. In order to have a model to test the putative effect of EDCs in thyroid cancer we undertook the characterization of a zebrafish animal model (developed previously by our group in collaboration with IGC institute), that harboured the $BRAF^{V600E}$ mutation specifically in the thyrocytes. Knowing the critical role of p53 loss in tumour progression, our group had also developed another zebrafish model expressing $BRAF^{V600E}$ specifically in thyroid cells of $tp53^{M214K}$ zebrafish.

6- As the zebrafish model did not inherently develop cancer, we wondered if EDC exposure could promote cancer development. And so, we investigated the effects of a well-known thyroid disruptor, BPA, to ascertain whether exposure to these disruptors could increase the susceptibility to cancer or exacerbate thyroid disease.

This model will serve as an invaluable platform for studying thyroid disease and contribute to a comprehensive understanding of the interplay between environmental factors, genetic predisposition, and thyroid health.

CHAPTER III | MATERIALS AND METHODS

The material and methods of this thesis are described in detail in the papers included in the Results section (**Chapter IV**). We present here a brief summary of the methods used in this thesis:

1. Patients' collection and clinicopathological data

Article I - In collaboration with the University of São Paulo, in Brazil, and Hospital São João, in Portugal, we obtained a series of thyroid specimens from autopsies of patients who died from COVID-19 in 2020. The series from Brazil included 14 patients and the Portugal series 2 patients. Clinicopathological data of the patients were collected and each of these cases had either a premortem or a post-mortem nasopharyngeal swab performed.

Article III - From the Institute of Pathology and Molecular Immunology of the University of Porto (IPATIMUP) medical files, 190 samples of frozen thyroid tissue, corresponding to 165 patients, were collected. The series of patients reported on in this work was already partially characterized by our group.

2. Molecular techniques

Techniques such as total RNA extraction of the samples, reverse transcription and cDNA synthesis, were performed in articles I, III, VI and VII. Real-time polymerase chain reaction (RT-PCR) was performed for SARS-CoV-2 genes, ACE2, TMPRSS2, Furin in thyroid specimens in articles I and III and for thyroid differentiation markers in zebrafish in articles VI and VII. *In situ* hybridization (ISH) was only performed for SARS-CoV-2 study, in article I and Immunohistochemistry (IHQ) technique was performed in articles I, III and V. Western blot technique was performed only for zebrafish thyroid in article VI.

3. Morphometric studies

We have performed morphometric studies using ImageJ software in the thyroid of rats and zebrafish animal models, and they are described in detail in articles V and VI.

4. Ecotoxicological studies

We have performed toxicological studies with TBT in a rat model in article V and performed preliminary assays with BPA in a zebrafish model in article VII.

5. Systematic Review

To conduct the systematic review, we used the methodology described in Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) as described in more detail in article IV.

6. Statistical analysis

We performed statistical analysis in all original works using SPSS statistics, and the tests applied are described in articles I, III, V, VI and VII.

CHAPTER IV | RESULTS

I. Detection of SARS-CoV-2 infection in thyroid follicular cells from a COVID-19 autopsy series

At the time of conducting this study, patients with SARS-CoV-2 infection often manifested clinical signs of thyroiditis and thyrotoxicosis, but whether this virus could infect directly the thyroid follicular cells, was still not known. This paper investigated the possibility of direct SARS-CoV-2 infection in thyroid cells and explored the expression of key proteins involved in viral entry in thyroid tissues from deceased COVID-19 patients.

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RESEARCH

Detection of SARS-CoV-2 infection in thyroid follicular cells from a COVID-19 autopsy series

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Abstract

Objective: To understand whether thyroid cells can be directly infected by the SARS-CoV-2 virus and to establish a putative correlation with the expression of the host entry machinery: ACE-2, TMPRSS2, and furin.
Methods: We assessed the presence of SARS-CoV-2 virus at the gene level by RT-PCR, viral RNA transcripts localization by *in situ* hybridization, and by detecting viral proteins by immunohistochemistry for the nucleocapsid and the spike proteins. Furthermore, we also described the immunoexpression of key host factors for virus entry in the COVID-19 thyroid samples.
Results: We performed RT-PCR for SARS-CoV-2 in all autopsy specimens and detected viral genome positivity in 13 of 15 thyroid tissues and in a lung specimen. In 9 of the 14 positive samples, we were also able to confirm SARS-CoV-2 signal by *in situ* hybridization. Immunohistochemistry for the viral nucleocapsid and spike protein was also positive for ten and nine of the RT-PCR-positive cases, respectively, but revealed a lower sensitivity. We also described, for the first time in a COVID-19 series, the immunohistochemical expression of ACE-2, TMPRSS2, and furin in the thyroid.
Conclusions: Our results obtained in thyroid specimens from deceased COVID-19 patients indicate that thyrocytes can be directly infected by SARS-CoV-2 since we detected the presence of SARS-CoV-2 genome in follicular cells. Nevertheless, we did not find a clear correlation between the presence of viral genome and the expression of the host factors for virus entry, namely ACE-2, TMPRSS2, and furin.

Key Words
- SARS-CoV-2
- thyroid
- ACE-2
- TMPRSS2
- furin

Introduction

Corona virus disease (COVID-19) dissemination first took place in 2019; in March 2020 the World Health Organization (WHO) officially declared it a pandemic. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus causing the disease and is highly infectious, having already been responsible for more than 6 million deaths worldwide (https://coronavirus.jhu.edu/). COVID-19 disease mainly affects the respiratory tract,

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yet, like other coronaviruses, it can also affect endocrine organs, such as the thyroid gland (1, 2).

Angiotensin-converting enzyme 2 (ACE-2) receptor was reported as the functional receptor of the SARS-CoV virus (3). Recent studies reported that SARS-CoV-2 also employs the same receptor (4, 5), with a much higher affinity in comparison with previous SARS-CoV virus (6), probably related to the higher spreading of the disease (6). ACE-2 is a metallo-carboxypeptidase type I transmembrane protein encoded by a gene localized in Xp22.2 (7) and is responsible for the cleavage of angiotensin (Ang) I and Ang II to generate Ang 1–9 and Ang 1–7, respectively (8). Interestingly, the thyroid gland constitutes one of the top ten organs expressing ACE-2 mRNA (9), and its expression in thyroid cells has been recently reported to be modulated by pro-inflammatory cytokines which ultimately can facilitate the entering of the virus into the host cells (10). Hence, it is reasonable to advance that the thyroid gland may represent a possible target for SARS-CoV-2 infection. Since the pandemic outbreak, some studies have been reporting, in COVID-19 patients', alterations in thyroid hormone levels (11, 12), thyrotoxicosis (13, 14), and subacute thyroiditis (2, 15). These effects are mostly transient during the infection period, but when comparing severe COVID-19 patients with severe non-COVID-19 pneumonia patients, it has been reported a significantly higher decrease in TSH serum levels for COVID-19 patients, reinforcing the putative (direct or indirect) role of SARS-Cov-2 on thyroid hormones' regulation (16).

In order for SARS-CoV-2 to infect the host cell, other key players besides ACE-2 are required. Furin and the transmembrane protease serine 2 (TMPRSS2) are two proteases involved in SARS-CoV-2 spike protein processing in the host cell, ensuring an efficient infection. The coronavirus is constituted by four main structural proteins: nucleocapsid (N), membrane (M), envelope (E), and spike (S) (17). The entry of the virus into the cells encompasses the cleavage of the subunit S1 of the S protein by the convertase furin, leading to the binding of S1 to the ACE-2 receptor. On the other hand, the S2 subunit connects the S protein to the membrane in order for fusion to occur (5). As reviewed by Jackson *et al.*, an additional trigger for SARS-CoV-2 glycoprotein entry is the cleavage of an additional internal site of the S2 subunit, the S2', that is cleaved at the cell surface by the TMPRSS2 protease, which is exposed when the binding to ACE-2 occurs (17). Interestingly, both TMPRSS2 and furin are thought to be highly expressed in the thyroid gland (18).

In this article, we aimed to understand if the thyroid cells could be directly infected by SARS-CoV-2 by detecting viral RNA molecules in the thyroid tissues from a series of autopsies of COVID-19 patients. Also, we intended to characterize protein expression of the cellular targets for virus entry, ACE-2, TMPRSS2, and furin, in the same series of thyroid tissues from patients deceased by COVID-19.

Materials and methods

Patients' collection and clinicopathological data

In collaboration with the University of São Paulo, in Brazil, and Hospital São João, in Portugal, we were able to obtain a series of thyroid specimens from autopsies of patients who died from COVID-19 in 2020. The series from Brazil was composed of 13 thyroids and 1 lung specimen ($n = 14$). The cases from Portugal were six thyroid samples from two different patients. Therefore, a total of 20 samples (including the lung) from 16 patients were obtained. All the slides were stained for hematoxylin and eosin (H&E) and reviewed by an experienced thyroid pathologist (M S-S). Clinicopathological data of the patients were collected (Table 1), and each of these cases had either a *pre-mortem* or a *post-mortem* nasopharyngeal swab performed. The lung specimen was used as a positive control for the experiments in the thyroid series. The period of sample collection occurred between March and September 2020, for both series. The Research Ethics Committee CHUSJ, Portugal, approved this study under the number 272/2020 under the project entitled 'Is thyroid gland a target of SARS-CoV-2 infection? Early identification and follow-up of thyroid dysfunction in COVID-19 patients', and for Brazil series, the study was approved under the project 'Studies of the fatal COVID-19 trough-guided ultrasound minimal invasive autopsy', process number #4.813.199, March 28, 2020.

Real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted from two slides of 10 µm thick formalin-fixed paraffin-embedded (FFPE) sections for each case, in an automatic extractor Maxwell® CSC RNA FFPE Kit, from Promega (AS1360). RT-PCR for SARS-CoV-2 gene amplification was performed with two different kits: Fosun COVID-19 RT-PCR Detection Kit (Cat. #PCSYHF03-a) and TaqPath™ COVID19 CEIVD RT-PCR Kit (Cat. #A48067). The run method was the same for both and started with a cycle of 15 min at 50°C and a cycle of 3 min at 95°C. The PCR program was 5 cycles of 5 s at 95°C and 40 s at 60°C,

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Table 1 COVID-19 patients' demographic and clinical data.

Case	Tissue	Series	Date of death	Days until *post-mortem* examination	Age	Sex	BMI	Interval of days from symptoms to death	Comorbidities
1	Lung	Brazil	n.a.	March 2020	83	F	24.56	5–10	–
2	Thyroid	Portugal	June 2020	+3 days	44	F	<25	10–20	N
3	Thyroid	Portugal	April 2020	+1 day	70	F	>25–30	n.a.	HT
4	Thyroid	Brazil	April 2020	+1 day	71	F	28.31	10–20	DM, HT, CP, A
5	Thyroid	Brazil	May 2020	+1 day	45	M	23.25	10–20	DM, HT, CKD
6	Thyroid	Brazil	May 2020	Same day	32	M	29.39	>30	–
7	Thyroid	Brazil	May 2020	+1 day	34	F	30.32	10–20	N
8	Thyroid	Brazil	May 2020	+1 day	38	F	20.42	10–20	N, CP
9	Thyroid	Brazil	May 2020	Same day	22	M	24.69	10–20	–
10	Thyroid	Brazil	July 2020	+1 day	51	F	29.41	<5	N
11	Thyroid	Brazil	July 2020	Same day	24	F	29.41	>30	–
12	Thyroid	Brazil	July 2020	Same day	49	F	35.91	20–30	HT
13	Thyroid	Brazil	July 2020	Same day	26	F	25.76	>30	–
14	Thyroid	Brazil	September 2020	+1 day	63	M	29.41	20–30	–
15	Thyroid	Brazil	August 2020	+1 day	66	M	24.09	10–20	DM, HT, CP
16	Thyroid	Brazil	August 2020	+1 day	70	M	24.08	<5	DM, HT

–, no major comorbidities reported; A, asthma; CKD, chronic renal disease; CP, cardiopathology; DM, diabetes mellitus; F, female; HT, hypertension; M, male; N, neoplasia; n.a., non-available.

followed by 40 cycles of 5 s at 95°C and 40 s at 60°C. The genes amplified in the Fosun kit were ORF1ab that encodes for orf1ab polyproteins (representing two-thirds of the virus genome (19)) and gene N and gene E that encode for nucleocapsid and envelope proteins, respectively. In the TaqPath™ kit, the genes amplified were ORF1ab, gene N, and gene S (that encodes for the viral spike protein). The human RnaseP (hRnaseP) was used as an endogenous control in both kits. About 50 ng of RNA was used for each RT-PCR test, and in three of the cases, a second RNA extraction was needed. Samples were considered positive when at least two of the four SARS-CoV-2 genes were amplified at maximum at cycle threshold (Ct) 37 (Supplementary Fig. 1A and B, see section on supplementary materials given at the end of this article). Samples were considered inconclusive when only one viral gene was amplified or there was amplification after Ct 37. A case was considered negative when hRnaseP was positive but none of the viral genes were amplified (Supplementary Fig. 1C). The reaction was only considered valid when hRnaseP control was amplified. RT-PCR was performed for all 16 patients plus 2 pre-COVID FFPE controls (Table 2) and 10 pre-COVID thyroid frozen samples (data not shown).

In situ hybridization (ISH)

RNAscope® ISH was performed with RNAscope 2.5 HD Reagent kit - Brown, (Advanced Cell Diagnostics, Inc., Newark, CA, USA) (20). Three different probes were used for each sample: SARS-CoV-2 spike probe (V-nCoV2019-S), DapB probe that targets the bacterium gene (negative control probe), and Ubiquitin C (UBC) (positive control probe) to evaluate RNA detection and integrity. Four-micrometer sections of FFPE blocks were deparaffinized and subjected to antigen retrieval for 20 min with a 1× target retrieval solution. The slides were then incubated with protease plus and treated for 20 min at 40°C using the HybEZ™ oven (Advanced Cell Diagnostics, Inc). Subsequently, the slides were incubated for 2 h at 40°C in the oven with the target probe. Finally, the signal was amplified using a series of the six amplifiers recommended by the manufacturer. The chromogen detection was performed with DAB for 10 min at room temperature and counterstained with Gill's hematoxylin.

Immunohistochemistry

Immunohistochemistry (IHQ) was performed in all the FFPE specimens. Dewaxing and rehydration, followed by 45 min of heat-induced antigen retrieval with EDTA buffer (pH 9.0) in the steamer, were performed. The IHQ protocol including the peroxidase and protein block, antibody amplifier, and polymer incubations was performed with the Ultravision Quanto Detection System HRP, Epredia®, TL-125-QHL, according to the manufacturer's instructions. The antibodies used were: mouse monoclonal anti-SARS/SARS-CoV-2 coronavirus nucleocapsid antibody (Invitrogen, MA1-7404, 1:100); mouse monoclonal SARS-CoV/SARS-CoV-2 spike antibody ((1A9), GTX632604, Genetex (Irvine, CA, USA), 1:200); mouse monoclonal anti-ACE-2 (Invitrogen, MA5-31395, 1:2500); rabbit monoclonal

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Table 2 RT-PCR for amplification of SARS-CoV-2 genes and RNAscope results.

Case	Tissue	Status	Amplification Cts					RNAscope
			Gene Orf1ab	Gene N	Gene E	Gene S	hRNAp	
1	Lung	Positive	25	23	24	22	19	++
2	Thyroid	Positive	No amplification	33	32	32	22	+
3	Thyroid	Positive	32	33	No amplification	No amplification	27	–
4	Thyroid	Positive	34	32	No amplification	36	20	+[a]
5	Thyroid	Positive	34	31	33	No amplification	21	–
6	Thyroid	Positive	No amplification	31	33	No amplification	20	–
7	Thyroid	Positive	33	30	30	32	20	+[a]
8	Thyroid	Positive	34	33	35	No amplification	20	–
9	Thyroid	Positive	32	33	No amplification	No amplification	19	++[a]
10	Thyroid	Positive	32	30	30	28	20	–
11	Thyroid	Inconclusive[b]	No amplification	34	33	No amplification	22	–
12	Thyroid	Negative	No amplification	No amplification	No amplification	No amplification	21	–
13	Thyroid	Positive	35	35	No amplification	No amplification	20	+[a]
14	Thyroid	Positive	No amplification	31	33	No amplification	19	+
15	Thyroid	Positive	32	30	31	33	20	+
16	Thyroid	Positive	No amplification	32	32	No amplification	20	+
Control 1	Thyroid	Negative	No amplification	No amplification	No amplification	No amplification	22	ND
Control 2	Thyroid	Negative	No amplification	No amplification	No amplification	No amplification	23	ND

[a]RNAscope positivity in the adjacent tissue; [b]Case positive for the Fosun Kit in the first RNA extraction and negative in the second extraction for both Fosun and TaqPath kits.
–, no signal detected; +, at least one focal area with signal; ++, several focal areas with signal; ND, not done.

anti-TMPRSS2 antibody ((EPR3862), Abcam, ab109131, 1:1000); rabbit polyclonal anti-furin (Invitrogen, PA5-96680; 1:200); and rabbit monoclonal anti-cleaved caspase-3 (Asp175, Cell Signalling, 9664, 1:1000). The detection was performed with DAB chromogen for all antibodies (Epedria®, TA-125-QHDX), with the exception of spike and furin which detection was performed with HIGHDEF® Red chromogen (Enzo Life Sciences (Farmingdale, NY, USA), NC1609433). All slides were counterstained with Gill's hematoxylin.

Results

Patients' clinicopathological data

The age of the 16 patients varied from 22- to 83-year-old (Table 1), with a median age of 47 years. The population was composed of ten females (62.5%) and six males (37.5%), and females were younger than males (median age: 46.5 vs 54.0, respectively). BMI was available for the 16 patients, and 50% were overweight. From these, two female patients were obese (BMI > 30). Major comorbidities in this population were 3/16 (19%) patients that had neoplasia and 5/16 (31%) patients who had any type of cardiac pathology (Table 1). None of these patients had reports of a previous history of thyroid disease, and the thyroid function of these patients was not evaluated during hospital admission.

Histology of the thyroid specimens

All the slides were evaluated by an experienced pathologist (MSS) and no major lymphocytic infiltrate or destruction was observed in the thyroid sections (Fig. 1A), besides expected from *post-mortem* autolysis. In some cases, we were able to identify slight follicle alterations (Fig. 1B). No signs of hyperplastic/neoplastic lesions were detected.

RT-PCR

We performed RT-PCR in samples corresponding to the 16 patients. All the cases amplified for hRnaseP, the gene used for control of RNA integrity. Of the 16 cases, 14 were positive (88%) on the RT-PCR reaction since amplification of at least two of the four viral genes tested was observed before Ct 37 (Table 2 and Supplementary Fig. 1). Four cases showed amplification for all the viral genes (the lung sample and three thyroid samples; Table 2). In four cases, three of the viral genes gave positive signals and in six cases, two viral genes were positive (Table 2). One case was negative (6%), since none of the viral genes were amplified, only the positive control hRnaseP produced a signal (Table 2). Another case was considered inconclusive since the result of a second RNA extraction was negative for all genes after first extraction had been positive for two of the genes (Table 2). In order to exclude some potential non-specificity of the viral genes amplified in the RT-PCR, we have also performed the RT-PCR in two different FFPE

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thyroids (control 1 and control 2; Table 2) plus a series of ten frozen samples of thyroid that were pre-pandemic samples (data not shown). No gene amplification was observed, except for the hRNAp gene.

ISH

To confirm the presence of viral material in the tissues, we have performed ISH using RNAscope®. For control of our experiment, SARS-CoV-2-infected (Fig. 2A) and not infected (Fig. 2B) monkey VERO cells (ATCC, CCL-81) were used. The results from RNA-ISH obtained with the positive control probe (UBC) revealed expression in all samples (Fig. 2C), including the lung (Fig. 2F), indicating that all samples had preserved mRNA. To monitor for non-specific signals, we used a negative control probe, the DapB gene from the *Bacillus subtilis bacterium* that, as expected, was negative for all samples (Fig. 2D and G). In the samples that revealed positivity for the SARS-CoV-2, the signal detected was very discrete and focal, as previously described in the thyroid (21). In 9/20 (45%) of the samples, it was possible to detect RNA-ISH signals in the slides (Fig. 2E), including the lung (Fig. 2H). In four of the samples, we detected a signal in the thyroid follicles (Fig. 2E), whereas, in the additional five, the signal was observed in the adjacent tissue (adipose tissue and/or muscle) (data not shown).

IHQ

Expression of SARS-CoV-2 N and S proteins

The thyroid samples displayed a focal, cytoplasmic, and granular-like staining for SARS-CoV-2 nucleocapsid antibody (Fig. 3A). In 4 out of 20 samples (20%), we observed >25% of the tissue with the expression of the protein on the thyrocytes and was mostly cytoplasmic granular-like staining. Eleven out of 20 (55%) had <25% of the tissue expressing the protein, and 5/20 (25%) were completely negative. As for the spike protein, the staining was scarcer, with small, focal areas of cytoplasmic staining (Fig. 3B). In 2 out of 20 samples (10%), we observed >10% of the tissue staining and 4/20 (20%) had <10%. Five samples (25%) had only one or two focal areas and the remaining seven (35%) were totally negative. Both proteins displayed focal and cytoplasmatic expression in the lung tissue (Fig. 3C and D) (see Supplementary Table 1).

Expression of cleaved caspase-3

The cleaved caspase-3 antibody detects the endogenous levels of activated caspase-3 in the tissue. The staining for cleaved caspase-3 was mainly cytoplasmic, with a granular pattern in some cases, and its expression throughout the series was high, indicating a high level of apoptosis in these cases, including the lung (Fig. 4A and B). In 5/20 (25%) cases, we observed >75% of the tissue staining, 11/20 (55%) cases had 50–75% of positive cells, 2 cases had 25–50% of positive cells, and 2 cases (10%) had less than 25% of the cells with cleaved caspase-3 expression (see Supplementary Table 1).

Expression of ACE-2, TMPRSS2, and furin

ACE-2 immunohistochemistry displayed mostly basal staining that seems to co-localize with the endothelial cells (Fig. 5A). No expression of ACE-2 was observed in the cytoplasm or apical membrane of the thyrocytes (Fig. 5A). The expression was variable among the cases and 6/19 (32%), including the lung tissue, had less than 10% of the tissue staining (Fig. 5B). The majority of the samples, 13/19 (68%), presented ACE-2 expression in >25% of the tissue, and two of those samples had >75% of the tissue expressing ACE-2. TMPRSS2 protein expression was very high in the thyroid specimens. Eleven out of the 20 samples (55%) had >75% of the tissue expressing cytoplasmic staining and occasionally, nuclear staining for TMPRSS2 was observed as well (Fig. 5C and D). Furin protein expression was generally low, and 5/19 (26%) of the samples had up to 25% of the tissue expressing cytoplasmic staining with a low to moderate intensity. Four samples (21%) showed positivity in up to 50% of

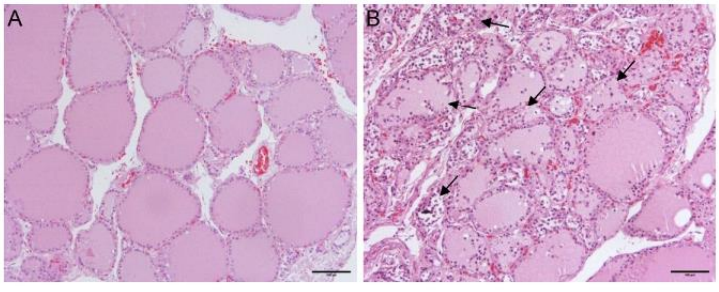

Figure 1
(A) H&E of thyroid specimens of COVID-19 patients: neither lymphocytic infiltration nor thyroid wide-spread alterations were evident; (B) in some cases, there were focal areas of follicle alterations (arrows). Scale bars: 100 μm.

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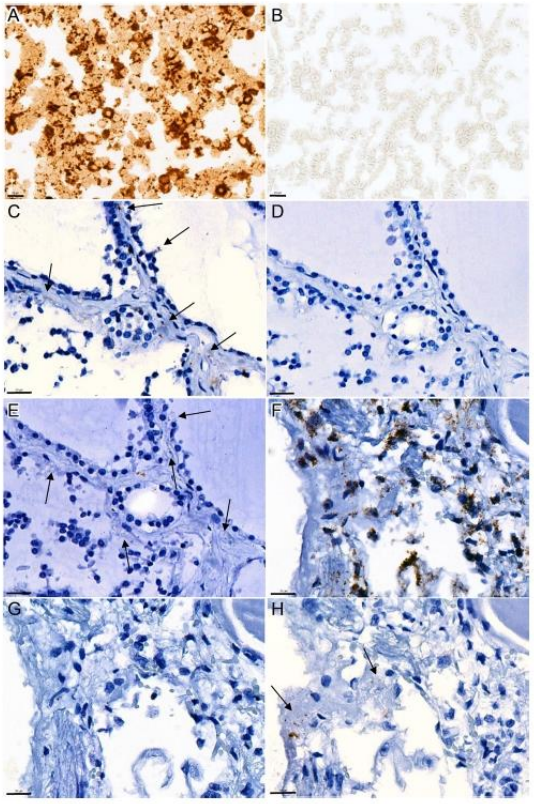

Figure 2
(A) Positive RNA-ISH signal in VERO cell lines infected with SARS-Cov-2; (B) negative RNA-ISH signal in VERO cell lines without infection of SARS-Cov-2; (C) representative section of a thyroid sample showing positivity for control UBC probe (arrows); (D) the respective negative control for DapB probe; (E) thyroid sample showing positivity for SARS-CoV-2 probe in the thyroid follicles (arrows); (F) lung tissue showing positive signal for control with UBC probe and (G) the negative control with DapB probe; (H) the lung for the SARS-CoV-2 probe (arrows). Scale bars: 20 μm.

the tissue, presenting moderate staining. The remaining samples, 10/19 (53%), were negative or with less than 10% of the cells staining (see Supplementary Table 1).

Discussion

COVID-19 infection has been revealed as a multisystemic disease, presenting several endocrine repercussions. In this study, we aimed to clarify if the thyroid cells could be directly infected by the SARS-CoV-2 through the analysis of a series of thyroid specimens from patients deceased by COVID-19, with histopathologic and molecular evaluation of the specimens.

In this series, we were able to detect, by RT-PCR, the expression of SARS-CoV-2 genes in 14/16 (88%) of the thyroid specimens. To our knowledge, 3 studies reported to date the presence of the virus genome in thyroid; Wang *et al.* in 1 thyroid sample (21), Poma *et al.* in 9 out 25 thyroids analyzed (36%) (22), and Wong *et al.* 2021 in 6 out of 8 thyroids (75%) (23). In the three previous studies, a sample was considered positive for SARS-CoV-2 when at least one of the tested genes was amplified by RT-PCR. We were more restricted in the present study; we performed RT-PCR with two different kits and considered positive only those cases with amplification for at least two viral genes. To evaluate the specificity of the signal, we also included 12 cases of thyroid tissue from the pre-pandemic period, and all were negative for all the viral genes tested. Taken together, these results suggest that thyroid cells can be infected by SARS-Cov-2 virus.

By RNA-ISH techniques, we were able to corroborate this observation since we could detect the presence of viral RNA transcripts in the thyrocytes in four cases *in situ* that were also positive for RT-PCR. In four other cases positive for RT-PCR, we were able to detect the RNA transcripts by ISH, but only in the adjacent tissue of the thyroid (muscle cells and adipocytes), and therefore, we cannot dismiss the possibility that the positivity detected in these cases by RT-PCR could be due to the infection of the adjacent tissue and not from the thyrocytes itself. Five cases that were positive for RT-PCR did not show positivity in the RNA-ISH. This discrepancy can be explained by the fact that ISH techniques are not as sensitive as RT-PCR and, consequently, miss some of the cases with low viral load. Similar results were also observed in a previous study (21).

The overall signals for RNA-ISH were very focal and discrete in our study. A similar pattern was described previously in studies reporting RNA-ISH in different human tissues (21, 23, 24, 25). So far, RNA-ISH data regarding SARS-CoV-2 in thyroid specimens are very scarce, and only two studies performed the aforementioned technique (21, 23). One study performed RNA-ISH in one thyroid sample, and, similar to our results, only rare positive clusters were detected in the specimen (21); as referred by the authors, this focal and discrete pattern can result from the possible low viral load in the thyroid samples. This possibility is in accordance with the relatively higher Ct values obtained in the RT-PCR for SARS-CoV-2 genes that we also observed in our study (Table 2). The other study uses fluorescent *in situ* hybridization (FISH)-based detection of SARS-CoV-2 RNA in six thyroids using two different probes: SARS-CoV-2 sense S gene probe and SARS-CoV-2 antisense of S gene probe that, according to Wong *et al.*, indicates viral

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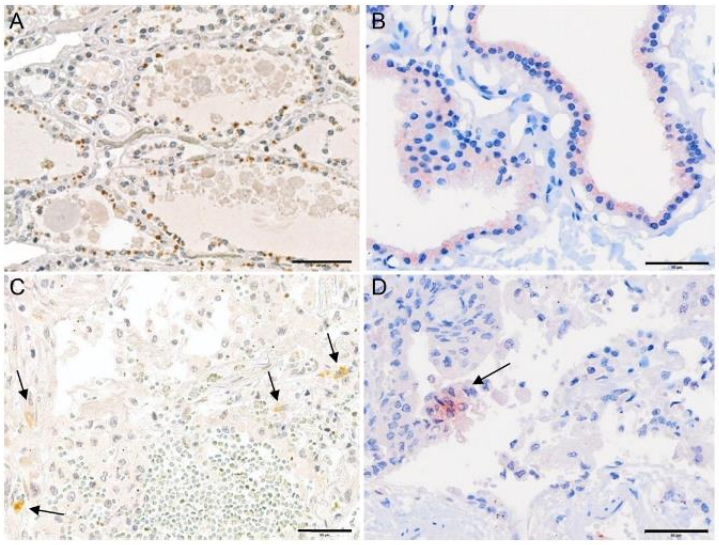

Figure 3
(A) SARS-CoV-2 nucleocapsid granular-like expression and (B) SARS-CoV-2 spike protein expressing cytoplasmic staining in the thyrocytes; (C) SARS-CoV-2 nucleocapsid immunohistochemical expression in the lung with focal cytoplasmic staining (arrows); (D) SARS-CoV-2 spike protein in the lung with focal cytoplasmic expression (arrows). Scale bars: 50 μm.

replication activity (23). They report positive signals from both genomic and replicating RNA, in follicular epithelial cells, as well as in some stromal cells and endothelial cells (23), as we detected in our samples.

In the present study, SARS-CoV-2 variates were not sequenced, but SARS-CoV-2 B.1 clade, subclade B.1.1.33, was reported as the most prevalent genetic group circulating in the Latin America and Caribbean region until January 2021 (26). So, we can admit that, at least for the Brazil series, most of the patients were infected with the SARS-CoV-2 B.1 clade.

We have also performed IHQ for SARS-CoV-2 nucleocapsid and spike proteins, but the results obtained were not as demonstrative as the ones obtained with the previous techniques. We detected granular-like staining for the nucleocapsid protein in the thyrocytes of the majority of the samples; similar data have been reported for this protein in another study (22) in which the authors present a staining pattern very similar to the one shown in Fig. 4A.

In our series, we detected positive staining for the N protein in 11 cases, 10 of them were positive for the RT-PCR and 1 was inconclusive. Interestingly, the inconclusive case was the one that was positive for SARS-CoV-2 in the first extraction and negative in subsequent extraction. Once more, it is possible that viral load in the tissue of the second RNA extraction could not be sufficient for positive amplification of more than one gene. Immunohistochemistry for spike protein in thyroid has not been documented yet, and in our series, we found a very focal, weak cytoplasmic staining, and it was not possible to correlate N and S proteins expression through IHC (Supplementary Table 1). Hence, we observed that the nine cases positive for S protein were also positive in the RT-PCR amplification for the SARS-CoV-2, but in five cases that were positive in the RT-PCR, we could not detect S protein expression. The difficulty in the detection of the viral protein through IHC techniques has already been reported in other studies (23); besides the problem of viral load, it can also reside in antibody specificity, even

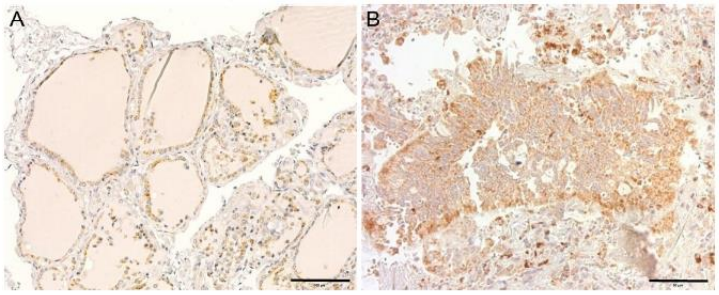

Figure 4
(A) Cleaved caspase-3 immunoexpression in the thyroid (scale bar: 100 μm) and (B) in the lung tissue (scale bar: 50 μm).

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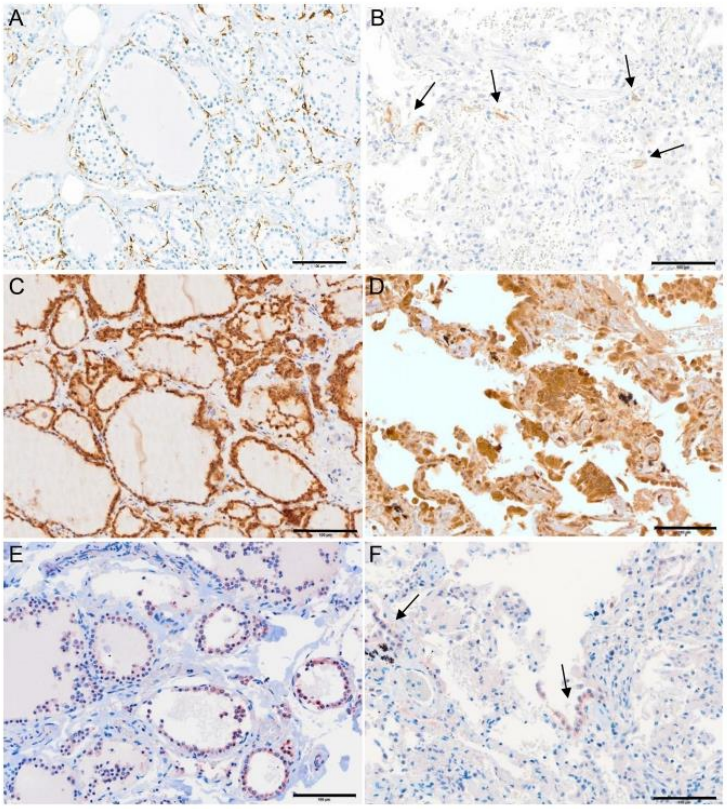

Figure 5
(A) ACE-2 expression in a thyroid specimen with extensive staining, and (B) in the lung, with staining in <10% of the cells (arrows) (scale bar: 100 μm); (C) TMPRSS2 expression in the thyrocytes with strong cytoplasmatic expression (scale bar: 100 μm) and (D) in the lung with strong cytoplasmatic/nuclear staining (scale bar: 100 μm); (E) Furin moderates cytoplasmatic expression in the thyrocytes (scale bar: 100 μm) and (F) in the lung with low cytoplasmatic staining (arrows) (scale bar: 100 μm).

more, when using FFPE material from autopsy. Finally, the negative case for the SARS-CoV-2 genome was also negative for both viral proteins N and S.

Another aim of our study was to evaluate the expression of cell entry factors such as ACE-2, TMPRSS2, and furin in the same specimens. ACE-2 expression was variable in the series, with RT-PCR-negative cases presenting high expression and RT-PCR-positive cases presenting low ACE-2 expression, and thus, it was not possible to establish any correlation. ACE-2 regulation upon SARS-CoV-2 entry is controversial and not yet well understood. A study using a cohort of COVID-19 patients detected high levels of Ang II in the blood of the patients, which was positively correlated with high viral load, thus, linking SARS-CoV-2 infection with ACE-2 downregulation (27). On the other hand, more recently, opposite results were observed in critically ill COVID-19 patients; van Lier *et al.* reported high levels of soluble ACE-2, low levels of Ang II and increased levels of Ang 1–7 when compared with healthy controls (28). Therefore, more studies are needed to clarify the putative relationship between ACE-2 expression and SARS-CoV-2 infection, especially since there are several other molecules that have been suggested as alternative cellular receptors for SARS-CoV-2 (e.g. lectins, phosphatidylserine receptors, CD147, neuropilin 1) (17). The aforementioned points may provide a justification for our variable data about ACE-2.

We observed in the present series a high expression of TMPRSS2, and this was also true for the negative cases in the RT-PCR for SARS-CoV-2 genes. On the contrary, furin protein expression was very low in our series, independent of the RT-PCR status. To our knowledge, no other study has reported IHQ protein expression of these two markers in thyroid of COVID-19 patients. Wang *et al.*, using RNA-ISH techniques, reported the presence of ACE-2 and TMPRSS2 mRNA in a series of pulmonary and non-pulmonary tissues of infected and deceased patients, and they reported that most tissues expressed both ACE-2 and TMPRSS2 transcripts, including the thyroid (21).

We observed high levels of apoptosis in the series, with high levels of expression of the activated caspase-3 in the thyroid and lung tissues. A co-localization of the viral RNA and activated caspase-3 was described in nasopharyngeal

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swabs, indicating that viral infected cells had increased apoptosis (29). In our study, the activated caspase-3 expression was relatively high throughout the cases, independently of the results for SARS-CoV-2 RT-PCR, so we cannot exclude that this increase in the expression could be due to a *post-mortem* autolysis process.

Although infection by SARS-CoV-2 virus has been associated with cases of subacute thyroiditis (2, 15), we could not identify in our series any major histological signs of lymphocytic infiltration or inflammatory damage. Nevertheless, it would be interesting to evaluate the clinical data in terms of thyroid function of these patients.

Using a unique series of thyroid of COVID-19-deceased patients, the present study has some limitations. Since we studied autopsy specimens, the degree of autolysis in the tissues is variable, which can restrict the detection of the virus or even raise non-specific signals. To downgrade that possibility, we have used several positive and negative controls and pre-pandemic cases in our experiments. Furthermore, for most patients, we had only access to a single sample; this restricts the possibility of selecting a more representative thyroid sample of the SARS-CoV-2 infection.

Summing up, our results support that thyroid gland can be directly infected by the SARS-CoV-2 virus. We detected the amplification of SARS-CoV-2 genes in the thyroid specimens of 88% of the COVID-19-deceased patients, which was possible to confirm in the follicular cells through hybridization techniques *in situ*. On the other hand, protein detection of the SARS-CoV-2 nucleocapsid or spike protein does not seem to be a robust strategy to detect viral infection in FFPE tissues. Finally, we did not find any correlation between SARS-CoV-2 infection and the putative cellular 'doors' for virus entry.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/ETJ-22-0074.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S M: performed ISH and IHQ staining, analysis of the results, reviewed the literature, and wrote the first version of the manuscript; A P: histological cuts, H&E staining, and IHQ staining; L S, C N, S G, F C: clinicopathological data and patients' material collection from Portugal; M D, P Sa: clinicopathological data and patients' material collection from Brazil; G A, R O, D C: SARS-CoV-2 RT-PCR amplification; MSS: histological evaluation of the H&E slides and wrote the manuscript; P So: study design and methodology, verification and discussion of data, and wrote the manuscript. All the authors contribute to the final version of the manuscript.

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Supplementary data

Table S1 – Summary of the immunohistochemistry results obtained on the series.

Case	Sample	Tissue	RT-PCR Status	Immunohistochemistry					
				SARS-CoV-2 N	SARS-CoV-2 S	ACE-2	TMPRSS2	Furin	Cleaved Caspase-3
1	1	Lung	POSITIVE	focal areas	focal areas	rare cells	+++++	rare cells	++++
2	2	Thyroid	ND	+	-	+++	+++++	+++	++++
	3	Thyroid	ND	+	focal areas	++	++++	+	++++
	4	Thyroid	ND	++	+	+++	+++++	++	++++
	5	Thyroid	POSITIVE	+	focal area	++++	+++++	+++	++++
3	6	Thyroid	ND	+++++	-	-	+++++	++	+++++
	7	Thyroid	POSITIVE	+++++	-	+++	+++++	-	++++
4	8	Thyroid	POSITIVE	+++	focal areas	++	+++	+	+++++
5	9	Thyroid	POSITIVE	+	-	+	rare cells	- (**)	+++
6	10	Thyroid	POSITIVE	++	+++	+++++	+++++	+++	+++++
7	11	Thyroid	POSITIVE	++	focal area	rare cells	++	rare cells	++++
8	12	Thyroid	POSITIVE	rare cells	-	+++	++++	-	+++
9	13	Thyroid	POSITIVE	+	++	++	++++	+++	+++++
10	14	Thyroid	POSITIVE	-	+	+	+++++	++	++++
11	15	Thyroid	INCONCLUSIVE*	+++	-	++++	+++++	- (**)	++++
12	16	Thyroid	NEGATIVE	-	-	++	+++++	++	++++
13	17	Thyroid	POSITIVE	-	+	+	++++	++	++
14	18	Thyroid	POSITIVE	++	-	+++	++	rare cells	+
15	19	Thyroid	POSITIVE	-	+	++++	+++++	ND	+++++
16	20	Thyroid	POSITIVE	-	-	rare cells	++++	focal areas	++++

***Legend**: **-** negative case; **+** <10% of the cells with expression; **++** 10-25%; **+++** 25-50%; **++++** 50-75%; **++++** >75%; *Case positive for the Fosun Kit in the first RNA extraction, and negative in the second extraction for both Fosun and TaqPath kits; **Case with thyroid negative but with staining on the adjacent tissue; **ND** - Not done.*

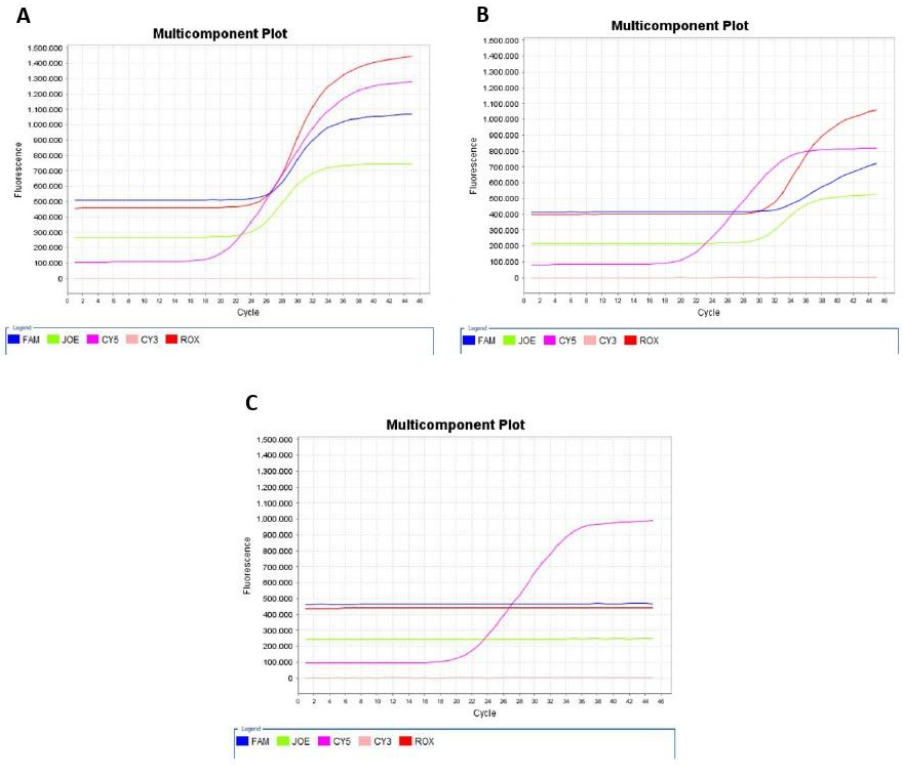

Figure S1 – Examples of RT-PCR multicomponent results for Sars-Cov-2 virus using the Fosun Kit in the control tissue, lung (A), and thyroid tissue (B and C) of COVID-19 patients. Samples A and B are positive for all the viral genes and sample C is negative for the presence of the viral genes. CY5 channel amplifies the positive control hRNAP and the CY3 channel amplifies a negative control; FAM, JOE, and ROX channels amplify the SARS-CoV-2 genes ORF1ab, Gene N, and Gene E, respectively.

II. Post-COVID-19 Condition: Where Are We Now?

In alignment with the work on SARS-CoV-2 infection of the thyroid, the following review critically addresses the systemic impact of COVID-19, emphasizing its potential implications on thyroid function and long-term complications. Through a comprehensive review, we searched the landscape of post-COVID symptoms, exploring the context of chronic complications. Given the high expression of ACE in the thyroid, a key target for SARS-CoV-2, our investigation seeks to understand the molecular mechanisms linking the virus to thyroid dysfunction. By unveiling the complex mechanisms, including direct cellular effects, inflammatory responses, and HPT axis dysfunction, we contribute to understanding COVID-19's role in post-viral syndrome.

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Review

Post-COVID-19 Condition: Where Are We Now?

Paula Boaventura [1,2,3,*], Sofia Macedo [1,2], Filipa Ribeiro [1,2], Sónia Jaconiano [4] and Paula Soares [1,2,3]

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Abstract: COVID-19 is currently considered a systemic infection involving multiple systems and causing chronic complications. Compared to other post-viral fatigue syndromes, these complications are wider and more intense. The most frequent symptoms are profound fatigue, dyspnea, sleep difficulties, anxiety or depression, reduced lung capacity, memory/cognitive impairment, and hyposmia/anosmia. Risk factors for this condition are severity of illness, more than five symptoms in the first week of the disease, female sex, older age, the presence of comorbidities, and a weak anti-SARS-CoV-2 antibody response. Different lines of research have attempted to explain these protracted symptoms; chronic persistent inflammation, autonomic nervous system disruption, hypometabolism, and autoimmunity may play a role. Due to thyroid high ACE expression, the key molecular complex SARS-CoV-2 uses to infect the host cells, thyroid may be a target for the coronavirus infection. Thyroid dysfunction after SARS-CoV-2 infection may be a combination of numerous mechanisms, and its role in long-COVID manifestations is not yet established. The proposed mechanisms are a direct effect of SARS-CoV-2 on target cells, an indirect effect of systemic inflammatory immune response, and a dysfunction of the hypothalamic-pituitary-thyroid (HPT) axis leading to decreased serum TSH. Only a few studies have reported the thyroid gland status in the post-COVID-19 condition. The presence of post-COVID symptoms deserves recognition of COVID-19 as a cause of post-viral fatigue syndrome. It is important to recognize the affected individuals at an early stage so we can offer them the most adequate treatments, helping them thrive through the uncertainty of their condition.

Keywords: post-COVID-19 condition; long COVID; SARS-CoV-2; thyroid

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1. Introduction

COVID-19 was first described as a respiratory disease, but presently it is considered a systemic infection comprising multiple systems and causing chronic complications [1–3] (Figure 1). The pathology results not only from the virus infection but from an aberrant inflammatory host immune response [4]. The immune response has been well described in acute COVID-19 patients, but the lasting consequences of the infection are still not well known [4]. Researchers have been exhaustively surveying the diverse symptoms of long COVID, but until now, no integrated explanation exists for their manifestation [5]. Sykes et al. [6] alerted us to the effects of this poorly known lethal virus, to the societal disruption it has caused, and to the importance it may have in the development of long-lasting physical and mental health symptoms. On the other hand, Sancak and Kilic [1] state that post-COVID-19 condition symptoms can most often be interpreted as somatization; however, the fact that we may not understand them does not mean they are purely psychosomatic [1]. In the study of Xiong et al. [7] in hospitalized patients from Wuhan, a non-infected control group from the general population was used in order to exclude the psychological effects

of the long and mandatory isolation period, which caused deconditioning, anxiety, and depression. The authors showed a significant difference between the group of COVID-19 "recovered" patients and the control group, with the latter reporting very few long-term symptoms [7].

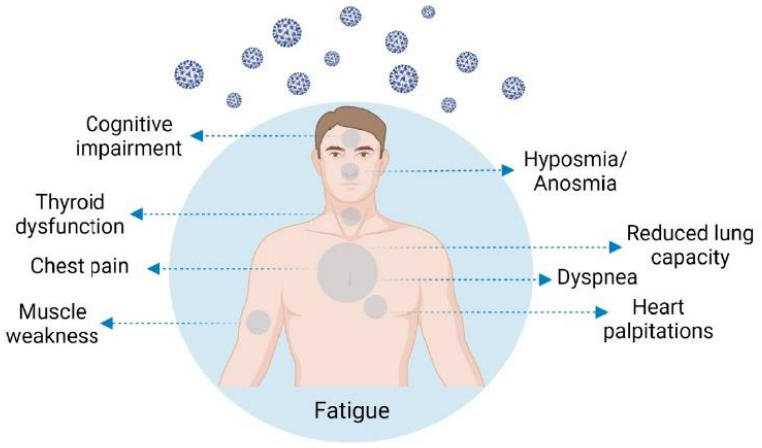

Figure 1. Examples of post-COVID-19 condition chronic complications.

2. Post-COVID-19 Condition Symptomatology and Prevalence

Protracted COVID-19 infection–related symptoms are common [8,9], but the post-COVID-19 condition [10] (previously referred to as long COVID] is a poorly understood aspect of the current pandemic [9,11]. Compared with other post-viral fatigue syndromes, the symptoms are wider and more intense [12]. An exact definition was recently published by the WHO [10]: typically, symptoms with duration ≥2 months [10,13,14] that cannot be explained by an alternative diagnosis are considered post-COVID-19 condition [10]. Post-acute manifestations may be divided into three categories: (1) residual symptoms continuing after recovery from acute infection; (2) organ dysfunction continuing after initial recovery; and (3) new symptoms or syndromes that appear after initial asymptomatic or mild infection [15].

Over several studies, the frequency of post-COVID-19 condition ranged from 4.7 to 80% (n = 25), occurring between 3 to 24 weeks after the acute phase or hospital discharge [16]. Yong [17], in a study on COVID-19 survivors (n = 10), reported that a post-COVID-19 condition persisted for one to six months in 30−80% of patients. Other studies reported a 35% prevalence of residual symptoms in non-hospitalized patients [18], but around 75–87% in hospitalized patients [6,18,19]. In a cohort of patients followed-up for three to nine months after infection, about 30% maintained persistent symptoms [20].

The most frequently reported symptoms, not restricted to severe acute disease [19,21], are profound fatigue [3,6,15,19,22–30] or muscle weakness [6,19,29,31], dyspnea [3,6,24,25,28,30], sleep difficulties [6,19,23,28], anxiety or depression [6,19,29], reduced lung capacity [22,32], memory/cognitive impairment ("brain fog") [23,28,29], hyposmia/anosmia [23], and the inability to fully exercise or work. The most frequent symptom of post-COVID-19 condition is fatigue, which is independent of the acute disease severity or the presence of respiratory problems [33]. A summary of post-COVID-19 condition symptoms, with the frequencies reported and number of patients evaluated, is presented in Table 1. The high variability found between studies is mostly attributable to acute COVID-19 severity, with more frequent symptoms in hospitalized patients compared with patients who suffered from mild or asymptomatic disease.

Table 1. Post-COVID-19 condition symptoms most frequently reported.

Post-COVID-19 Symptoms	Number of Patients Included in the Study	% Patients Suffering from Symptom/References
Fatigue	596, 177, 538, 270, 138, 3065, 134, 242, 115, 143, 96, 1733, 5440, 384, 287	13.1% [34], 13.6% [20], 28.3% [7], 34.8% [25], 39.0% [27,30], 39.6% [6], 41.7% [35], 47% [26], 53.1% [36], 56.3% [28], 63% [19], up to 65% [16], 69% [37], 72.8% [38]
Persistent breathlessness /dyspnea	596, 3065, 287, 270, 96, 138, 143, 384, 134, 5440, 35	6.0% [34], 23.2% [30], 28.2% [38], 34.0% [25], 37.5% [28], 40.0% [27], 43.4% [36], 53.0% [37], 60% [6], up to 61% [16], 80% [23]
Myalgia /muscle weakness	277, 242, 134, 1733	19.6% [25], 35,1% [35], 51.5% [6], 63% [19]
Anxiety	287, 402, 134	38.0% [38], 42% [39], 47.8% [6]
Sleep disturbance	1733, 96, 134, 35, 138	21.1% [35], 26.0% [19], 26.0% [28], 35.1% [6], 40.0% [39], 46% [23], 49% [27]
Joint pain	277, 143, 287	19.6% [25], 27.3% [36], 31.4% [38]
Headache	242, 270, 3065, 287	19.0% [35], 19.8% [25], 23.4% [30], 28.6% [38]
Chest pain	596, 242, 538, 143, 287, 35, 5440	0.8% [34], 10.7% [35], 12.3% [7], 21.7% [36], 28.9% [38], 34.8% [23], up to 89% [16]

To better examine this issue, Gaber et al. [27] looked at the effects of COVID-19 infection in healthcare workers, a population with an expected high level of exposure to the virus. They reported a high incidence of infection and a high prevalence of incapacitating post-COVID-19 symptoms, with fatigue commonly reported [27]. Nonetheless, these health workers were unwilling to either seek medical help or take sick leave, despite their struggle to cope with the symptoms [27].

Taquet et al. [40] found a higher incidence of numerous psychiatric disorders in COVID-19 survivors compared with matched patients with influenza or other respiratory tract infections, in a retrospective cohort study using 236,379 electronic health records. The estimated incidence of a neurological or psychiatric diagnosis in the six months following a COVID-19 diagnosis was 33% (95% CI; 33.17–34.07) [40]. Post-COVID-19 condition presents neurological symptoms similar to chronic fatigue syndrome (CFS) and functional neurological disorder (FND) (except for hypogeusia) [41].

Davis et al. [42] conducted an online survey to characterize post-COVID-19 condition in an international cohort (56 countries), tracing the symptoms over 7 months. They found for 91% of the respondents that the time to recovery exceeded 35 weeks; the most frequent symptoms after six months were fatigue, post-exertional malaise, and cognitive dysfunction [42]. According to the authors, their study represents the largest collection of symptoms recognized in post-COVID-19 condition individuals to date (June 2021). More recent studies have shown that persistent symptoms can be found 12 [43] or up to 15 months after recovery from the acute phase of COVID-19 [44]; symptoms are common both in ambulatory and hospitalized patients [44].

3. Post-COVID-19 Condition Risk Factors

Post-COVID-19 condition is associated with a weak anti-SARS-CoV-2 antibody response [45], severity of illness [19,34,46,47], female sex [3,5,6,18,19,34,35,45], presence of more than five symptoms in the first week of the disease [3,18,48], older age [18], and presence of comorbidities [18]. Concretely, Fernández-de-las-Peñas et al. [49] reported that the most significant risk factor for developing more post-COVID symptoms was the number of symptoms at hospital admission, which supports the idea that a higher symptom burden in the acute phase of the disease is associated with a higher probability of the post-COVID-19 condition.

Early dyspnea, prior psychiatric disorders, and specific biomarkers (e.g., D-dimer, C- reactive protein, and lymphocyte count) have also been reported as risk factors, even though more research is needed to validate them [3]. Peghin et al. [34] suggested that the

constantly elevated titers of the serological response against SARS-CoV-2 may constitute an independent risk factor for the post-COVID-19 condition, since the presence of SARS-CoV-2 IgG antibodies is significantly associated with the condition. Contrarily, Seessle et al. [28] reported that patients presenting at least one post-COVID-19 symptom 12 months after infection did not significantly differ in their SARS-CoV-2 antibody levels when compared with patients without symptoms, although their physical and mental quality of life had significantly decreased.

Interestingly, Townsend et al. [26] showed that significant illness persistence after the COVID-19 acute phase of the disease, affecting health perception, ability to return to work, and the existence of lasting fatigue, appears to be unrelated to the severity of the acute phase, though one would expect to see a difference in post-COVID symptoms between hospitalized and non-hospitalized patients; this hypothesis needs to be verified in upcoming studies [50]. In fact, one puzzling feature of post-COVID-19 condition is that it affects COVID-19 patients at all disease severity levels [3], often affecting patients with a mild acute illness [51]. Studies have shown that post-COVID-19 condition affects even mild to moderate cases [3,52,53] and younger adults (or even children) who did not need respiratory support or hospital or intensive care [3]. Post-COVID-19 condition in children is similar to that seen in adults [54], with symptoms such as a fatigue, dyspnea, myalgia, cognitive impairments, headache, palpitations and chest pain [3,55].

In general, it appears that the ratio for post-COVID-19 condition development is 2:1 in women compared with men, but only until around age 60, when the ratio between women and men becomes similar [14].

Post-COVID-19 condition in patients with comorbidities may result from their comorbidity worsening [56].

4. Post-COVID-19 Condition Pathophysiology

Different lines of research are trying to explain these protracted symptoms. A persistent immune activation and/or inflammation may contribute to post-COVID-19 condition, which could explain why many patients with mild COVID-19 disease experience chronic persistent symptoms, involving the cardiovascular, nervous, and respiratory systems [57]. In fact, the persistently elevated inflammatory markers observed in long-COVID patients point towards chronic persistence of inflammation [18,58].

Shuwa et al. [4] observed lasting alterations in the functional potential of CD8+ T cells from recovering COVID-19 patients up to six months following hospital discharge, which may imply a sustained change in cytokine potential, contributing to a constant inflammatory status [4]. Contrarily, B cell changes seem to be largely restored in convalescence [4]. In a more recent study, Glynne et al. [51] reported that CD8+ EM T cells are diminished for up to 400 days following infection, regardless of symptoms, and CD4+ and CD8+ CMT cell PD-1 levels are augmented following COVID-19 (more marked in post-COVID-19 condition). T-cell dysfunction may promote post-COVID-19 condition pathophysiology similarly to what occurs in autoimmune diseases [3]. It remains to be determined if SARS-CoV-2–specific T cells have the capacity to react against self-antigens [57].

Seessle et al. [28] observed several neurocognitive symptoms that were associated with antinuclear antibody titer elevation, pointing to autoimmunity as a cofactor in the etiology of post-COVID-19 neurologic conditions [28]. The autoimmune hypothesis could explain the greater incidence of this condition in women [14,57]. Since thyroid is closely linked to T-cell-mediated autoimmunity, thyroid dysfunction may be important in the pathophysiology of post-COVID-19 condition, as discussed in more detail below [3].

Post-COVID-19 condition has been related to additional characteristics of the innate and adaptive response, involving a weaker initial inflammatory response, with lower baseline levels of C-reactive protein and ferritin [45]. The participation of the immune system in post-COVID-19 condition has been reported in other studies [8,21,57,59,60]. Symptoms such as cognitive dysfunction, persistent fatigue, muscle aches, depression, and

other mental health issues are highly associated with an initial immune challenge and/or with a constant dysregulation of the immune system [29,60].

Many neurological anomalies have been described in patients with COVID-19 [41,61], comprising the central and peripheral nervous systems, ranging from mild to fatal, and occurring in patients with severe or asymptomatic SARS-CoV-2 infection [61]. These deferred manifestations may be significant, because they likely affect patients not presenting neurological symptoms in the acute phase [62]. Neurocognitive post-COVID-19 condition symptoms can last for at least one year subsequent to acute infection, diminishing life quality considerably [28].

The involvement of inflammatory cytokines in the etiology of the neuropsychiatric symptoms, reported in current large-scale population-based epidemiological and genetic studies, indicates that these cytokines may have a role in the etiology of the neuropsychiatric symptoms usually observed in patients with post-COVID-19 condition [3,29,60]. This cytokine storm must also be considered as a possible driving factor for the expansion of neuropathies after severe COVID-19 infection, contributing to the chronic pain that appears after acute infection recovery [62]. The augmented cytokine activity, which drives the inflammatory process, disrupts T cell responses, and imposes limitations on neuronal metabolism, may also be an adequate therapeutic target for management and prevention of post-COVID-19 condition [60].

Altered tryptophan absorption and tryptophan-disrupted metabolism have been suggested as key contributors to the enduring symptoms in COVID-19–recovered patients, with numerous studies showing low levels of tryptophan and serotonin in individuals infected with SARS-CoV-2 [63]. Tryptophan is a precursor of melatonin and serotonin, molecules implicated in sleep control and mood disorders, respectively; it is also involved in skeletal muscle mass regulation, a notorious lasting symptom of post-COVID-19 condition [63].

Some symptoms may be related to virus- or immune-mediated disruption of the autonomic nervous system, leading to transient or longstanding orthostatic intolerance syndromes [8,31,64,65]. In orthostatic intolerance, the release of epinephrine and norepinephrine causes pronounced tachycardia, which is experienced as palpitations, breathlessness, fatigue, and chest pain, which are common symptoms of post-COVID-19 condition [8]. Alterations in the autonomic nervous system can promote each of these symptoms, theoretically providing a uniting pathobiology for acute, subacute, and lasting sequelae of the infection, and may also be considered as a target for intervention [31].

Studies have shown that patients with severe symptoms may have more severe autonomic dysfunction when compared with patients presenting mild symptoms, as indicated by the heart rate variability (HRV) analysis [2], which is a reliable non-invasive tool used to evaluate autonomic modulation [2,64]. Patients with severe symptoms presenting amelioration in autonomic parameters also show enhancements in immune and coagulation functions, as well as in cardiac injury biomarkers [2].

Townsend et al. [66] conducted research to assess if fatigue, the most common symptom following infection, was associated with autonomic dysfunction. No association was found with autonomic dysfunction; the authors found an intense association of fatigue with increased anxiety ($p < 0.001$) in patients without pre-existing diagnoses of anxiety [66].

Another potential cause of post-COVID-19 condition could be the SARS-CoV-2 tropism from the olfactory system into the brainstem, and the consequent persistent, low-grade brainstem dysfunction [17]. SARS-CoV-2 may damage the brainstem through viral invasion, inflammation, and vascular activation [17]. Interestingly, functions of the brainstem and post-COVID-19 condition symptoms have a great degree of overlap [17].

SARS-CoV-2 RNA was found in the brain during autopsy of deceased COVID-19 patients in some studies, but in other studies no SARS-CoV-2 materials were found [17]. This suggests that SARS-CoV-2 neurotropism or brain invasion may happen but not in every patient [17]. The presence of SARS-CoV-2 in the central nervous system has not been directly related to the severity of the neuropathological findings, suggesting that neuronal infection may be only one of the pathways through which SARS-CoV-2 could influence

brain function and contribute to some of the long-lasting symptoms of post-COVID-19 condition [29].

Hypometabolism has been reported in post-COVID-19 condition patients; specifically, hyposmia/anosmia was associated with cerebellar hypometabolism [23]. In general, areas of hypometabolism comprised the bilateral rectal/orbital gyrus (including the olfactory gyrus], the right temporal lobe (including the amygdala and the hippocampus extending to the right thalamus], the bilateral pons/medulla brainstem, and the bilateral cerebellum [23]. These metabolic groups allowed distinguishing between patients and healthy subjects with a high power of discrimination.

Long-term cardiovascular effects of COVID-19 have been described [67]. Vascular events can happen unpredictably in fit patients with mild or asymptomatic COVID-19 infection, even several weeks after the infection [68]. This means that clinicians should remain attentive for post-infective thrombotic sequelae and carefully manage cardiovascular risk factors in convalescent patients, irrespective of the infection severity and the absence of co-morbidities [68]. In post-COVID-19 condition management it is essential to control blood pressure, lipid levels, and obesity after infection with SARS-CoV-2 [53].

Immunological memory of SARS-CoV-2 is not easy to predict [19]. Neutralizing antibody titers at six-month follow-up are significantly lower compared with the acute phase [8,45]. Contrarily, Sette et al. [22] reported data indicating that T and B cell memory and antibodies probably remain for years in most SARS-CoV-2 infected patients.

As previously mentioned, an additional possibility is that post-COVID-19 condition is caused by an immune system dysfunction that leads the immune system to attack the body, meaning that this condition could be an autoimmune disease [5]. Still, it is precocious to affirm which hypothesis is right and, in fact, it might be the case that each is true in different individuals; preliminary data suggest that post-COVID-19 condition could be various disorders grouped into one [5]. These various disease courses may be traced back to the initial phases of the infection, as shown by the fundamental role of type I IFN responses during the acute phase of SARS-CoV-2 infection [13]. As previously mentioned, the autoimmune hypothesis could explain women's higher susceptibility to this syndrome [14]. Indeed, women present a stronger immune response for genetic and hormonal factors compared with men; this is a double-edged sword, leading to a more severe outcome of acute infection in men, but to more common autoimmune reactions in women [14].

5. Thyroid Involvement in COVID-19

Due to the reported high expression of ACE2, the thyroid may become a target of coronavirus infection, and thyroid involvement in COVID-19 patients has been demonstrated [69]. In fact, SARS-CoV-2 uses ACE2, combined with the transmembrane protease serine 2 (TMPRSS2), as the main molecular complex for the host cell infection [70]. Interestingly, ACE2 and TMPRSS2 expression levels are higher in the thyroid gland than in the lungs [70]. Scappaticcio et al. [70], in their literature review on thyroid dysfunction in COVID-19 patients, presented strong evidence that the thyroid gland and the entire hypothalamic–pituitary–thyroid (HPT) axis may be important targets for SARS-CoV-2 damage.

Coperchini et al. [71] showed that IFN and, to a minor degree TNF-alpha, regularly increase ACE-2 mRNA levels in normal human thyroid primary cultures. As stated by these authors, the increased pro-inflammatory cytokine levels may enable SARS-CoV-2 penetration in the cells through an additional increase of ACE-2 expression and/or account for the diverse grades of severity of the infection [71]. Nevertheless, additional specific studies are needed to validate this hypothesis [71]. Two main mechanisms account for thyroid function alterations in COVID-19 patients: a direct effect of SARS-CoV-2 on target cells and an indirect effect of the systemic inflammatory immune response [70,72–75]. A third hypothesis is that dysfunction of the HPT axis causes centrally a decreased level of serum TSH in the infected patients [74]. Changes in thyroid function tests, mostly defined by a TSH level decrease, were described during the acute phase of the infection [69]. These

changes have been associated with either destructive thyroiditis or non-thyroidal illness syndrome (NTIS) [69,76]. NTIS, which is defined by low T3 levels, may be caused by any severe systemic disease [76,77]. It occurs due to the diminished conversion of T4 to T3, which is likely elicited by the same factors that cause a decrease in TSH (increase in cytokines and other inflammatory factors) [77,78]. T3 reduction was observed even in mild COVID-19 disease severity, with increased conversion of T4 to reverse T3 [79].

Since manifestations of post-COVID-19 condition include fatigue, and immune dysregulation is one of the proposed mechanisms involved in the condition development, Lui et al. [80] decided to investigate whether thyroid function and autoimmunity play a role in post-COVID-19 condition. They showed, following-up COVID-19 patients, the spontaneous recovery of most thyroid dysfunction observed in the acute phase of the disease, and that incident thyroid dysfunction was a rare situation. Subgroup analysis revealed that symptom recovery occurred more among patients with positive anti-TPO at the time of re-evaluation, suggesting a potential protective role of anti-TPO in post-COVID-19 condition [80].

Only a few studies have evaluated the thyroid gland condition in the convalescent stage of COVID-19 [81]. Clarke et al. reported that adrenal and thyroid function was maintained $\geq$3 months after COVID-19 diagnosis, even though an important proportion of patients suffered from chronic fatigue [82].

Campi et al. [69] found a temporary situation of low TSH with normal T4 and low T3 levels in patients hospitalized for SARS-CoV-2 infection, which was inversely associated with C-reactive protein, cortisol, and IL-6, and positively associated with normal Tg levels. These authors stated that this temporary change was probably due to the cytokine storm induced by the virus, with a direct or mediated impact on TSH secretion and deiodinase activity, and probably not to a destructive thyroiditis. The THYRCOV study offers early evidence that patients with acute SARS-CoV-2 infection with thyrotoxicosis have statistically significantly higher levels of IL-6 [83]. In a short-term follow-up, Pizzocaro et al. [84] showed a spontaneous normalization of thyroid function in most infected patients with SARS-CoV-2-related thyrotoxicosis. Nevertheless, these authors stated that long-lasting studies are needed, since they found a frequent thyroid hypoecogenicity pattern in the ultrasonographic evaluation of these patients, which may predispose them to late-onset thyroid dysfunction development [84].

Subacute thyroiditis related to COVID-19 typically presents without pain and with thyrotoxicosis, which in some cases is followed by hypothyroidism [73,85]. Subacute thyroiditis was reported in 13 cases (in 10 papers), detected 7 weeks before to 7 weeks after the diagnosis of COVID-19 [76], so only some of these cases could be compatible with post-COVID-19 condition.

Dworakowska et al. [86] stated that clinicians should be aware of subacute thyroiditis likelihood, particularly in the early weeks or months after even mild COVID-19 infection. Subacute thyroiditis might be considered as a late complication of SARS-CoV-2 infection, since it frequently arises a few weeks after the upper respiratory tract infection [75,87]. It may be difficult to promptly diagnose this due to a potential lack of classic symptoms and to shared clinical features between COVID-19 and thyrotoxicosis [88].

Recently, Trimboli et al. [89] conducted a systematic review of subacute thyroiditis in COVID-19 patients, concluding that the size and quality of published data are poor, with only case reports and case series being available. According to the authors, and based on these evidence-based data, subacute thyroiditis cannot yet be considered as a direct or common complication of SARS-CoV-2. Still, this assumption might change in the future, considering the fast worldwide diffusion of SARS-CoV-2 and its variants [89].

Even though clear evidence is missing, infection of the thyrocyte, thyrotroph, and corticotroph may lead to a decrease in T3, T4, TSH, ACTH, and cortisol levels [76]. HPT dysregulation has been considered, at least in part, responsible for hypothyroidism in COVID-19 [74,76]. Low FT3 levels are independently associated with increased mortal-

ity [72,76,90] and disease severity [74,91–93] and may be used as a surrogate prognostic biomarker [72,76,90].

Sick euthyroidism is the most common thyroid-related issue in COVID-19 follow-up, especially in patients who were hospitalized or were admitted to intensive care units [73]. Euthyroid sick syndrome is a condition characterized by low serum levels of thyroid hormones in patients with nonthyroidal systemic illness who are clinically euthyroid. These alterations were transitory and recovered during follow-up, although long-term follow-up studies on thyroid function are still needed [73]. Asghar et al. [94], who analysed 54 COVID-19 patients, reported severe COVID-19 patterns in those patients who appeared to have euthyroid sick syndrome. They also reported that the precise clinical importance of a low TSH was uncertain. The authors included a cut-off estimation of TSH decline, predicting disease severity; patients with low TSH levels (<0.996 uIU/mL) showed significantly low survival, whereas patients with sufficient TSH (>0.996 uIU/mL) had a higher cumulative survival proportion [94]. One main limitation of this study was its small sample size. Gong et al. [95] reported that critical illness rates (74.07% vs. 37.40%, $p = 0.001$) and mortality rates (51.85% vs. 22.76%, $p = 0.002$) were significantly higher in the low TSH group compared with a normal TSH group. Zou et al. [96] also reported that euthyroid sick syndrome was significantly associated with the disease severity and inflammatory parameters in COVID-19 patients.

An altered thyroid function is a common situation in COVID-19 patients [72,74,97], especially in critically ill patients [92]. Lui et al. [90] reported that approximately 15% of patients with mild to moderate COVID-19 had thyroid dysfunction. Nevertheless, with the data published so far, it is not possible to assume that thyroid diseases are a risk factor for COVID-19 disease [77,98]. Likewise, a higher occurrence of thyroid disease in patients with COVID-19 has not been observed [77,98]. Although COVID-19 is linked to NTIS, it is not clear if it also raises the risk of developing autoimmune hypothyroidism [98]. It is hypothesized that SARS-CoV-2 might directly influence thyroid morphology and function, leading to an aggravation of a pre-existing autoimmune thyroid disease [98]. Additionally, COVID-19 may worsen autoimmune thyroid disease due to its repercussions on the immune system, which may lead to the development of the cytokine storm [98]. Thyroid autoimmunity, evaluated through the presence of anti-TPO antibodies, was common in COVID-19 patients as compared with pre-pandemic controls [99].

Recent data demonstrate that thyroid hormones have an important role in protecting the lungs from damage, including those related to SARS-CoV-2 infection [98]. The lung is one of many organs that responds to the thyroid hormone, and the T3 receptor is present in alveolar type II cells [98]. T3 increases cell size and number, stimulates surfactant release, and elevates the sodium- and potassium-ATPase pump activity, increasing the cell capacity to translocate fluid and therefore absorb alveolar oedema fluid [98].

Our knowledge of the thyroid patterns of COVID-19 is still incomplete, as is the etiologic view of COVID-19 and thyroid insults [76,100]. To find direct evidence concerning the nature and cause of thyroid SARS-CoV-2 injury, and the full immune response in those patients with thyroid dysfunction, we need a histologic and cytological examination of the thyroid gland in a wide number of patients [74,76]. Poma et al. [101] detected SARS-CoV-2 in a small number of thyroid specimens (9/25, 36%). Currently, there is no clear statement on the importance of SARS-CoV-2-induced apoptosis in the thyroid dysfunction [102], but in the SARS-CoV-2 outbreak, it was shown that apoptosis plays an important role in thyroid injury [102]. Summing up, thyroid dysfunction secondary to SARS-CoV-2 infection is probably a combination of various mechanisms [74], and its role in post-COVID-19 condition is not yet established. Tutal et al. [103], who performed a systematic review of COVID-19 and autoimmune thyroiditis, considered it reasonable to routinely assess thyroid function, both in the acute phase of the infection and during the convalescence, through serum TSH, T4, and T3 evaluation. Contrarily, based on the assumption that thyroid function usually normalizes on follow-up, Pat et al. [104] did not recommend a widespread thyroid function screening.

6. Post-COVID-19 Condition Health Burden and Patient Management

Post-COVID-19 condition (or long COVID) first gained extensive credit among social support groups, and then in scientific and medical communities [3,5,105,106]. It is probably the first illness to be cooperatively identified by patients discovering one another using Twitter and other social media [105]. The term "post-COVID condition" comprises a wide range of organ impairment, and at the moment we do not have enough information to perform a clear diagnosis, to elect a specific treatment, or to indicate a probable prognosis [107]. Some patients may never recover from the illness [52,56], and all age groups are vulnerable [52]. Patients with post-COVID-19 condition are a heterogeneous group, which makes it difficult to advise treatment [108,109]. It is crucial for each patient to find the correct equilibrium between mild activity to avoid deconditioning and not triggering post-exercise malaise [108]. Strategies tackling our levels of stress and/or the stress response, comprising psychosocial intervention, physical exercise, or possibly dietary interventions could be a good approach to counteract some of the negative effects of chronic inflammation [29]. Rebello et al. [110] advanced that physical exercise may counter the neuropsychiatric and endocrine sequelae of post-COVID-19 condition, through the release of circulating factors that mediate the anti-inflammatory response, support brain homeostasis, and increase insulin sensitivity .

Management of post-acute COVID-19 syndrome requires a comprehensive team, including physicians of various specialties (primary care, pulmonology, cardiology, and infectious disease), physiatrists, behavioural health experts, physical and occupational therapists, and social workers, which will address the clinical and psychological aspects of the disease [111].

Although still speculative at the present time, there is a considerable body of literature supporting the anti-stress and anti-inflammatory role of certain seated meditations, yoga asanas, and pranayama practices [112]. The possible benefits of these practices encompass wider neuroimmune systems, which is an advantage since we are facing a systemically dysregulating disease in COVID-19 [112].

Lastly, we may refer to the role of COVID-19 vaccines in post-COVID-19 condition. Although vaccines prevent death and severe illness, it is not yet clear if they may also prevent post-COVID-19 condition [5]. Small studies have shown that AstraZeneca and Pfizer-BioNTech vaccines were associated with overall improvements in post-COVID-19 condition symptoms [113]. Recently, Antonelli et al. [114] found that the odds of having symptoms for 28 days or more after post-vaccination infection were approximately halved by having two doses of the vaccine.

7. Conclusions

It is urgent to better understand this emerging, complex, and puzzling medical condition [16,115]. Post-COVID-19 condition can become a crisis for health systems, which are already facing the challenge of the pandemic [116]. It is essential to be able to better deal with the symptoms of this condition in terms of clinical care, public health, and health resource planning [116]. At the population level, it is necessary to evaluate the burden of post-COVID-19 condition in order to evaluate its impact on the healthcare system and distribute resources in an adequate way [18,48,107,117]. The primary care services, which represent the first approach for patient diagnosis, still have little information or resources to deal with these patients [118]. Patients with post-COVID-19 condition may have a variety of positive and negative healthcare experiences, which can be useful for the creation or adaptation of the healthcare services [119].

The existence of post-COVID symptoms is leading to the recognition of COVID-19 as a cause of post-viral fatigue syndrome, even when the disease acute phase was mild [20,35]. This can help clinicians to organize patient care, namely follow-up visits, rehabilitation, cognitive behavioral therapy, and even simple actions like inclusion of counseling sessions at discharge to diminish patient anxiety about prolonged symptoms [35]. The patients need to be monitored with a systematic protocol, including symptoms of mental and physical

health, and specific healthcare programs to support a healthier lifestyle after SARS-CoV-2 infection need to be implemented [120].

There is an urgent need to identify affected individuals early so the most appropriate and efficient treatments may be provided [111,115], helping them to thrive through the uncertainty of their condition [15,121].

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III. Significance of Furin Expression in Thyroid Neoplastic Transformation

The complex relationship between viral infections and tumourigenesis has been an area of growing interest, especially due to the global health crisis caused by SARS-CoV-2 pandemic. Considering the thyroid's susceptibility to viral infection and following the previous work rationale, this study aimed to unravel the expression patterns of ACE2, TMPRSS2, and Furin in a pre-covid series of thyroid benign and malignant lesions. Recognizing the significance of these molecules in the viral entry and host cell infection, our investigation focused on understanding potential associations between their expression profiles and thyroid tumourigenesis.

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Significance of Furin Expression in Thyroid Neoplastic Transformation

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Simple Summary: Angiotensin-Converting Enzyme 2 (ACE2), Transmembrane Serine Protease 2 (TMPRSS2), and Furin are highly expressed in the normal thyroid gland. The putative role played by these molecules in thyroid tumours has not been thoroughly explored to date. Our study shows that the downregulation of *ACE2* mRNA and overexpression of *Furin* mRNA may play a role in thyroid neoplastic transformation. *ACE2* mRNA expression was lower in thyroid neoplasms, as for Furin an increased expression in neoplastic lesions was observed. Furin revealed a high discriminative power between normal adjacent and neoplastic thyroid tissue. Its high expression was significantly correlated with poor prognostic features for thyroid neoplasia.

Abstract: *Angiotensin-Converting Enzyme 2* (ACE2), *Transmembrane Serine Protease 2* (TMPRSS2), and Furin were known to be key players in the SARS-CoV-2 infection, and the thyroid gland was revealed to be one of the relevant targets of the virus. Regardless of the viral infection, the expression of these molecules in the thyroid gland and their putative role in the neoplastic transformation of the thyrocytes has not been thoroughly explored. In this work, we aimed to characterize the mRNA and protein expression pattern of ACE2, TMPRSS2, and Furin in a series of patients with thyroid lesions. Our main results revealed a significantly decreased expression of *ACE2* mRNA in the thyroid neoplasms in comparison to normal adjacent tissue. *Furin* mRNA was significantly increased in thyroid neoplasms when compared to normal adjacent tissue. In addition, a higher *Furin* mRNA level in thyroid carcinomas was associated with the presence of lymph node metastasis. *Furin* mRNA expression revealed a high discriminatory power between adjacent tissue and neoplasms. Protein expression of these molecules did not correlate with mRNA expression. Our study shows the mRNA downregulation of *ACE2* and overexpression of *Furin* in thyroid neoplasms. Further studies are required to clarify if *Furin* expression can be a potential diagnostic indicator in thyroid neoplasia.

Keywords: ACE2; TMPRSS2; Furin; thyroid; thyroid neoplasms

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1. Introduction

Angiotensin-Converting Enzyme 2 (ACE2), Transmembrane Serine Protease 2 (TMPRSS2), and Furin recently gained relevance for being key players in the SARS-CoV-2 infection [1]; the thyroid gland was shown to be one of the targets of the virus [2], and it was advanced that this tropism is related to the mRNA expression levels of these molecules in the gland.

The ACE2 molecule is found at the surface of cells in many different organs, including the lungs, heart, kidneys, and intestines. It is essential in the regulation of the renin–angiotensin–aldosterone system (RAAS) [3–5]. ACE2 converts angiotensin II (Ang II) into angiotensin 1–7, resulting in blood vessel dilation and the lowering of blood pressure, acting as a counterbalance to another enzyme, ACE (Angiotensin-Converting Enzyme). ACE converts angiotensin I (Ang I) into Ang II, whose effect is to constrict blood vessels and raise blood pressure [5,6]. *ACE2* mRNA expression was described to be increased in renal papillary cell carcinoma and colon and pancreatic adenocarcinomas, and decreased in kidney chromophobe, testicular germ cell tumours, and thyroid cancer [7–9]. Regarding ACE2 protein expression in cancer, the information is scarce. It is mostly described as expressed in renal and colorectal cancers [8–11].

The TMPRSS2 protein belongs to the type II transmembrane serine proteases (TTPs) family, which comprise nearly one-third of all proteases [12]. Deregulation of TTP activity is a common characteristic in cancers leading to tumour cell proliferation, invasion, and metastasis [13–16]. TMPRSS2 plays an important role in the activation of proteins that are involved in blood clotting, and it is implicated in the normal development of the prostate gland [17]. *TMPRSS2* expression was described to be higher in neoplastic and hyperplastic prostate tissue when compared to normal epithelium [18,19]. TMPRSS2 mRNA and protein expression have been described as highly expressed in gastrointestinal tissues (small intestine, stomach, and colon), lung, salivary gland, oesophagus, prostate, liver, and thyroid [10,11,20–23].

Furin is an enzyme that plays an important role in processing several proteins, cleaving proteins at specific sites known as Furin cleavage sites [24–26]. These sites are characterized by a specific amino acid sequence, which includes the amino acid sequence Arg-X-(Arg/Lys)-Arg' [25,27,28]. Furin cleavage is important for the activation of many proteins, including growth factors, receptors, and enzymes [26]. Its overexpression, at the mRNA level, has been associated with several human tumours such as breast [29], head, and neck tumours [30], non-small cell lung carcinoma [31], and ovarian cancer [32], whereas in clear cell renal cell carcinoma and renal papillary carcinoma, the *Furin* mRNA expression was described as relatively low [24].

At the protein level, thyroid cancer has been described as one of the tumours with the highest expression of Furin. Moderate expression was present in lung, liver, prostate, and urothelial cancers [10,11,33]. Some functional studies modulating Furin activity showed the relevance of the protein in the processing of many cancer-related substrates and indicated that high Furin activity promotes the malignant phenotype of cancer cells, namely thyroid cancer cells [34,35].

The expression of these three molecules in abnormal thyroid tissue is not clearly established. Furthermore, the genetic expression of ACE2, TMPRSS2, and Furin has been shown to be altered and implicated in the pathogenesis of several tumours, as previously shown. However, despite a great focus on these molecules in recent years, the data about the roles of ACE2, TMPRSS2, and Furin on the neoplastic transformation of the thyrocytes are still scarce. Therefore, we aim to study the expression of ACE2, TMPRSS2, and Furin in a series of patients with benign and malignant thyroid neoplasms and correlate their expression with clinicopathological features.

2. Materials and Methods

2.1. Biological Samples

From the Institute of Pathology and Molecular Immunology of the University of Porto (IPATIMUP) medical files, 190 samples of frozen thyroid tissue, corresponding to 165 patients, were collected. The expression patterns of ACE2, TMPRSS2, and Furin in the series of thyroid lesions were characterized by the quantification of the mRNA expression by real-time quantitative PCR (qPCR) that included samples of adjacent thyroid tissue (n = 36), goitres (n= 2), benign neoplasms, specifically Follicular Thyroid Adenomas (FTAs) (n = 76), and malignant neoplasms (n = 76) that comprised classical Papillary Thyroid Carcinomas (cPTCs) (n = 35), Follicular Variant of Papillary Thyroid Carcinomas (FVPTCs) (n = 15), Diffuse Sclerosing Variant of Papillary Thyroid Carcinomas (DSVPTCs) (n = 5), Solid Variant of Papillary Thyroid Carcinomas (SVPTCs) (n = 1), Oncocytic Variant of Papillary Thyroid Carcinomas (OVPTCs) (n = 2), Oncocytic Variant of Papillary Thyroid Carcinomas with other components (n = 1), Follicular Thyroid Carcinomas (FTCs) (n = 10), Oncocytic Thyroid Carcinomas (OCAs) (n = 2), Poorly Differentiated Thyroid Carcinomas (PDTCs) (n = 4), and Medullary Thyroid Carcinomas (MTCs) (n = 1).

In addition, the protein expression pattern of these molecules was evaluated in situ by immunohistochemistry (IHC) on a smaller series of 75 formalin-fixed paraffin-embedded (FFPE) thyroid tissues, correspondent to 75 patients. The protein expression was evaluated in the adjacent thyroid tissue, when present (n = 60), in goitres (n = 5), Grave's disease (n = 1), FTAs (n = 15), cPTCs (n = 13), FVPTCs (n = 19), OVPTCs (n = 6), DSVPTCs (n = 2), Tall Cell Variant of Papillary Thyroid Carcinomas (TCVPTCs) (n = 2), SVPTCs (n = 1), Hobnail Variant of Papillary Thyroid Carcinoma (HVPTC) (n = 1), FTCs (n = 2), OCAs (n = 3), and MTCs (n = 1). In 4 of the FFPE tissues, only normal adjacent thyroid tissue was available.

2.2. Patients' Characteristics and Clinicopathological Data

The series of patients reported on in this work was partially characterized by Pestana et al. [36]. The clinical and pathological information available for the patients included: age, sex, number of lesions per patient, tumour size, the presence of tumour capsule, capsule invasion/infiltration, lymphovascular invasion, lymph node metastasis, microscopical extrathyroidal extension, lymphocytic infiltrate, and chronic lymphocytic thyroiditis (CLT).

Some of the cases were also previously characterized for TERT mRNA expression and hotspot mutations in BRAF (exon 15), NRAS (exon 2), and TERT (promoter region) [36].

2.3. Reverse Transcription of the RNA from Frozen Tissues

For cDNA conversion, 1 µg of total RNA from the thyroid tissue frozen samples was treated with DNase and reverse transcribed using the RevertAid RT Reverse Transcription Kit (REF: K1691, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

2.4. Real-Time Quantitative Polymerase Chain Reaction (qPCR)

The mRNA expression of ACE2, TMPRSS2, and Furin was evaluated by qPCR. The PCR reaction was performed with Taqman® Universal PCR Master Mix (REF: 4324018, Applied Biosystems, Foster City, CA, USA). The probes used for the genes of interest were ACE2 qPCR assay (REF: 228806911, IDT, Coralville, IA, USA), TMPRSS2 (REF: 28806907, IDT, Coralville, IA, USA), and Furin (REF: 228806915, IDT, Coralville, IA, USA). The endogenous control Human Tata Box Binding protein (TBP) (REF: 222242490, IDT, Coralville, IA, USA) was used for the normalization of the mRNA expression.

The qPCR method was as follows: an initial step at 95 °C for 10 min, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Triplicates and non-template controls (NTC) were performed for all the samples. Samples were considered positive when genes were amplified at maximum at cycle threshold (Ct) 37.

2.5. Immunohistochemistry

To evaluate the protein expression of ACE2, TMPRSS2, and Furin at the subcellular localization, immunohistochemistry (IHC) was performed in the FFPE samples. First, dewaxing and rehydration of the slides was performed, followed by heat-induced antigen retrieval with EDTA buffer (pH 9.0) using a steamer. The protocol included peroxidase and protein block, antibody amplifier, and polymer incubation with the Ultravision Quanto Detection System HRP (Epredia®, Kalamazoo, MI, USA, TL-125-QHL) following the manufacturer's instructions.

Mouse monoclonal anti-ACE2 (Invitrogen, Waltham, MA, USA, MA5-31395, 1:2500); rabbit monoclonal anti-TMPRSS2 antibody (Abcam, Boston, MA, USA, ab109131, clone EPR3862, 1:1000); and rabbit polyclonal anti-Furin (Invitrogen, PA5-96680; 1:250) were used. The detection was performed with DAB chromogen for all antibodies (Epedria®, TA-125-QHDX, Kalamazoo, MI, USA) and slides were counterstained with Gill's hematoxylin.

2.6. Immune Reactive Score (IRS)

Immunohistochemistry evaluation was performed blindly by an endocrine pathologist (S.C.) who evaluated the slides semi-quantitatively using the immunoreactive score (IRS) method [37]. This method consists in the multiplication of the staining intensity score (absent (0), weak (1), moderate (2), and strong (3)) by the percentage of positively stained cells (<10% (0), 10–25% (1), 26–50% (2), 51–75% (3) and>75% (4)). The results obtained ranged from 0 to 12.

Each tissue was analysed entirely. Most of the slides evaluated contained both tumour and adjacent thyroid tissue, and scores were attributed separately.

2.7. Statistical Analysis

Statistical analysis and graphical construction were performed using IBM SPSS Statistics 29.0 (IBM, Armonk, NY, USA), GraphPad Prism 8 software version 8.0.2 (GraphPad, San Diego, CA, USA), and Seaborn data visualization library version 0.12.0 [38]. Outlier analysis was performed in both series and only extreme outliers, i.e., values located 3 times the interquartile range value (above the third quartile or below the first quartile) were excluded. The distribution of the data was accessed by Shapiro–Wilk tests. For multiple comparisons, Kruskal–Wallis tests with Bonferroni correction were used, whereas for dichotomic variables, the Mann–Whitney test was applied. Variables such as age and tumour size were categorized according to the cut-off established by the 8th edition of the American Joint Committee on Cancer (AJCC) [39] and median size, respectively (Table 1).

Table 1. Patients' characteristics and clinicopathological data for frozen and FFPE tissue series.

Patient Characteristics	Frozen Tissue	FFPE Tissue
	***n* = 165**	***n* =75**
Age	***n*= 155**	***n*= 71**
Mean ± SD	43.4 ± 15.4	42.2 ± 15.4
Median ± IQR	43.0 ± 21.0	44.0 ± 22.0
Min–Max	11–82	11–76
Age	***n*= 155**	***n*= 71**
<55 years	121 (78.1%)	58 (81.7%)
≥55 years	34 (21.9%)	13 (18.3%)
Sex	***n*= 158**	***n*= 71**
Female	130 (82.3%)	59 (83.1%)
Male	28 (17.7%)	12 (16.9%)

Table 1. *Cont.*

Clinicopathological Features	Frozen Tissue	FFPE Tissue
	***n*= 168**	***n*= 75**
Number oflesions	***n*= 137**	***n*= 35**
1	82 (59.9%)	24 (68.6%)
>1	55 (40.1%)	11 (31.4%)
Tumour size (cm)	***n*= 130**	***n*= 35**
Mean ± SD	3.3 ± 1.8	3.6 ± 1.7
Median ± IQR	3.0 ± 2.5	3.5 ± 2.8
Min–Max (cm)	0.5–10.0	0.5–7.0
Tumour size	***n*= 130**	***n*= 35**
≤3 cm	77 (59.2%)	16 (45.7%)
>3 cm	53 (40.8%)	19 (54.3%)
Tumour capsule	***n*= 142**	***n*= 33**
Presence	95 (66.9%)	23 (69.7%)
Absence	47 (33.1%)	10 (30.3%)
Capsule invasion/infiltration	***n*= 94**	***n*= 22**
Presence	13 (13.8%)	3 (13.6%)
Absence	81 (86.2%)	19 (86.4%)
Lymphovascular invasion	***n*= 139**	***n*= 34**
Presence	28 (20.1%)	9 (26.5%)
Absence	111 (79.9%)	25 (73.5%)
Lymph node metastases	***n*= 35**	***n*= 8**
Presence	17 (48.6%)	4 (50.0%)
Absence	18 (51.4%)	4 (50.0%)
Microscopical extrathyroidal extension	***n*= 102**	***n*= 22**
Presence	23 (22.5%)	4 (18.2%)
Absence	79 (77.5%)	18 (81.8%)
Lymphocytic infiltration	***n*= 140**	***n*= 36**
Presence	50 (35.7%)	16 (44.4%)
Absence	90 (64.3%)	20 (55.6%)
Chronic lymphocytic thyroiditis	***n*= 132**	***n*= 72**
Presence	43 (32.6%)	13 (18.1%)
Absence	88 (66.7%)	59 (81.9%)
***TERT*p**	***n*= 151**	***n*= 38**
Wild-Type	147 (97.4%)	37 (97.4%)
Mutated	4 (2.6%)	1 (2.6%)
***TERT* expression**	***n*= 159**	***n*= 38**
Positive	107(67.3%)	25 (65.8%)
Negative	52 (32.7%)	13 (34.2%)
***NRAS* mutation**	***n*= 152**	***n*= 38**
Wild-Type	136(89.5%)	33 (86.8%)
Mutated	16 (10.5%)	5 (13.2%)
***BRAF* mutation**	***n*= 89**	***n*= 27**
Wild-Type	69 (77.5%)	23 (85.2%)
Mutated	20 (22.5%)	4 (14.8%)

Note: For some of the characteristics the information was not available in all the cases.

3. Results

3.1. Clinical Series Characterization

The population of this study was composed of two series; a series of 165 patients bearing thyroid lesions that had RNA extracted from frozen thyroid tissues and a series of 75 patients with FFPE thyroid tissues. The clinicopathological data of the patients is summarized in Table 1.

From the frozen thyroid tissue series, 82.3% of the patients were females (n = 130/158), and 17.7% were males (n = 28/158). The patients' median age was 43.0 ± 21 years (median ± IQR), ranging from 11 to 82 years (n = 155). Most patients (59.9%) presented a single lesion (n = 82/137), and the median tumour size was 3.00 ± 2.50 cm (median ± IQR) (n = 130). The majority of the tumours, 66.9% (n = 95/142), were encapsulated; 13.8% (n = 13/94) had capsule invasion/infiltration, and lymphovascular invasion was present in 20.1% of the cases (n = 28/139). Microscopical extra-thyroidal extension (ETE) was observed in 22.5% of the cases (n = 23/102), and lymphocytic infiltrate was present in 35.7% of the cases (n = 50/140). Information about lymph node metastasis (LNM) was only available for 35 cases, and it was present in almost half of the cases, 48.6% (n = 17/35). The mutational status of these tumours was also accessible for *TERTp*, *NRAS*, and *BRAF* genes. Most of the patients were wild-type (WT) for all genes, only 2.6% (n = 4/151) presented *TERTp* mutation, 10.5% (n = 16/152) presented *NRAS* mutation, and 22.5% (n = 20/89) were *BRAF*-mutated; 67.3% (n = 107/159) of the cases were positive for *TERT* mRNA expression.

From the FFPE tissue series, 83.1% of the patients were females (n = 59/71) and 16.9% males (n = 12/71). The patient's median age was 44 ± 22 years (median ± IQR), ranging from 11 to 76 years (n = 71). Most patients presented a single lesion, 68.6% (n = 24/35), and the median tumour size was 3.5 ± 2.8 cm (median ± IQR) (n = 35). The majority of tumours were encapsulated, 69.7% (n = 23/33); capsule invasion occurred in 13.6% (n = 3/22), and lymphovascular invasion was present in 26.5% of the cases (n= 9/34). Microscopical ETE was observed in 18.2% of the cases (n = 4/22), and lymphocytic infiltrate was present in 44.4% of the cases (n = 16/36). Half of the cases (n = 4/8) had lymph node metastasis. For the hotspot mutations, 2.6% (n = 1/38) were mutated for TERTp, 13.5% (n = 5/38) were mutated for *NRAS*, and 14.8% (n = 4/27) were mutated for *BRAF*. *TERT* mRNA expression was positive in 34.2% of the cases (n = 13/38).

3.2. mRNA and Protein Analysis in the Thyroid Series

The results obtained from both mRNA and protein expression were stratified and analysed.

1. First, data were analysed by comparing ATs with benign lesions, which included goitres and FTAs, and with carcinomas, which included all malignant thyroid neoplasms;
2. Data were then stratified and separated according to the main histological groups: goitres, FTAs, PTCs, FTCs, OCAs, PDTCs, and MTCs;
3. Finally, all the sub-histological groups were compared: goitres, FTAs, cPTCs, FVPTCs, DSVPTCs, OVPTCs, FTCs, OCAs, and PDTCs.

3.2.1. mRNA Expression in Thyroid Tissues

The results concerning *ACE2* mRNA expression revealed a statistically significant decrease ($p < 0.0001$) in benign and malignant thyroid lesions when compared with AT (Figure 1a). When divided according to the histological groups, the decreased expression was still evident, but only significant when comparing ATs to FTAs ($p = 0.001$) and PTCs ($p < 0.0001$) (Figure 1b). The statistical significance of the *ACE2* decrease prevailed for the FTAs ($p = 0.001$) and classical PTCs ($p = 0.001$) when data were divided according to neoplasm subtype (Figure 1c). The clinicopathological data of the series was then compared according to *ACE2* mRNA expression. We observed a significant decrease of *ACE2* in the benign neoplasms that were bigger than 3 cm ($p = 0.017$), with absent lymphocytic infiltration ($p = 0.005$), which corresponded to the absence of CLT ($p = 0.007$) (Figure 2a, 2b and 2c, respectively). *ACE2* was also decreased in follicular adenomas that were negative for *TERT* mRNA expression ($p = 0.017$) (Figure 2d). *ACE2* expression did not reveal any statistically significant differences in the clinicopathological data of the patients with malignant neoplasms.

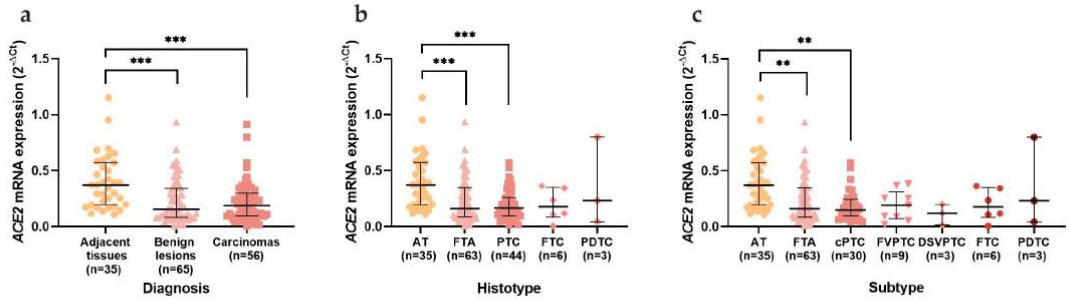


Figure 1. *ACE2* mRNA expression in the thyroid series. Analysis of *ACE2* mRNA expression according to the (**a**) diagnosis; (**b**) histotype; (**c**) subtype. Results are shown as median ± IQR. ** *p*-value ≤ 0.01 and *** *p*-value ≤ 0.001. Abbreviations: AT (Adjacent Thyroid Tissues), FTA (Follicular Thyroid Adenomas), PTC (Papillary Thyroid Carcinomas), FTC (Follicular Thyroid Carcinomas), PDTC (Poorly Differentiated Thyroid Carcinomas), cPTC (classical Papillary Thyroid Carcinomas), FVPTC (Follicular Variant of Papillary Thyroid Carcinomas) and DSVPTC (Diffuse Sclerosing Papillary Thyroid Carcinomas).

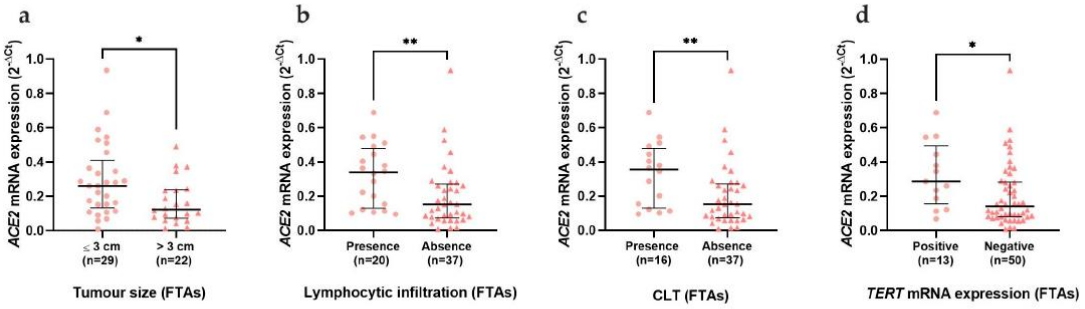


Figure 2. *ACE2* mRNA expression correlation with clinicopathological data. Analysis of *ACE2* mRNA expression in FTAs comparing (**a**) tumour size; (**b**) presence of lymphocytic infiltration; (**c**) presence of CLT and (**d**) *TERT* expression. Results are shown as median ± IQR. * *p*-value ≤ 0.05 and ** *p*-value ≤ 0.01. Abbreviations: CLT (Chronic lymphocytic thyroiditis).

The mRNA expression of *TMPRSS2*, among the different histotypes and subtypes of thyroid neoplasms, did not show any significant differences (Figure 3). When comparing the patients' clinicopathological data, *TMPRSS2* was significantly increased ($p = 0.042$) in PTCs that were larger than 3 cm (Figure 4a), a difference that was maintained when only the cPTC ($p = 0.039$) was considered (Figure 4b), despite the low number of cases available.

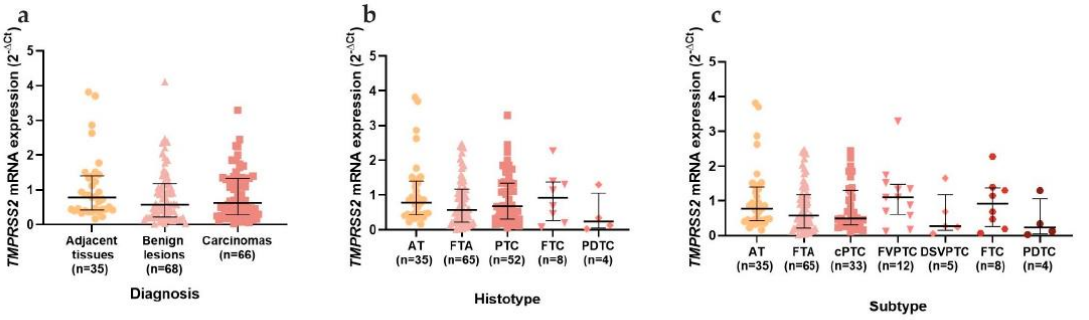


Figure 3. *TMPRSS2* mRNA expression in the thyroid series. Analysis of *TMPRSS2* mRNA expression according to (**a**) diagnosis; (**b**) histotype; (**c**) subtype. Results are shown as median ± IQR. Abbreviations: AT (Adjacent Thyroid Tissues), FTA (Follicular Thyroid Adenomas), PTC (Papillary Thyroid Carcinomas), FTC (Follicular Thyroid Carcinomas), PDTC (Poorly Differentiated Thyroid Carcinomas), cPTC (classical Papillary Thyroid Carcinomas), FVPTC (Follicular Variant of Papillary Thyroid Carcinomas) and DSVPTC (Diffuse Sclerosing Papillary Thyroid Carcinomas).

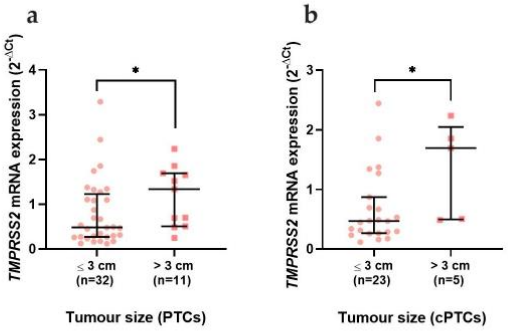


Figure 4. *TMPRSS2* mRNA expression correlation with clinicopathological data. Analysis of *TMPRSS2* mRNA expression in PTCs comparing the: (**a**) tumour size in PTCs and (**b**) tumour size in classical PTCs. Results are shown as median ± IQR. * *p*-value ≤ 0.05. Abbreviations: PTC (Papillary Thyroid Carcinomas) and cPTC (classical Papillary Thyroid Carcinomas).

Furin mRNA expression revealed an opposite expression pattern from *ACE2*, since it was significantly increased in benign ($p < 0.0001$) and malignant ($p = 0.002$) thyroid lesions when compared with the adjacent tissue (Figure 5a). In the histotypes category, this increase was significant in adenomas ($p < 0.0001$) and in PTCs ($p = 0.001$) (Figure 5b). The increase in PTCs was mainly due to the classical subtype, which maintained the statistical significance ($p = 0.002$) (Figure 5c). *Furin* revealed statistical differences in the malignant neoplasms of the thyroid. *Furin* was significantly increased in carcinomas with lymph node metastasis ($p = 0.037$) and with wild-type *NRAS* status ($p = 0.028$) (Figure 6a and 6b, respectively). In the PTCs histotype, *Furin* expression was higher in male patients ($p = 0.002$), which was mainly due to the classical PTC subtype ($p = 0.046$) (Figure 6c and 6d, respectively).

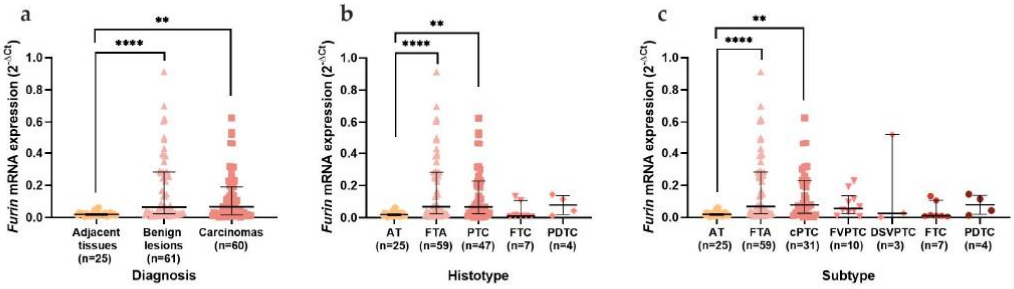


Figure 5. *Furin* mRNA expression in the thyroid series. Analysis of *Furin* mRNA expression according to (**a**) diagnosis; (**b**) histotype; (**c**) subtype. Results are shown as median ± IQR. ** p-value ≤ 0.01 and **** p-value ≤ 0.0001. Abbreviations: AT (Adjacent Thyroid Tissues), FTA (Follicular Thyroid Adenomas), PTC (Papillary Thyroid Carcinomas), FTC (Follicular Thyroid Carcinomas), PDTC (Poorly Differentiated Thyroid Carcinomas), cPTC (classical Papillary Thyroid Carcinomas), FVPTC (Follicular Variant of Papillary Thyroid Carcinomas) and DSVPTC (Diffuse Sclerosing Papillary Thyroid Carcinomas).

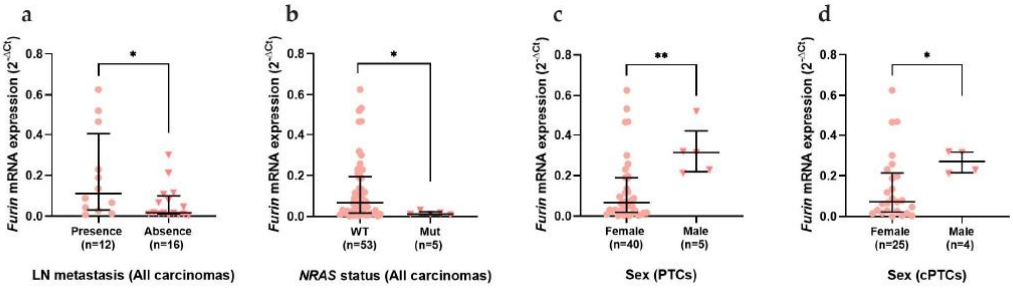


Figure 6. *Furin* mRNA expression and correlation with clinicopathological data. Analysis of *Furin* mRNA expression comparing the (**a**) presence of LNM; (**b**) *NRAS* mutational status in thyroid carcinomas; (**c**) patient sex in PTCs and (**d**) in classical PTCs. Results are shown as median ± IQR. * p-value ≤ 0.05 and ** p-value ≤ 0.01. Abbreviations: PTCs (Papillary Thyroid Carcinomas) and cPTC (classical Papillary Thyroid Carcinomas).

3.2.2. ACE2, TMPRSS2, and Furin mRNA Expression Distribution in Adjacent Thyroid Tissue and Thyroid Neoplasms and Furin Discriminative Power

To access the distribution of mRNA expression levels of each transcript, the series was divided into two major groups: adjacent thyroid tissues and thyroid neoplasms. The latter includes all lesions present in the series, with the exception of goitres. *ACE2* and *Furin* transcripts showed an unequal distribution of mRNA expression levels. Most thyroid neoplasms had lower *ACE2* expression than adjacent tissue, and the expression levels seemed to be more accumulated in the lower tercile, i.e., the second tercile (2T) of thyroid neoplasms was 0.257 when compared to the 1T of adjacent tissue of 0.253 (Figure 7 Top Panel), whereas *Furin* mRNA expression levels in thyroid lesions seemed to be more accumulated in the third tercile (Figure 7 Lower Panel) compared to the distribution observed in adjacent tissue, i.e., 1T was 0.029 and 2T was 0.023, respectively. Regarding *TMPRSS2* mRNA expression, the distribution was similar between thyroid neoplasms and adjacent normal tissues (Figure 7 Middle Panel).

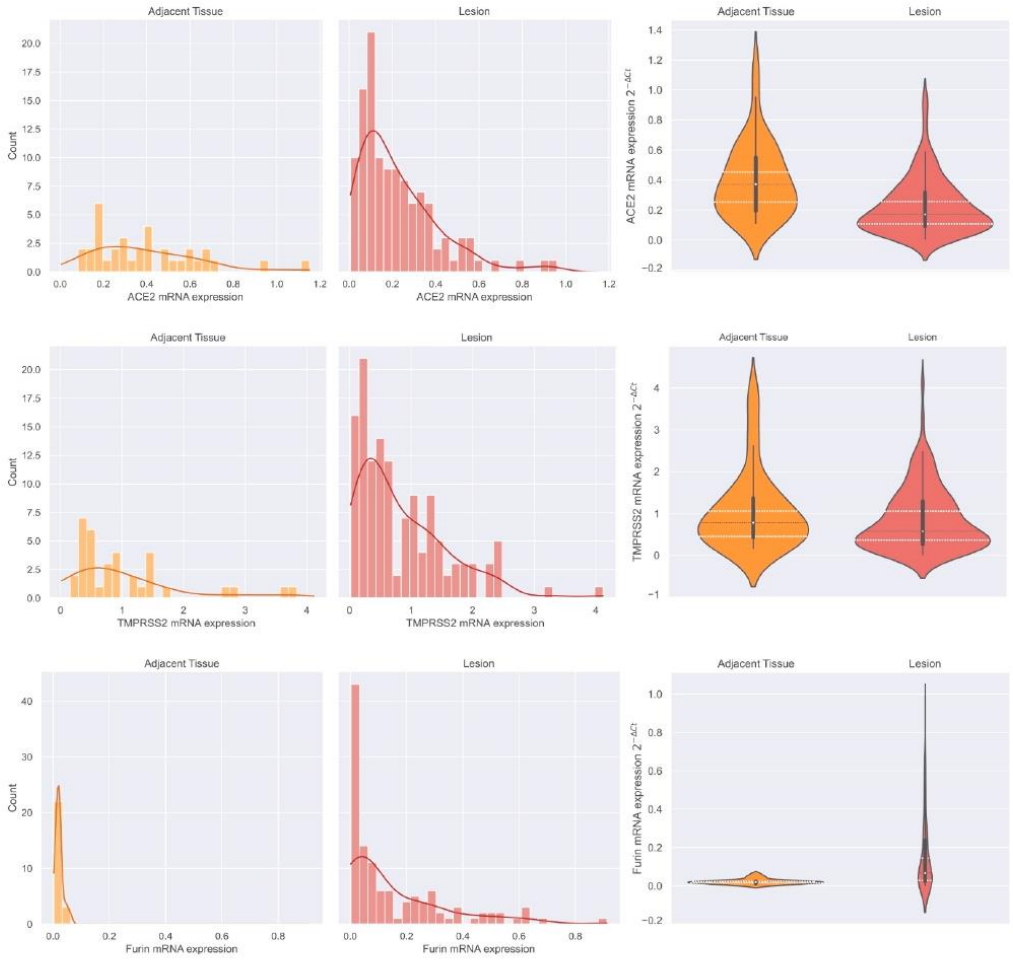
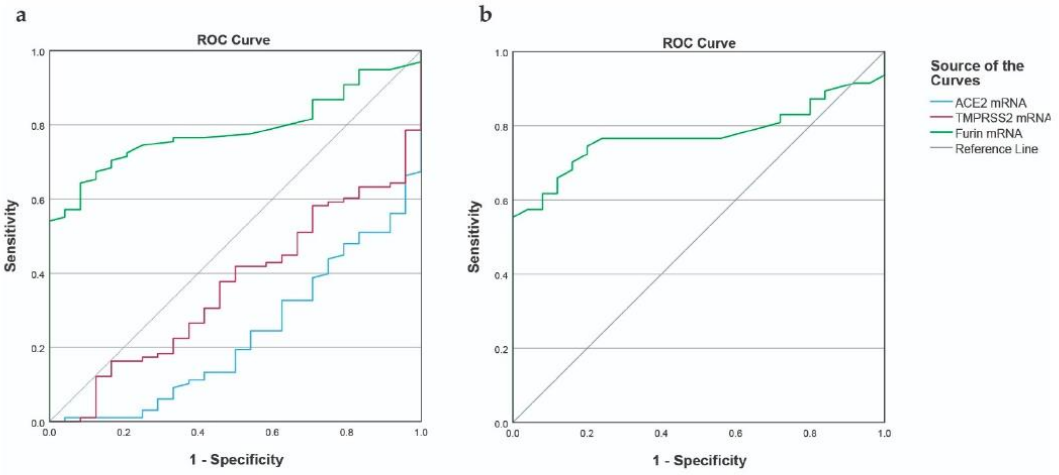


Figure 7. mRNA expression distribution in adjacent thyroid and thyroid neoplasms. Analysis of mRNA expression distribution of *ACE2* (**Top** Panel), *TMPRSS2* (**Middle** Panel), and *Furin* (**Lower** Panel).

The ROC curve analysis was performed in order to investigate if any of the transcripts would exhibit any discriminative power in the identification of thyroid neoplasms. In our series, *Furin* stood out as the transcript that presented the highest discriminatory power, displaying an area under the curve (AUC) of 0.786 (Figure 8a,c), thus allowing the identification of thyroid neoplasms with a sensitivity of 70% and a specificity of about 80%. An mRNA expression of *Furin* equal to or greater than 0.025 proved to be the most precise cut-off value for the identification of thyroid neoplasms (Table S1).

A similar relationship was observed when the data were restricted to PTCs, exhibiting an AUC of 0.774 (Figure 8b,c) allowing the identification of this histotype with a sensitivity and a specificity of nearly 80% when the considered cut-off value for *Furin* is equal or greater than 0.0250 (Table S2).



c

	Test result variables	AUC	Standard Error	*p*-value
Thyroid neoplasms vs AT	*ACE2*	0.230	0.049	<0.0001
	TMPRSS2	0.362	0.057	0.0360
	Furin	0.786	0.040	<0.0001
PTCs vs AT	*Furin*	0.774	0.055	<0.0001

Figure 8. ROC curve analysis. (**a**) AUC representation for *ACE2*, *TMPRSS2*, and *Furin* mRNA expression in adjacent thyroid tissues and thyroid neoplasms; (**b**) *Furin* AUC when data were restricted to PTCs; (**c**) AUC values for *ACE2*, *TMPRSS2*, and *Furin* mRNA expression in both analyses. Abbreviations: AUC (area under the curve).

3.2.3. Protein Expression in Thyroid Tissues

The staining pattern observed for ACE2 in thyroid lesions was predominantly membranous and mainly positive in vascular cells surrounding the thyroid follicular cells (Figure 9a,b). No statistically significant differences in protein expression were found among the different groups and categories of the thyroid lesions ($p > 0.05$) (Figure S1). The clinicopathological data did not reveal any statistically significant differences.

In a small number of cases, we were able to detect the ACE2 immunostaining in follicular cells in an apical position, in 12 thyroid neoplasms (2 FTAs and 10 PTCs) and 9 thyroid adjacent tissues. ACE2 apical expression was detected specifically in the papillae of thyroid carcinomas (Figure S2) and was also observed in some tumours with microfollicular histologic patterns.

The staining pattern observed for TMPRSS2 was observed in follicular cells, with a predominantly cytoplasmic and, occasionally, nuclear localization (Figure 9c,d). Cytoplasmic and nuclear staining were analysed separately. No statistical differences were found in TMPRSS2 protein expression among the different histotypes and subtypes of the thyroid lesions, for both cytoplasmic and nuclear expression (Figures S3 and S4). Concerning the clinicopathological data, TMPRSS2 protein expression was higher in males, but only in the adjacent tissue (Figure S5a). In thyroid carcinomas, TMPRSS2 cytoplasmic expression

was decreased in the absence of CLT ($p = 0.031$) (Figure S5b), which was also observed, but restricted to the PTC histotype (Figure S5c).

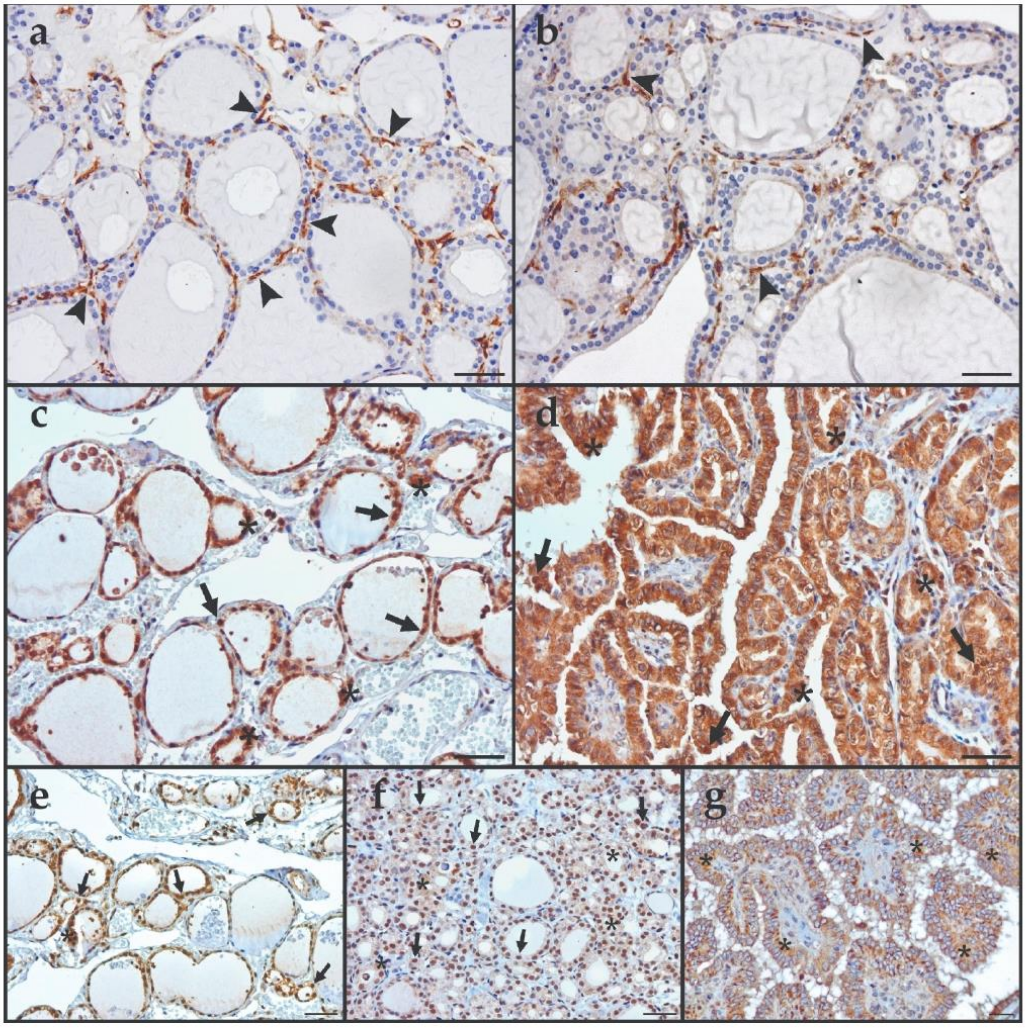


Figure 9. The immunohistochemical staining pattern of ACE2, TMPRSS2, and Furin. Representative images of ACE2 staining in (**a**) adjacent thyroid tissue and (**b**) follicular variant of PTC. The staining is mainly membranous, located in endothelial cells (arrowheads). Representative images of TMPRSS2 staining in (**c**) AT and (**d**) cPTC. The staining is mainly cytoplasmic (asterisks), and occasionally nuclear (arrows), located in follicular cells. Representative images of Furin staining in (**e**) AT, (**f**) FTA, and (**g**) cPTC. The staining is both cytoplasmic (asterisks) and nuclear (arrows), located in follicular cells. Scale bars: 50 µm. Abbreviations: AT (Adjacent Thyroid Tissue), FTA (Follicular Thyroid Adenoma), and cPTC (classical Papillary Thyroid Carcinoma).

Furin protein expression was predominantly cytoplasmic and occasionally nuclear, localized in follicular cells (Figure 9e–g). No statistically significant differences in the protein expression among the different histotypes and subtypes were detected ($p > 0.05$)

(Figure S6). Regarding the clinicopathological data, in thyroid-adjacent tissue, an increase in Furin protein expression was observed in younger patients (<55 years) (Figure S7a) and for the negative *TERTp* mRNA expression (Figure S7b).

4. Discussion

The ACE2, TMPRSS2, and Furin molecules became notorious during the SARS-CoV-2 pandemic, since their fundamental role in the mechanism of the virus infection was perceived. This seemed particularly true for the thyroid gland, since this organ is reported to highly express ACE2, *TMPRSS2*, and *Furin* mRNA [24,40–42].

Nevertheless, on what concerns ACE2, TMPRSSS2, and Furin expression in thyroid neoplastic lesions, the information available in the literature is scarce. In this work, we aimed to primarily characterize these molecules in a series of thyroid lesions composed of goitres and benign and malignant neoplasms with the intent of understanding their potential role in thyroid carcinogenesis.

The expression of ACE2, TMPRSS2, and Furin was analysed at mRNA and protein levels; the thyroid tissues adjacent to the lesions were also evaluated and used as control.

The first approach was to characterize the mRNA expression of these molecules in the lesions, comparing them with normal adjacent tissues. Regarding the *ACE2* mRNA expression levels, our results showed that benign and malignant thyroid lesions presented significantly decreased expression compared to adjacent thyroid tissue.

Several studies reported high *ACE2* mRNA expression levels in normal thyroid tissues and in thyroid cell lines [43–47]. Our results are consistent with findings in the literature. Bao et al. reported a significant reduction of *ACE2* gene expression in a pooled set of different tumour types, but specifically for thyroid cancer; *ACE2* gene expression was significantly decreased when compared with matched normal tissue [48]. Similarly, Chai et al., using the GEPIA2 tool, reported that thyroid cancer presented decreased *ACE2* expression in comparison to normal tissue [7].

On the other hand, a study encompassing a series of 26 benign and 35 malignant thyroid lesions reported an increase in *ACE2* mRNA expression in malignant lesions such as PTCs and FTCs when compared to FAs and goitres [49]. The authors also observed that undifferentiated thyroid carcinomas presented a decrease in *ACE2* expression when compared to PTCs [49]. In our study, we found lower *ACE2* mRNA in thyroid lesions compared to the normal adjacent tissue, but did not detect significant differences ($p > 0.05$) in *ACE2* mRNA expression between benign and malignant thyroid lesions, since both were diminished in comparison with the normal adjacent tissue.

ACE2's role in tumorigenesis is still controversial, but several studies claim its association with a more favourable prognosis [7,48,50,51]. A higher expression of *ACE2* predicted better outcomes for disease-free survival and overall survival in tumours such as renal clear cell carcinoma, hepatocellular carcinoma, and ovarian serous cystadenocarcinoma [7]. In breast cancer, patients with higher *ACE2* expression were reported to have longer relapse-free survival [50]. In vitro, ACE2 has also been reported to be involved in tumorigenesis inhibition [50]. In breast cancer cells, *ACE2* expression reduced cell migration and human umbilical vascular endothelial cell proliferation, and downregulated the expression of VEGFa [50]. Cheng et al. reported that *ACE2* overexpression was associated with angiogenesis suppression in non-small cell lung cancer after the development of acquired platinum resistance in human lung cancer xenografts [29].

In our study, we additionally detected, in follicular adenomas, a positive association between *ACE2* expression and smaller tumours (<3 cm) and with the presence of lymphocytic infiltration, which directly correlated with the presence of CLT. Furthermore, *ACE2's* increased expression was associated with positive *TERT* mRNA expression ($p < 0.05$). These results were concordant with the work by Pestana et al., who also described a significant correlation between *TERT* mRNA expression and lymphocytic presence in smaller tumours [36]. The authors found that most of the FTAs positive for *TERT* mRNA expression presented concomitant lymphocytic thyroiditis, as demonstrated long before [52].

Expression of *ACE2* in lymphocytes has already been described in oral mucosa, the digestive system, and the lungs [53–55]; furthermore, *ACE2* has been reported to be positively modulated by cytokines in the thyroid [56]. In the study by Pestana et al., the authors hypothesized that the expression of *TERT* mRNA in FTAs resulted from the presence of lymphocyte infiltration of the tumours [36]. Taking this into account, it is not surprising that there is a concomitant expression of *TERT* and *ACE2* mRNA in tumours with lymphocytic infiltration. No significant differences were observed with *ACE2* expression in malignant neoplasms.

Regarding ACE2 IHC protein expression, the staining was mainly present in small vessels and not specifically in thyroid follicular cells. The fact that ACE2 protein is not expressed by the thyrocytes, but instead by the pericytes of the endothelial cells, has been reported [45]. However, in a few cases, apical expression of the protein was detected in follicular cells of the tumours, specifically in PTC and in tumours with microfollicular patterns. Still, this pattern of expression was a rare event in our series, and to our knowledge, apical expression of ACE2 in the thyroid cells was not described before. We did not find any common feature in these tumours associated with this pattern of expression.

TMPRSS2 showed elevated mRNA levels of expression; however, we did not observe significant differences between expression in adjacent tissue and in thyroid lesions. The information available in the literature regarding this transcript is also limited, with a few studies reporting relatively high mRNA expression levels of *TMPRSS2* in normal thyroid and in a follicular thyroid cell line [23,45,47,57]. *TMPRSS2* has been reported to be dysregulated in other tumours, such as prostate and lung adenocarcinomas [17,19,58]. A few in silico studies pointed to a downregulated expression of *TMPRSS2* in thyroid carcinoma compared to healthy thyroid tissues [48,58–60]; we were not able to observe this, since according to our data, *TMPRSS2* did not seem to play a role in thyroid tumorigenesis.

Concerning *Furin* expression, contrary to what is reported in the GTex database, in our series, *Furin* mRNA in normal thyroid tissue was virtually negative in all samples. We observed a significant increase in *Furin* mRNA expression in benign and malignant lesions compared to adjacent tissue expression ($p < 0.001$). Interestingly, this difference was more pronounced in FTAs ($p < 0.0001$). Different from *ACE2*, *Furin* mRNA revealed significant associations with malignant lesions, in particular the papillary histotype. *Furin* mRNA was significantly higher in tumours from male patients, tumours that were wild -type for the *NRAS* gene, and tumours that presented lymph node metastasis. Interestingly, these parameters seem to be associated with more aggressive behaviour in thyroid tumours.

Men with thyroid cancer are considered to have a more guarded prognosis. In a series including more than 60,000 patients with thyroid cancer, the male sex presented significantly more aggressive histological subtypes, independently of the age group, and significantly more advanced disease at presentation [61]. Our results revealed *Furin* to be significantly increased in male patients with Papillary Thyroid Carcinomas ($p = 0.002$).

Regarding *NRAS* mutation, our results revealed a significantly increased expression of *Furin* in thyroid carcinomas wild-type for the *NRAS* mutation that were mostly Papillary Thyroid Carcinomas. *RAS* mutations in thyroid cancer have generally been associated with less aggressive behaviour, specifically when present in the papillary histotype [62,63]. Given our results and the fact that *Furin* seems to be associated with a more aggressive signature in carcinogenesis of the thyroid, it would be reasonable to hypothesize that *Furin* would not be associated with *NRAS* mutation.

Our study revealed *Furin* to be increased in thyroid cancer patients with lymph node metastasis ($p < 0.05$). *Furin* is increased in several types of cancers, and a recent report shows *Furin* to be significantly associated with more aggressive clinicopathological characteristics and poorer patient outcomes in PTC [34]. In a large cohort of PTC patients, *Furin* was found to be highly expressed in almost 45% of the series. In addition, it was significantly associated with clinicopathological characteristics such as advanced stage, tall cell variant, extrathyroidal extension, and high American Thyroid Association risk score [34]. Furthermore, the authors also report a significant association between *BRAF* mutation and

Furin expression [34]. These authors also described that *Furin* ectopic expression increased the invasive and migratory potential of a PTC cell line [34], supporting the hypothesis that *Furin* is involved in thyroid tumorigenesis and associated with poor prognostic features.

In our series, we could not identify any association with *BRAF* or other genetic alteration, however, *Furin* was revealed to be the only molecule that could discriminate normal thyroid adjacent tissue from the thyroid neoplasms (AUC = 0.786), with a sensitivity of 72% and a specificity of 79%, assuming a cut-off value of 0.025. These values were similar when the series was restricted to PTCs, differentiating the malignant lesions from normal tissue with an AUC = 0.774; 77% sensitivity, and 76% specificity, assuming the same cut-off value. Our results are consistent with the findings of Poyil et al., who observed a higher expression of Furin protein in PTCs when compared to normal thyroid tissue [34]. These results suggest *Furin* as a possible candidate biomarker in thyroid disease.

Furin immunoexpression did not reveal any significant differences concerning the different thyroid lesions. Only a few studies described Furin protein expression in the thyroid [2] and in thyroid carcinomas, more specifically in PTCs [34,64]. In one of these studies, a higher expression of Furin in metastatic tissue was observed when compared to the primary PTC [34]. In our study, we could not detect such an association, although at the mRNA level, tumours with lymph node metastases showed a significantly higher expression of *Furin* mRNA than tumours without lymph node metastases.

A limitation in the interpretation of some of our results is the lack of correlation between Furin mRNA and protein expression. Discrepancies between mRNA and protein levels have been reported in the literature for several molecules, reflecting biologic and/or technical issues [65,66]. The levels of mRNA can be informative, but not predictive of protein expression, which makes it essential to always evaluate the two parameters.

5. Conclusions

Our work aimed at the characterization of ACE2, TMPRSS2, and Furin in thyroid lesions, including benign and malignant lesions.

We are able to disclose that *ACE2* mRNA expression was decreased in neoplasms when compared to normal tissue; it was more associated with benign lesions, rather than malignant ones, being only significantly increased in smaller adenomas and in the presence of lymphocytic infiltrate, and associated with CLT. On the other hand, *Furin* is significantly more expressed in thyroid neoplasms when compared to the normal tissue, and its high expression was correlated with the malignant histotype and clinicopathological parameters such as male sex, lymph node metastasis, and *NRAS* wild-type status.

Our work revealed that *ACE2* and *Furin* are modulated in thyroid tumorigenesis, and in an opposite manner. Further studies with larger series are needed to confirm *Furin's* potential utility as a candidate biomarker in thyroid neoplasia.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15153909/s1, Table S1. *Furin* mRNA expression cut-off values for all thyroid neoplasms; Table S2. *Furin* mRNA expression cut-off values considering only PTCs; Figure S1. ACE2 immune reactive staining score (IRS) in thyroid series; Figure S2. ACE2 apical staining pattern; Figure S3. TMPRSS2 cytoplasmic IRS score in thyroid series; Figure S4. TMPRSS2 nuclear IRS score in thyroid series; Figure S5. Correlation of TMPRSS2 cytoplasmic IRS with clinicopathological data in adjacent thyroid tissue and carcinomas; Figure S6. Furin cytoplasmic IRS score in thyroid series; Figure S7. Furin IRS score and correlation with clinicopathological data.

Author Contributions: Conceptualization, P.S., A.P. and S.M.; methodology, M.T.A., S.M., S.C., A.P., R.B. and M.S.-S.; software and image analysis, M.T.A., L.C. and T.B.G.; investigation, M.T.A. and S.M.; resources, P.S.; writing—original draft preparation, M.T.A. and S.M.; writing—review and editing, P.S., S.C., M.S.-S., L.C. and T.B.G.; supervision, P.S.; funding acquisition, P.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study follows the recommendations of the Helsinki and Tokyo Declarations; the WHO and the European Community were respected. The study protocol was approved in 2013 by the Ethical Committee of the Centro Hospitalar e Universitário de São João (CHUSJ, approval number CES284-13). Since it was an anonymized retrospective study, it was exempted from requiring the informed consent from each patient. Ethical approval for the RESEARCH4COVID-19 Special Programme was obtained in 2020.

Informed Consent Statement: Patient consent was waived due to the retrospective nature of the study, and anonymous clinical data was used for the analysis, according to national ethical guidelines.

Data Availability Statement: The data presented in this study are available in this article and supplementary material. Additional information would be available upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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Significance of Furin expression in thyroid neoplastic transformation

Supplementary data

Table S1. *Furin* mRNA expression cut-off values for all thyroid neoplasms.

***Furin* mRNA expression ($2^{-\Delta Ct}$) (Positive if greater than or equal to)**	**Sensitivity**	**Specificity**
0.024	74.5%	75.0%
0.025	72.4%	79.2%
0.026	71.4%	79.2%
0.027	70.4%	83.3%

Table S2. *Furin* mRNA expression cut-off values considering only PTCs.

***Furin* mRNA expression ($2^{-\Delta Ct}$) (Positive if greater than or equal to)**	**Sensitivity**	**Specificity**
0.024	76.6 %	72.0 %
0.025	76.6 %	76.0 %
0.027	74.5 %	80.0 %
0.028	72.3%	80.0 %

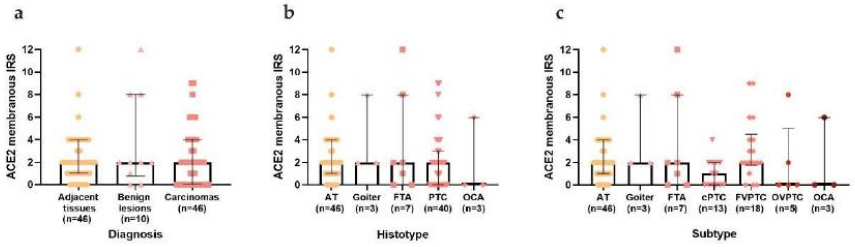

Figure S1. **ACE2 immune reactive staining score (IRS) in thyroid series**. Analysis of ACE2 protein expression score according to the **(a)** Diagnosis; **(b)** Histotype; **(c)** Subtype. Results are shown as median ± IQR. **Abbreviations**: **AT** (Adjacent Thyroid Tissues), **FTA** (Follicular Thyroid Adenomas), **PTC** (Papillary Thyroid Carcinomas), **OCA** (Oncocytic Thyroid Carcinomas), **cPTC** (classical Papillary Thyroid Carcinomas), **FVPTC** (Follicular Variant of Papillary Thyroid Carcinomas) and **OVPTC** (Oncocytic Variant of Papillary Thyroid Carcinoma).

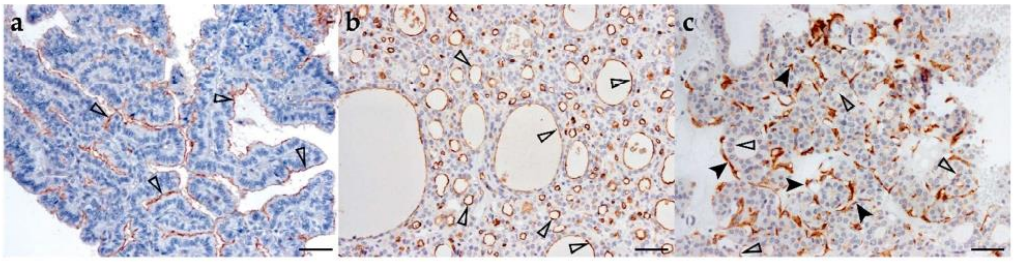

Figure S2. ACE2 apical staining pattern. Representative images of ACE2 apical staining in **(a)** cPTC **(b)** FVPTC and **(c)** a mixed pattern of endothelial (basal) and apical staining in a OVPTC. The apical staining is mainly located in the apical pole of thyrocytes (triangles) surrounded by the basal staining present in endothelial cells (arrowheads). Scale bars: 50 µm. **Abbreviations: FVPTC** (Follicular Variant of Papillary Thyroid Carcinomas) and **OVPTC** (Oncocytic Variant of Papillary Thyroid Carcinoma).

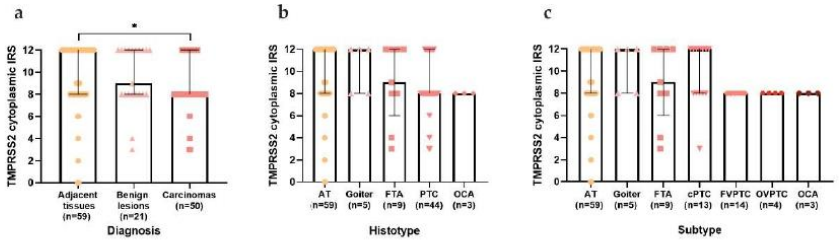

Figure S3. **TMPRSS2 cytoplasmic IRS score in thyroid series**. Analysis of TMPRSS2 cytoplasmic protein expression score according to the **(a)** Diagnosis; **(b)** Histotype; **(c)** Subtype. Results are shown as median ± IQR. **Abbreviations**: **AT** (Adjacent Thyroid Tissues), **FTA** (Follicular Thyroid Adenomas), **PTC** (Papillary Thyroid Carcinomas), **OCA** (Oncocytic Thyroid Carcinomas), **cPTC** (classical Papillary Thyroid Carcinomas), **FVPTC** (Follicular Variant of Papillary Thyroid Carcinomas) and **OVPTC** (Oncocytic Variant of Papillary Thyroid Carcinoma).

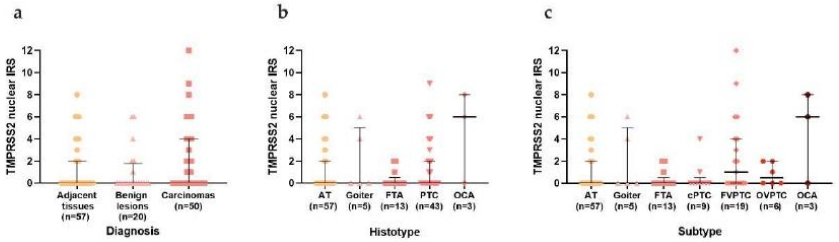

Figure S4. **TMPRSS2 nuclear IRS score in thyroid series**. Analysis of TMPRSS2 nuclear protein expression score according to the **(a)** Diagnosis; **(b)** Histotype; **(c)** Subtype. Results are shown as median ± IQR. **Abbreviations**: **AT** (Adjacent Thyroid Tissues), **FTA** (Follicular Thyroid Adenomas), **PTC** (Papillary Thyroid Carcinomas), **OCA** (Oncocytic Thyroid Carcinomas), **cPTC** (classical Papillary Thyroid Carcinomas), **FVPTC** (Follicular Variant of Papillary Thyroid Carcinomas) and **OVPTC** (Oncocytic Variant of Papillary Thyroid Carcinoma).

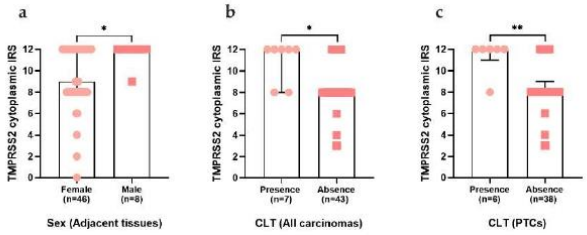

Figure S5. Correlation of TMPRSS2 cytoplasmic IRS with clinicopathological data in adjacent thyroid tissue and carcinomas. Analysis of TMPRSS2 protein expression score comparing the expression on the adjacent tissue according to (a) patient sex; (b) CLT in carcinomas and (c) in PTCs. Results are shown as median ± IQR. **p*-value ≤ 0.05 and ***p*-value ≤ 0.01. **Abbreviations: CLT** (Chronic lymphocytic thyroiditis) and **PTC** (Papillary Thyroid Carcinoma).

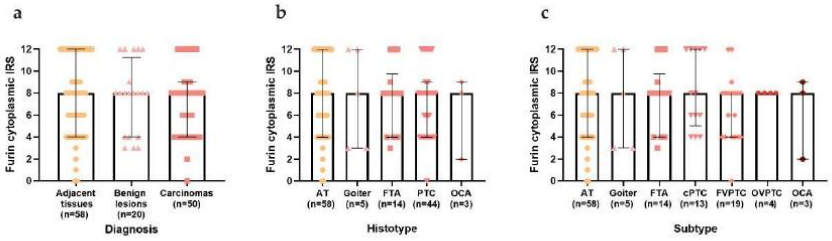

Figure S6. **Furin cytoplasmic IRS score in thyroid series**. Analysis of Furin cytoplasmic protein expression score according to the **(a)** Diagnosis; **(b)** Histotype; **(c)** Subtype. Results are shown as median ± IQR. **Abbreviations**: **AT** (Adjacent Thyroid Tissues), **FTA** (Follicular Thyroid Adenomas), **PTC** (Papillary Thyroid Carcinomas), **OCA** (Oncocytic Thyroid Carcinomas), **cPTC** (classical Papillary Thyroid Carcinomas), **FVPTC** (Follicular Variant of Papillary Thyroid Carcinomas) and **OVPTC** (Oncocytic Variant of Papillary Thyroid Carcinoma).

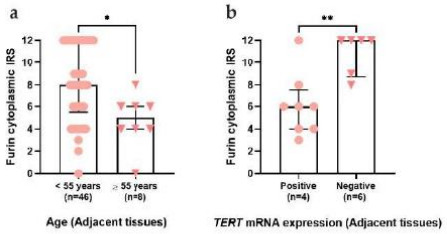

Figure S7. Furin IRS score and correlation with clinicopathological data. Analysis of Furin protein expression score in adjacent tissue comparing the **(a)** Patient age; **(b)** *TERT* mRNA expression. Results are shown as median ± IQR. **p*-value ≤ 0.05 and ***p*-value ≤ 0.01

IV. Endocrine-disrupting chemicals and endocrine neoplasia: A forty-year systematic review

In the recent years, the increasing incidence of thyroid diseases, particularly, cancer cases, has raised significant questions in the endocrinology field. The thyroid, belonging to the endocrine system, is susceptible to various environmental factors, with EDCs emerging as potential contributors to the rising prevalence of thyroid disorders.

Driven by the need to comprehend the complex relationship between environmental exposures to chemicals and thyroid health, we conducted a comprehensive systematic review with the aim to clarify the impact of these chemicals on the increasing cancer incidence on thyroid, but also in other endocrine organs such as breast, ovary, uterus, prostate and testis.

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Review article

Endocrine-disrupting chemicals and endocrine neoplasia: A forty-year systematic review

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ABSTRACT

Introduction: Endocrine disrupting chemicals (EDCs) are exogenous substances recognised as relevant tumourigenic chemicals. Studies show that even EDCs which were long abolished are still contributing to the increasing incidence of neoplasia.

Aim: To investigate the association between human exposure to EDCs and the risk of endocrine-related tumours: breast, prostate, thyroid, uterus, testis, and ovary.

Methods: A systematic review using PubMed, Scopus, and Embase was conducted, searching for original observational studies published between 1980 and 2020, approaching EDCs exposure and endocrine tumourigenic risk in humans. We comprised neoplasia of six endocrine organs. We included all the studies on EDCs reporting tumour odds ratio, risk ratio, or hazard ratio. Study levels of confidence and *risk of bias* were accessed applying accredited guidelines. Human-made accidents and natural EDCs were not considered in the present study.

Results: Our search returned 3271 papers. After duplicate removal and screening, only 237 papers were included (corresponding to 268 records). EDCs were grouped from the most frequently (pesticides) to the least frequently studied (salts). The most tumourigenic EDC groups were phthalates (63%), heavy metals (54%), particulate matter (47%), and pesticides (46%). Pesticides group comprised the highest number of retrieved studies (n = 133). Increased neoplasia risk was found in 43–67% of the studies, with a lower value for ovary (43%) and a higher value for thyroid (67%).

Conclusions: The innovative nature of our review comes from including human studies of six endocrine-related neoplasia aiming to understand the contribution of specific EDCs groups to each organ's tumourigenesis. Thyroid was the organ presenting the highest cancer risk after EDC exposure which may explain the increasing thyroid cancer incidence. However, detailed and controlled works reporting the effects of EDCs are scarce, probably justifying conflicting results. Multinational and multicentric human studies with biochemical analysis are needed to achieve stronger and concordant evidence.

Abbreviations: ApC, alkylphenolic compound; BC, breast cancer; CC, cervical cancer; DIE, deep infiltrating endometriosis; ED, endocrine-disrupting; EDC, endocrine-disrupting chemical; EndC, endometrial cancer; ES, effect size; FR, flame retardant; HM, heavy metal; HR, hazard ratio; OC, organochlorine; OE, ovarian endometrioma; OP, organophosphate; OR, odds ratio; OSPs, organic solvents & phenols; OvC, ovarian cancer; PBP, petroleum-based product; PC, prostate cancer; PCB, polychlorinated biphenyl; PM, particulate matter; POP, persistent organic pollutant; RR, risk ratio; TC, testicular cancer; ThC, thyroid cancer; UL, uterine leiomyoma.

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1. Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous substances (or a mixture of substances) that interfere with any aspect of hormone action, disrupting the endocrine system homeostasis of intact organisms, their progeny or (sub)populations (Zoeller et al., 2012; Gore et al., 2015; Demeneix and Slama, 2019a). Unfavourable health consequences have been described to emerge from EDCs exposure. These include reproductive disorders (namely reduced fecundity/fertility, spontaneous abortion, and reproductive tract abnormalities), neurobehavioural changes, immunological malfunction, and neoplastic lesions (McKinlay et al., 2008; World Health et al., 2013; Encarnacao et al., 2019).

As proposed by the European Commission (EC), EDCs should be considered as such when exhibiting three effects: (1) endocrine activity, (2) deleterious and/or pathologic endocrine mediated-activity, and (3) cause-effect relationship between substance exposure and endocrine activity (Lauretta et al., 2019; EFSA, 2013).

EDCs can interplay with multiple targets (*e.g.*, hormone receptors, hormone synthesis and clearance, hormone transportation, signalling pathways, epigenetic alterations) and display different mechanisms of action (*e.g.*, activation/inactivation of hormone receptors, stimulation/inhibition of hormone synthesis, alteration of signal transduction in hormone-responsive cells, induction of epigenetic changes in hormone-producing or hormone-responsive cells) (Supplementary Table A1). Binding to the hormone receptor (HR), and consequent activation of its signalling pathway, is the only hormone-type mechanism of action. The other mechanisms result in homeostasis disruption (Combarnous and Nguyen, 2019; La Merrill et al., 2020).

EDCs had their first burst in the second half of the 20th century, especially after World War II. Only in the 90's, did EDCs begin to receive the due consideration on their environmental and health consequences (McKinlay et al., 2008; Demeneix and Slama, 2019b). In 2019, the total production of hazardous chemicals was estimated to be 211 million tons within all European Union (EU) (Eurostat, 2019). Nearly 1000 chemicals have been classified as EDCs; this is a small fraction of the 80,000 known chemicals in the environment (Schug et al., 2016).

EDCs comprise a huge number of very heterogeneous chemicals; their sources of exposure are diverse and vary widely around the world. Based on their occurrence, and according to Kabir et al. (2015), EDCs can be classified as pesticides (*e.g.*, DDT), chemicals in products we use in everyday life such as products for children, electronics, personal care products, textile/clothing to building contact materials (*e.g.*, phthalates, heavy metals, flame retardants), and food contact materials (*e.g.*, bisphenols) (Kabir et al., 2015; Gore et al., 2014). The most common exposure routes to EDCs are direct contact (cutaneous), diet (digestive), and/or inhalation (respiratory); diet is a major source (Gore et al., 2015; Lauretta et al., 2019; Kabir et al., 2015).

Some EDCs are considered persistent organic pollutants (POPs), which upraises worries on their long-term effects on the world's population, clearly establishing them as a relevant environmental health problem. POPs are characteristically highly lipophilic, with extensive half-lives that can go up to a few decades (Gore et al., 2015; Heindel et al., 2015), leading to their accumulation over time in the environment and organisms (*e.g.*, animal fat tissue). The result of a lifelong exposure to multiple compounds (POPs and others) can lead to a "cocktail effect" resulting in a potential combination of cumulative, additive, and/or synergic effects (Lauretta et al., 2019; Mantovani, 2016; Zhang and Ho, 2011). These issues have been periodically addressed in the Stockholm Convention (Stockholm Convention on persistent organic, 2019).

The increased incidence of hormone-related neoplasia (Sung et al., 2021) over the last decades has raised the question of the putative role of EDCs in this occurrence (Pierozan et al., 2020), since about 80% of the EDCs are potentially tumourigenic (Yoon et al., 2014; Rocha et al., 2021; Brauner et al., 2021).

The focus of the present review is to investigate the evidence on endocrine neoplasia risk in adult patients exposed to EDCs. We solely focused on endocrine tumours of the breast, prostate, thyroid, uterus, testis, and ovary, because these are the most studied tumours concerning EDCs exposure and their cancer burden is well documented at the Global Cancer Observatory website (Sung et al., 2021). Breast, prostate, uterus, and thyroid cancers are in the top 10 of the most incident cancers, being breast, prostate, and uterus also in the top 10 of the most mortal cancers (Supplementary Table A2). The 40-year time frame selected is in accordance with the first-time recognition of chemicals' impact on health, which only occurred in 1980, despite the large usage of DDT and DES since 1940 (Schug et al., 2016).

2. Methodology

To conduct this systematic review, we used the methodology described in Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Page et al., 2021).

2.1. Search and eligibility criteria

2.1.1. Electronic databases

Studies were searched in three electronic databases [(Medline (PubMed), Embase, and Scopus)] using the terms and keywords detailed in Supplementary Table A3.

2.1.2. Inclusion criteria

We only included original observational studies (case-controls, cohorts, nested case-controls, nested cohorts, case-series, cross-sectional, and ecological studies) of adult humans exposed to any EDC, published online between 1980 and 2020. To be included the studies had to present clear data on odds ratio (OR), risk ratio (RR), or hazard ratio (HR) concerning the development of benign or malignant neoplasia of the breast, prostate, thyroid, uterus, testis, or ovary. Studies about other endocrine-related organs (hypothalamus, pituitary, thymus, pancreas, adrenal gland, pineal body, and parathyroid) are scarce, so they were excluded from this review.

Tumour cases and controls/referents characteristics were identified through medical records (hospital-based data) and/or population-based sources (*e.g.*, national databases).

2.1.3. Exclusion criteria

Exclusion criteria were the following: (1) not available, (2) not English, (3) non-original (*e.g.*, review articles), (4) not human, (5) not EDC, (6) EDC not specified (personal care products), (7) natural EDC or drug (*e.g.*, contraceptives), (8) transgenerational, (9) not neoplasia, (10) human-made accident (*e.g.*, Chernobyl), (11) mortality assessment, (12) methodology (other than RR, OR and HR). The discrimination of exclusion criteria by tumour location may be consulted in Supplementary Table A4.

2.2. Data collection and analysis

2.2.1. Data extraction and management

Records obtained in the initial search were screened for duplicates. Studies with overlapping populations exposed to the same EDC were rejected as duplicates, unless additional comparisons had been performed. Data extraction was performed by four researchers working independently (SM, ET, TBG, and MAS) applying the 12 exclusion criteria (Supplementary Table A4).

Collected data were organised by: a) experimental design (*e.g.*, time of data collection and follow-up, type of study, data source, inclusion of controls, sample size, *initial confidence*, *confidence in the body of evidence*; b) clinicopathological data (*e.g.*, tumour type, hormonal receptor status, presence of polymorphisms, menopausal status, age, residence, source of exposure); c) features of exposure (*e.g.*, specific EDC, exposure time, source and route of exposure, EDC concentration); and d) outcome definition (*e.g.*, statistical significance, adjustment variables and *level of*

evidence). Data regarding *exposure contrast* – i.e., comparison analyses (*e.g., ever exposed* vs *never exposed* or *highest quartile of exposure* vs *lowest quartile of exposure*) – were collected reproducing the exact criteria reported by the authors.

2.2.2. Study quality assessment and risk of bias

We created an evaluation system based on the "Handbook for Conducting a Literature-Based Health Assessment Using OHAT Approach for Systematic Review and Evidence Integration" guidelines to assess the study quality assessment and the *risk of bias*. The *initial confidence* of each study was classified as *very low*, *low*, *moderate*, or *high*, according to the number of the following verified categories: controlled exposure, individual outcome data, comparison group, and assessment of exposure prior to outcome. Controlled exposure data were not reported in any study evaluated, so a 0 was used in this category. The *confidence in the body of evidence* was evaluated after detailed analysis of the papers retrieved, based on the aforementioned guidelines, as a) *definitely high*, b) *probably high*, c) *probably low*, and d) *definitely low*. No study presented a *definitely low risk of bias* (Supplementary Figure A1).

2.2.3. Data synthesis

Effect size (ES) ratios (OR, RR, HR) and their respective confidence intervals (CIs) were retrieved. An association between EDC exposure and neoplasia risk was considered if the value 1 was not contained in the CI, but in this systematic review, only ES $\geq$ 1.01 was considered as showing a positive effect of EDCs on tumourigenesis. In accordance with the OHAT handbook (National Toxicology Program, 2019), the level of *evidence for tumourigenic effect* was equal to the level of *confidence in the body of evidence* (Supplementary Figure A1) in the presence of a positive effect on tumourigenesis. In its absence, *confidence in the body of evidence* was neglected (i.e., considered *inadequate*). The *magnitude effect* was considered *large* every time ES > 2.0.

The retrieved articles covered several EDCs. To group the information obtained, the ECDs were gathered into 12 groups from the most frequently (pesticides) to the least frequently studied (salts): pesticides, polychlorinated biphenyls (PCBs), heavy metals (HMs), petroleum by-products (PBPs), organic solvents & phenols (OSPs), perfluorinated compounds (PFCs), phthalates, particulate matter (PM), flame retardants (FRs), dioxins, alkylphenolic compounds (ApCs), and salts.

3. Results

3.1. Search results

The database search returned 3271 results. After eliminating duplicates, studies with other tumour locations, or overlapping series, 2324 studies remained. By applying the exclusion criteria, only 237 studies were included in the final review. Considering that 13 studies analysed more than one tumour location, 268 records were included. The paper selection detailed flowchart is presented in Fig. 1.

The descriptive statistics of the included studies is shown in Table 2.

For ovary and uterus, reports for benign tumours such as ovarian endometrioma (OE) and uterine leiomyoma (UL), were also found.

Most representative EDCs reported in our study are shown in Table 1.

3.2. Study quality and risk of bias

Study quality and *risk of bias* were independently analysed for each study/organ site as described in the methodology section. Final assessment is shown in Table 2. Detailed information on the assessment of each study *initial confidence*, *risk of bias* and *confidence in the body of evidence* by organ location is presented in the Supplementary Tables A5-

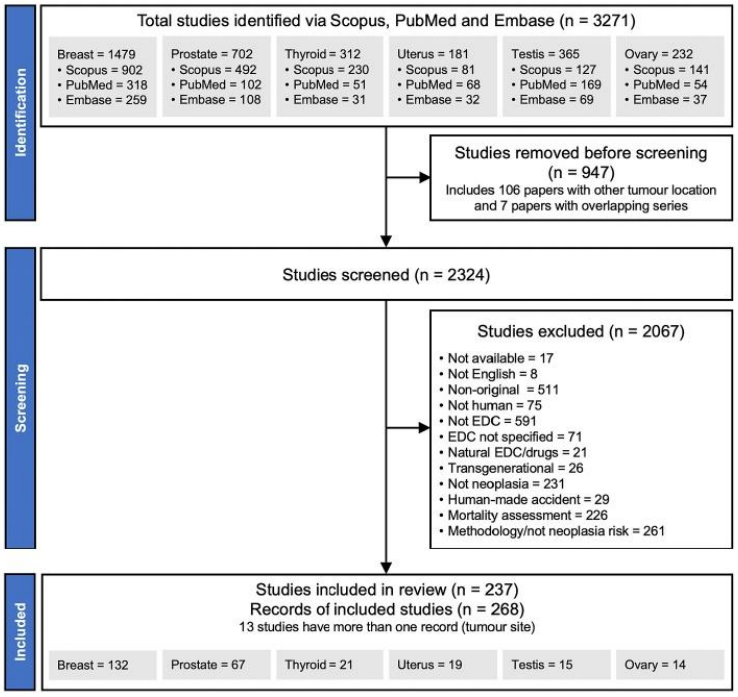

Fig. 1. PRISMA flowchart of the paper selection process according to Page et al. (2021) (Page et al., 2021).

Table 1
Most representative EDCs. BDE bromodiphenyl ether, BPA bisphenol A, BPAF bisphenol AF, BPE bisphenol E, DEHP di (2-ethylhexyl), phthalate, DDT dichlorodiphenyltrichloroethane, DDE dichlorodiphenyldichloroethylene (DDT metabolite), MEHHP mono-(2-ethyl-5-hydroxyhexyl) phthalate (DEHP metabolite), MEHP mono-2-ethylhexyl phthalate (DEHP metabolite), MEOHP mono (2-ethyl-5-oxohexyl) phthalate (DEHP metabolite), NPE nonylphenol epoxide, PCB polychlorinated biphenyl, PFOA perfluorooctanoic acid, PFOS perfluorooctanesulfonic acid, TCEP tris(2-chloroethyl) phosphate.

EDC group	Substance		
Pesticides	Agent Orange Atrazine Chlordane	DDE/DDT Heptachlor Lindane	Malathion Metalaxyl Nonachlor
Polychlorinated biphenyls (PCBs)	PCB 118 PCB 138	PCB 153 PCB 180	
Heavy metals (HMs)	Arsenic Cadmium	Mercury Strontium	
Petroleum-based products (PBPs)	Plastic	Polyvinyl chloride (PVC)	
Organic solvents & phenols (OSPs)	Benzene Bisphenol A (BPA) Bisphenol AF (BPAF)	Bisphenol E (BPE) Perchloroethylene Styrene	Tetrachloroethylene Toluene Xylene
Perfluorinated compounds (PFCs)	PFOA	PFOS	
Phthalates	DEHP MEHHP	MEHP MEOHP	
Particulate matter (PM)	Silica dust		
Flame retardants (FRs)	BDE-28 BDE-47	BDE-153 BDE-209	PBB-153 TCEP
Dioxins	Hexachlorodibenzo-furan	Octachlorodibenzo-furan	
Alkylphenolic compounds (ApCs)	Nonylphenol epoxide (NPE)		
Salts	Perchlorate		

A10.

Overall, most studies (67%) showed *moderate initial confidence*, with prostate reports having the highest frequency of *moderate initial confidence*. No studies had *high initial confidence* since none reported controlled exposure data (Table 2). *Risk of bias* was classified as *probably low* in approximately half of the reports (49%). On the other half, *risk of bias* was *definitely low* (21%), *probably high* (10%), and *definitely high* (20%). Most studies presented either *low* (36%) or *moderate* (39%) *confidence in the body of evidence*, however, a few studies also presented either *very low* (11%) or *high* (14%) levels. Testis was the organ with the most studies presenting a *higher* or *moderate confidence in the body of evidence*, compared with the other organs. Most reports with *high confidence in the body of evidence* concerned breast (17%) and prostate (16%) cancers (Table 2).

3.3. Neoplasia risk upon EDCs exposure

The percentage of reports showing an association of EDC exposure and increased risk of endocrine-related neoplasia is presented in Fig. 2. Considering the six endocrine-related neoplasia, the EDC groups most often reported has having a tumourigenic effect are phthalates (63%), followed by HMs (54%), PM (47%), and pesticides (46%).

3.3.1. EDCs exposure and breast cancer

Breast was the most studied organ (n = 132 studies) (Table 2) and with the highest diversity of EDC groups reporting a positive ES (Fig. 3A). The top 3 EDC groups with the highest contribution to breast cancer (BC) risk were HMs, PFCs, and ApCs. Despite the high number of records, pesticides and PCBs were not the EDC groups most frequently associated with BC risk, as ~60% of such studies did not report any association.

Eleven studies on HM exposure showed a higher risk for BC, with strontium [OR = 10.9 (3.53–33.77)] (Chen et al., 2012) and arsenic [OR = 13.1 (4.02–43.03)] (Marciniak et al., 2020) presenting the highest magnitude effect. Five studies reported a positive association concerning cadmium exposure and BC risk (Strumylaite et al., 2019; Peng et al., 2015; Nagata et al., 2013; Gallagher et al., 2010; Kresovich et al., 2019). In addition, three out of six studies on PFCs reported that exposure to these compounds increased the risk for BC. PFOS [OR = 6.4 (1.08–38.30)] and PFOA [OR = 7.2 (1.00–52.00) and [OR = 7.1 (6.97–7.17)] were the most highlighted with 6- and 7-fold increased risk, respectively (Ghisari et al., 2017; Omoike et al., 2021). The ApC nonylphenol epoxide (NPE) was also shown to have an association with BC (Peremiquel-Trillas et al., 2019). Of note, out of the four studies about the effect of FRs in BC, none reported any association. Complete data concerning all the 132 records are presented in Supplementary Tables A5 and Supplementary Table B1.

Some studies have analysed the correlation of EDC exposure and BC histopathological type: a) hormone receptor (HR) positive (HR+) [oestrogen receptor (ER) and/or progesterone receptor (PR) positive (ER+/PR+)], b) tyrosine kinase receptor human epithelial growth factor receptor 2 (HER2) positive (HER2+), and c) triple negative cases (HR-/HER2-). Studies on HMs have established an association between exposure and HR + BC development. Chen et al. (2012) found that exposure to strontium puts women at a 10-fold higher risk for HER2+ BC, while no effect was reported for HER2- BC (Chen et al., 2012). This suggests that the effect of strontium may be dependent on the HER2 status, which has been related to increased invasiveness and tumourigenicity. Contrarily, studies on pesticides found higher ES related to organochlorine (OC) (Hoyer et al., 2001; Woolcott et al., 2001) and

Table 2
Studies descriptive statistics by tumour location.
Description of all studies. Tumour locations are organised by decreasing order of records, from the highest (breast = 132) to the lowest (ovary = 14). * others include case-cohorts, case-series, cross-sectional, and ecologic studies.

Characteristic		Breast		Prostate		Thyroid		Uterus		Testis		Ovary		Subtotals
		n	%	n	%	n	%	n	%	n	%	n	%	
Number of records		132	49.3	67	25	21	7.8	19	7.1	15	5.6	14	5.2	268
Location	Africa	1	0.8	0	0	0	0	0	0	0	0	0	0	1
	Asia	8	6.1	7	10.4	5	23.8	5	26.3	0	0.0	2	14.3	27
	America	**87**	**65.9**	**46**	**68.7**	**11**	**52.4**	**8**	**42.1**	5	33.3	**6**	**42.9**	163
	Europe	31	23.5	13	19.4	5	23.8	6	31.6	**10**	**66.7**	**6**	**42.9**	71
	Oceania	5	3.8	1	1.5	0	0	0	0	0	0	0	0	6
Year of publication	1980-2000	24	18.2	4	6.0	0	0	1	5.3	3	20.0	1	7.1	33
	2001-2011	42	31.8	**33**	**49.3**	4	19.0	4	21.1	**8**	**53.3**	5	35.7	96
	2012-2021	**66**	**50.0**	30	44.8	**17**	**81.0**	**14**	**73.7**	4	26.7	**8**	**57.1**	139
Study duration	5-	55	41.7	13	19.4	10	47.6	**9**	**47.4**	**7**	**46.7**	3	21.4	97
	5+	**73**	**55.3**	**51**	**76.1**	**11**	**52.4**	**9**	**47.4**	**7**	**46.7**	**11**	**78.6**	162
	Not reported	4	3.0	3	4.5	0	0	1	5.3	1	6.7	0	0	9
Type of study	Case control	**102**	**77.3**	**37**	**55.2**	**12**	**57.1**	**10**	**52.6**	**13**	**86.7**	**5**	**35.7**	179
	Cohort	21	15.9	27	40.3	6	28.6	7	36.8	1	6.7	**5**	**35.7**	67
	Others*	9	6.8	3	4.5	3	14.3	2	10.5	1	6.7	4	28.6	22
Sample size	100-	2	1.5	4	6.0	2	9.5	1	5.3	2	13.3	1	7.1	12
	100+	**61**	**46.2**	14	20.9	**10**	**47.6**	**7**	**36.8**	5	33.3	2	14.3	99
	1,000+	42	31.8	22	32.8	2	9.5	5	26.3	**6**	**40.0**	2	14.3	79
	10,000+	18	13.6	**25**	**37.3**	4	19.0	4	21.1	0	0	**5**	**35.7**	56
	100,000+	9	6.8	2	3.0	3	14.3	2	10.5	2	13.3	4	28.6	22
Sex	Male	3	2.3	67	100	2	9.5	n.a.	n.a.	15	100	n.a.	n.a.	87
	Female	**126**	**95.5**	n.a.	n.a.	5	23.8	19	100	n.a.	n.a.	14	100	164
	Both	3	2.3	n.a.	n.a.	**14**	**66.7**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	17
Patient age (cases)	Mean	54	n.a.	64	n.a.	49	n.a.	52	n.a.	41	n.a.	54	n.a.	n.a.
	STD	9	n.a.	9	n.a.	7	n.a.	14	n.a.	14	n.a.	14	n.a.	n.a.
Laboratory tests	Yes	**72**	**54.5**	22	32.8	**13**	**61.9**	**11**	**57.9**	6	40.0	4	28.6	128
	No	60	45.5	**45**	**67.2**	8	38.1	8	42.1	**9**	**60.0**	**10**	**71.4**	140
Initial confidence	Very low	3	2.3	0	0.0	2	9.5	0	0	1	6.7	2	14.3	8
	Low	48	36.4	10	14.9	9	42.9	7	36.8	5	33.3	1	7.1	80
	Moderate	**81**	**61.4**	**57**	**85.1**	**10**	**47.6**	**12**	**63.2**	**9**	**60.0**	**11**	**78.6**	180
	High	0	0	0	0	0	0	0	0	0	0	0	0	0
Confidence in the body of evidence	Very low	12	9.1	8	11.9	3	14.3	4	21.1	1	6.7	2	14.3	30
	Low	45	34.1	**29**	**43.3**	**8**	**38.1**	**7**	**36.8**	4	26.7	3	21.4	96
	Moderate	**52**	**39.4**	19	28.4	**8**	**38.1**	**7**	**36.8**	**10**	**66.7**	**8**	**57.1**	104
	High	23	17.4	11	16.4	2	9.5	1	5.3	0	0	1	7.1	38
Paper with positive effect	Yes	**73**	**55.3**	**37**	**55.2**	**14**	**66.7**	**12**	**63.2**	**8**	**53.3**	6	42.9	150
	No	59	44.7	30	44.8	7	33.3	7	36.8	7	46.7	**8**	**57.1**	118

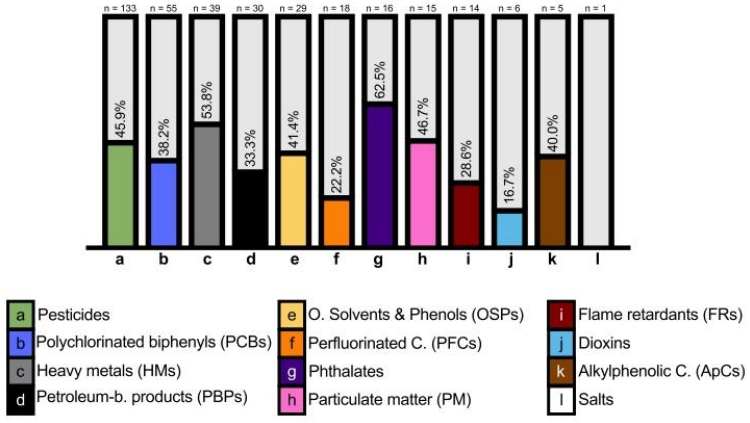

Fig. 2. Percentage of reports of EDCs with increased risk of endocrine-related neoplasia (all tumour sites). Colours and percentages in each bar represent the proportion of records with a positive effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

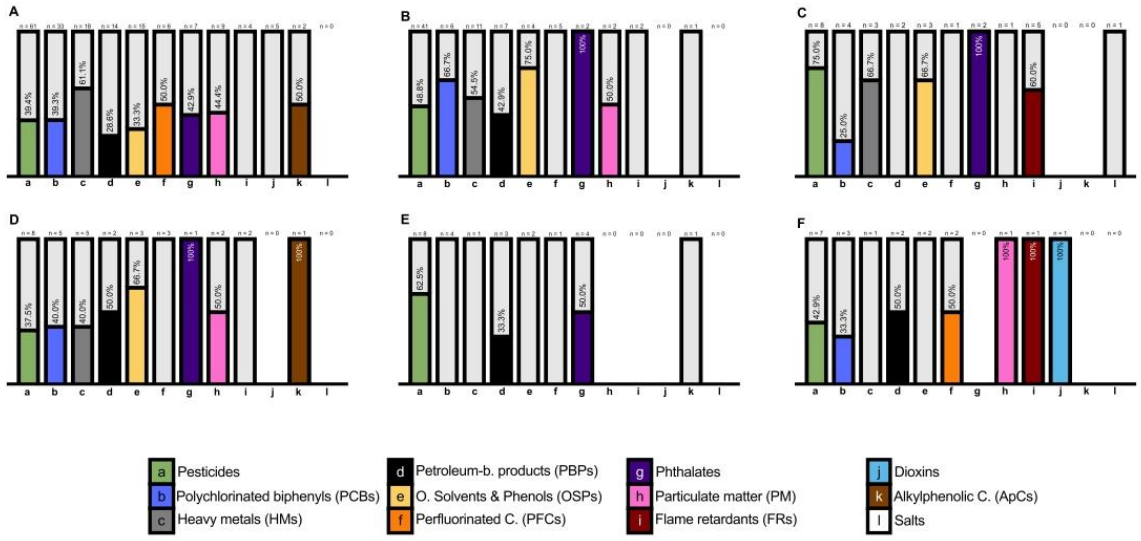

Fig. 3. Percentage of reports of EDCs with increased risk of endocrine-related neoplasia in (A) Breast, (B) Prostate, (C) Thyroid, (D) Uterus, (E) Testis, and (F) Ovary. Colours and percentages in each bar represent the proportion of records with a positive effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

organophosphate (OP) pesticides (Lerro et al., 2015a) exposure and HR-BC. Thirteen out of 37 (35%) reports studying dichlorodiphenyltrichloroethane (DDT) and its metabolites described an increased risk for BC (Woolcott et al., 2001; Olaya-Contreras et al., 1998; Demers et al., 2000; Hoyer et al., 2000; Romieu et al., 2000; Charlier et al., 2004; McCready et al., 2004; Cohn et al., 2007, 2019; White et al., 2013; Tang et al., 2014; Arrebola et al., 2015; Eldakroory et al., 2017) (Supplementary Table B1). Cohn et al. (2019) advocated that female exposure to DDT before or after menopause is determinant to the development of BC (Cohn et al., 2019).

3.3.2. EDCs exposure and prostate cancer

A total of 67 studies on prostate cancer (PC) risk and EDCs exposure were found (25%); this was the second most studied organ (Table 2). There were 41 studies on pesticides and 49% of them showed a positive association between exposure and PC. Besides pesticides, HMs and PBPs were also frequently studied, but most associations with PC risk were found in phthalates, OSP, PCBs, and HMs (Fig. 3B). In two studies, phthalates concentration in urine and blood was evaluated, with both studies showing an association with PC risk (Chang et al., 2018; Chuang et al., 2020), but one of the studies presented an *inadequate level of evidence* (Chang et al., 2018) (Supplementary Table B2). Three out of four studies reported associations between OSPs and PC risk (Tse et al., 2018; Blanc-Lapierre et al., 2018; Christensen et al., 2013). Bisphenol A (BPA) exposure showed a 57% increase in PC risk [OR = 1.57 (1.01–2.44)] (Tse et al., 2018). Blanc-Lapierre et al. (2018) showed, with a *high level of evidence*, that benzene was one of the most relevant EDCs for PC risk, with ES ranging from 1.24 to 2.32 [OR = 1.24 (1.01–1.54); OR = 2.32 (1.25–4.31)], and a 12-fold higher risk in individuals exposed over ≥25-year period (Blanc-Lapierre et al., 2018). The same study verified that styrene, toluene, and xylene were also relevant EDCs in prostate tumourigenesis, with different ES depending on patient's age and duration of exposure. Another study, by Christensen et al. (2013), indicated an association between perchloroethylene exposure and PC risk, showing a 6-fold increase with *moderate level of evidence for tumourigenic effect* [OR = 6.0 (1.2–30.0)] (Supplementary Table B2) (Christensen et al., 2013).

PCBs were also a relevant group in prostate carcinogenesis. Hardell et al. (2006) showed a 3-fold higher risk of PC upon PCB 153 exposure [OR = 3.15 (1.04–9.54)] (Hardell et al., 2006). This risk was even higher in patients with high levels of prostate-specific antigen (PSA) (>16.5 ng/mL) [OR = 30.3 (3.24–284)], a biomarker of PC progression. Lim et al. (2017) reported an almost 4-fold increased risk for PC upon exposure to PCB 153 [OR = 3.90 (1.43–10.60)] (Lim et al., 2017). In contrast, three studies found no increased risk with PCB 153 (Emeville et al., 2015; Pi et al., 2016; Aronson et al., 2010) (Supplementary Table B2). Only one out of three studies reported association between PC and PCB 118 and PCB 180 exposure [OR = 3.44 (1.01–11.69) and OR = 5.66 (1.53–21.00), respectively] (Lim et al., 2017; Pi et al., 2016; Aronson et al., 2010).

The most studied HM was cadmium; five studies were retrieved (Vinceti et al., 2007; Nyqvist et al., 2017; Platz et al., 2002; Armstrong and Kazantzis, 1985; van der Gulden et al., 1995), and two showed a positive ES (Nyqvist et al., 2017; van der Gulden et al., 1995). Apart from van der Gulden et al. (1995) study, which showed a positive ES with moderate *level of evidence for tumourigenic effect*, the other studies had an *inadequate confidence* (Supplementary Table B2). Other HMs, such as arsenic [OR = 5.07 (2.07–12.40)] (Roman et al., 2018), and mercury [OR = 42.86 (1.09–1684.00)] (Chang et al., 2018) were reported to have large *magnitude effects* on the association with PC risk. Complete data concerning all the 67 records on prostate search can be consulted in Supplementary Tables A6 and B2.

3.3.3. EDCs exposure and thyroid cancer

The most studied groups of EDCs in thyroid were pesticides, FR and PCBs. Phthalates, pesticides, HMs, and OSPs were the EDCs groups with more studies reporting a positive association with ThC (Fig. 3C). Even though only two studies reported exposure to phthalates as a risk for ThC, both studies found several significant associations, although with a low *level of evidence* Supplementary Table B3).

Phthalates showed the highest risk for ThC: the parental compound DEHP with a 14-fold increased risk [OR = 14.4 (1.69–122.98)] (Marotta et al., 2019) whereas its metabolite MEHP showed 16-fold increased risk for the same output [OR = 15.5 (1.2.20–109.40)] (Miao et al., 2020).

Six out of eight studies (75%) reported an increased risk of ThC upon pesticide exposure. From the 52 studied pesticides, increased risk of ThC

was reported for *trans*-nonachlor, chlordane, malathion, lindane, metalaxyl, and atrazine (Lerro et al., 2015b, 2018, 2021; Freeman et al., 2011; Han et al., 2019a). Exposure to atrazine was the most significant, showing almost five times increased risk of ThC [OR = 4.8 (1.31–17.93)] (Freeman et al., 2011).

Two out of three (67%) studies on HMs exposure showed increased risk of ThC (Lope et al., 2009; Zhang et al., 2019a; Park et al., 2021). Two studies reported that cadmium exposure was associated with ThC risk with ES ranging from 2- [OR = 2.3 (1.09–4.78)] (Park et al., 2021) to 3-fold [OR = 2.9 (1.5–5.59)] (Zhang et al., 2019a), and with at least one of the studies showing a *level of evidence* with *high confidence* (Park et al., 2021) (Supplementary Table B3). However, lead was the HM with the largest *magnitude effect* in papillary thyroid cancer (PTC) [OR = 4.7 (2.41–9.30)] (Zhang et al., 2019a).

Two studies out of three demonstrated OSPs exposure as relevant to ThC incidence risk. The only OSPs showing association with ThC were bisphenols. A cross-sectional study on bisphenol AF (BPAF) and bisphenol E (BPE), measuring EDC concentration in blood, reported a large *magnitude effect* for ThC risk [OR = 14.4 (1.69–122.98) and OR = 4.9 (1.17–20.47), respectively] (Marotta et al., 2019). BPA exposure was also reported to have positive ES, showing 3.5 times higher risk of outcome [OR = 3.5 (1.37–9.30)] (Zhou et al., 2017). Thyroid was the organ most studied for FRs effects, with three out of five studies (60%) showing and increased risk for ThC; specifically, five compounds were measured in blood: BDE-28, BDE-47, BDE-153, BDE-209, and TCEP (Hoffman et al., 2017a; Huang et al., 2020; Han et al., 2019b). In fact, BDE-28 exposure showed a 10-fold increased risk for PTC [OR = 10.7 (1.93–59.72)], in a study with moderate *confidence in the body of evidence* (Huang et al., 2020). Finally, only one study assessed the association of salt exposure (perchlorate) with ThC risk, without finding any effect (Li et al., 2001). Complete data concerning the 21 records retrieved are presented in Supplementary Table A7.

3.3.4. EDCs exposure and uterine neoplasia

Out of 19 studies, 12 reported an increased risk for uterine neoplasia. The most studied EDC groups were pesticides, PCBs, and HMs (Fig. 3D). Less than 40% of the studies on pesticides reported a positive association with uterine leiomyoma (UL) (Trabert et al., 2015), cervical cancer (CC) (Parron et al., 2014), and endometrial cancer (EndC) (Weiderpass et al., 2000).

One work reported an increased risk for EndC [OR = 1.8 (1.0–3.4)] allegedly after consumption of fish contaminated with DDT (Weiderpass et al., 2000) (Supplementary Table B4). PCBs and HMs were associated with increased risk of uterine neoplasia. Cadmium was reported in two studies as showing a 20–40% increased risk for uterine cancer, [OR = 1.2 (1.03–1.44)] and [OR = 1.4 (1.02–2.04)] (McElroy et al., 2017; Johnstone et al., 2014), respectively. The only study reporting phthalates described a positive association with UL. The presence of DEHP metabolites (MEHP, MEHHP, and MEOHP) in urine was associated with UL, with up to a 10-fold increased risk [OR = 10.78 (1.24–93.81)] (Kim et al., 2016). Silica dust also seemed to play a role in EndC occurrence [HR = 7.4 (1.4–39.7)] (Wernli et al., 2008a). Complete data concerning the 19 records can be consulted in Supplementary Table A8.

3.3.5. EDCs exposure and testicular cancer

Only 15 studies on EDCs exposure and testicular cancer (TC) were found (Fig. 3E). All reported tumours were malignant, namely seminoma, non-seminoma, and embryonal carcinoma. About half of the studies reported an increased risk for TC upon EDCs exposure. The top 3 EDC groups with more studies were pesticides, PCBs, and phthalates. Most studies were on pesticides (n = 8) and 63% of them reported an increased risk for TC. Three studies showing pesticide tumourigenic effect on testis performed laboratory tissue analyses (Chia et al., 2010; McGlynn et al., 2008; Giannandrea et al., 2011), while two were based only in questionnaires/interviews (Parron et al., 2014; Bullman et al., 1994). Agent Orange (2,4-D + 2,4,5-T) was the pesticide with the highest reported risk of TC development [OR = 2.60 (1.08–6.24)] (Bullman et al., 1994), followed by *trans*-nonachlor, [OR = 1.92 (1.03–3.58)] (Chia et al., 2010) (Supplementary Table B5).

From the four studies on PCBs, none retrieved a positive ES. One third of the studies on PBPs observed a positive association with TC risk. Two studies showed increased risk of TC with exposure to phthalates (DEHP), with estimated risks ranging from a 30% increase (Westberg et al., 2005) [OR = 1.3 (1.05–1.69)] to a 7-fold increased risk upon exposure to PVC [OR = 6.6 (1.4–32.0)], a main compound of DEHP (Hardell et al., 1997). On the other hand, two studies on phthalates found no association with TC risk (Paoli et al., 2015; Hardell et al., 2004); but only one performed laboratory analyses in semen and serum samples (Paoli et al., 2015). Out of three studies on PBPs (Hardell et al., 1997, 1998; Behrens et al., 2012), one found that exposure to oil-based cutting fluids almost doubled TC risk [OR = 1.87 (1.05–3.34)] (Behrens et al., 2012). Complete data concerning all the 15 records on testis are presented in Supplementary Table A9 and B5.

3.3.6. EDCs exposure and ovarian neoplasia

Ovary was the least studied organ; pesticides and PCBs were the most studied EDCs (n = 7 and n = 3, respectively) (Fig. 3F).Atrazine exposure was reported in three out of seven studies (43%), but none reported an increased risk of ovarian cancer (OvC) (Inoue-Choi et al., 2016; Hopenhayn-Rich et al., 2002; Young et al., 2005). Only one paper reported increased cancer risk, a 6-fold increase [HR = 5.6 (1.4–23.6)] after exposure to the PM silica dust (Wernli et al., 2008b) (Supplementary Table B6).

Similarly, only one study reported risk for a benign neoplasia, the ovarian endometrioma (OE) (Ploteau et al., 2017). Large *magnitude* positive effects on increased risk were reported for several compounds, in deep infiltrating endometriosis (DIE) with concurrent OE (Ploteau et al., 2017). The OC pesticide *cis*-heptachlor epoxide and the FR PBB-153 presented an 8- to 9-fold increased risk for OE with DIE [OR = 9.49 (2.62–57.74), and OR = 8.26 (2.27–44.41), respectively] (Ploteau et al., 2017) (Supplementary Table B6).

Ovary was the only organ with information on dioxins. Ploteau et al. (2017) assessed the risk of 10 different dioxins, all presenting a positive ES for OE, ranging from OR = 1.92 (1.03–3.79) (hexachlorodibenzofuran) to OR = 6.98 (2.94–22.27) (octachlorodibenzofuran) (Ploteau et al., 2017) (Supplementary Table B6).

Complete data concerning all the 14 records on ovary search can be consulted in Supplementary Table A10 and B6.

4. Discussion

The present systematic review included 237 studies assessing the association between human exposure to EDCs and the risk of endocrine-related neoplasia arising from breast, prostate, thyroid, uterus, testis, and ovary, over a period of 40 years. This work improves previous reviews on the subject by gathering the most important endocrine-related neoplasms, selecting all the specific EDCs reported in the literature, and considering a very broad period of data searching. Exposure to EDCs was associated with increased neoplasia risk in 43–67% of the studies, meaning that a considerable part of the studies did not show any association. This may be related to low levels of *initial confidence*, and *confidence in the body of evidence*, of a considerable number of studies, due to lack of controlled exposure. Moreover, most studies did not rely on the quantification of the EDCs in biological samples, which is considered the gold standard for assessing exposure to toxic substances. However, considering each specific EDC, we did not find a significant difference in neoplasia risk between studies with or without EDC quantification (data not shown). Thyroid was the endocrine organ with the highest frequency of studies with a positive effect (67%). Thyroid chemically attracts iodide and selenium to produce its hormones, and this chemical attraction can also occur between other substances and thyroid hormones, which may lead to an increased effect of the EDCs in this endocrine organ.

Breast was the endocrine organ with more individual EDCs showing an increased risk of neoplasia, something that may be expected due to the high number of included studies. This high number of studies is probably related to the high incidence of BC (Sung et al., 2021). Moreover, the mammary gland develops throughout a woman's life with phases of intense morphological changes and cell proliferation (including prenatal development, puberty, pregnancy, and menopausal transition), representing windows of increased susceptibility to hazardous environmental exposure (Rodgers et al., 2018). The great majority of the studies were case-control studies (77%), as also reported in a recent systematic review on BC risk and EDC (Wan et al., 2021). According to these authors, the relationship between EDC exposure and BC risk is not always clear-cut, and genetics could play a vital role determining whether EDC exposure affects host susceptibility and risks (Wan et al., 2021). Prostate was the second organ with the highest number of available studies. Like BC in women, PC is the second deadliest cancer in men worldwide, and the most common cancer in men. Prostate is the most studied organ of the male genitourinary system concerning EDC exposure (Houston and Ghosh, 2020). Prostate was also the endocrine organ whose studies showed the highest *initial confidence* (85% with moderate *initial confidence*) (Supplementary Figure A1). This may be explained by the Agricultural Health Study (AHS) cohorts with large sample sizes (>10,000) and appropriate choice of comparison groups concerning american farmers and their spouses (AHS. Agricultural Health Study, 2022). EDCs are associated with sex-steroid receptors, namely the two oestrogen receptors (ERα and ERβ) and the androgen receptor (AR) (Lacouture et al., 2022). EDCs may influence several molecular mechanisms involved in prostate homeostasis and disease, including PC, whose onset and progression is characterised by deregulation of several cellular pathways including AR signalling (Corti et al., 2022

For the other organs, less than 20 studies were retrieved (except for thyroid with 21 studies), resulting in a small number of studies for several EDC groups. This strongly implies a cautious interpretation of the results.

Pesticides are used to prevent, control, or eradicate pests, which somehow interfere with production, processing, storage or transport of agricultural products, animal feedstuffs and wood products (Akashe et al., 2018; Del Pup et al., 2016). Although pesticide usage and disposal has become highly regulated and monitored through the Environmental Protection Agency (EPA) and Food and Drug Administration (FDA), many of these compounds can persist for years after previous usage, affecting human health and development (Houston and Ghosh, 2020). In developing countries these adverse effects may be even more astounding due to little or even lacking regulatory controls (Alavanja et al., 2013). Pesticides were the most important group contributing to EDCs increased risk of TC and ThC (63 and 76% of the studies, respectively). Han et al. (2019) reported that pesticide exposure showed positive, statistically significant association with ThC; the sensitivity analysis performed indicated their data were robust (Han et al., 2019a). While agricultural exposure had a significant effect on ThC risk, industrial exposure was not associated with ThC in their meta-analysis (Han et al., 2019a). In PC we found a positive ES in only about half of the studies retrieved (49%) which is not in accordance with a previous systematic review published in 2016 reporting that most studies found a positive association between PC and exposure to pesticides or agricultural occupations (Silva et al., 2016). Overall, occupational exposure to pesticides has been consistently associated with PC in the American AHS (Koutros et al., 2010, 2013; Christensen et al., 2010), and in studies from Canada (Aronson et al., 2010) and France (Dieye et al., 2014). Usually, pesticides with ED activity act by interacting with HR, interfering with its function or expression (Supplementary Table A1).

PCBs are manufactured chemical products composed of carbon, hydrogen, and chlorine atoms, produced since 1929, and banned fifty years later in USA (1979) (Calaf et al., 2020) and worldwide by the Stockholm Convention on POPs (Stockholm Convention on persistent organic, 2019). However, we are still exposed to PCB-containing products, namely plastic, several electrical equipment, oil-based paints, fluorescent light ballasts, thermal insulation material, transformers and capacitors, adhesives, and tapes (Kodavanti et al., 2017). PCBs are highly persistent due to their lipophilic characteristics and have accumulated in the environment throughout the years in water and food chains (Calaf et al., 2020). PCBs have been shown as important in increasing the risk of PC (67% of the studies) (Fig. 3B); in no other organ an ES above 50% was observed. It has been reported that exposure to PCB congeners 118, 138, 153, and 180 was associated with PC incidence (Lim et al., 2017). Interestingly, we did not find any studies reporting a positive association of PCBs with TC, contrary to what was observed in all the other endocrine organs, including prostate. Houston and Ghosh (2020) have stated that there is an immense knowledge gap regarding the effects of EDCs on testis (Houston and Ghosh, 2020).

HMs are being discarded in water, soil, and atmosphere due to the rapidly growing agriculture and metal industry and inadequate waste disposal (Briffa et al., 2020). Metalloids tend to form covalent bonds which confer toxicological properties and consequently allow covalently binding to organic groups, namely non-metallic elements of cellular macromolecules (Briffa et al., 2020). HMs were the most relevant EDC group for increased risk of BC (61% of the studies) and ThC (67% of the studies), although for the latter only three studies were retrieved. HMs accumulate in the thyroid gland to a greater degree than in other tissues, but the association between HM exposure and the development of ThC remains not fully understood (van Gerwen et al., 2022). Romaniuk et al. (2017) established a relationship between the HMs accumulation in the foci of breast neoplasia and the degree of BC malignancy (Romaniuk capital A et al., 2017).

An increased risk of PC after HM exposure was observed in 55% of the included studies (Fig. 3B). Within the HMs group, cadmium has been one of the most studied HM, and an association was observed concerning high cadmium exposure and PC risk (Nyqvist et al., 2017; van der Gulden et al., 1995; Vella et al., 2017). This non-essential metal presents a half-life of 10–30 years, being stored mostly in the liver, kidneys, and muscles; no protocol for assessing metal retention (rate of assimilation/excretion) has been determined (McElroy et al., 2017). Moreover, there is increasing epidemiological evidence linking exposure to cadmium with an increased risk of BC development (Byrne et al., 2013). Strumylaite et al. (2019) have shown the oestrogenicity of cadmium and its ability to activate ER pathways (Strumylaite et al., 2019). Cadmium induces proliferation of oestradiol (E2) dependent BC cells, increases transcription of the *PR* gene (Strumylaite et al., 2019).

Studies on PBPs showed increased risk of endocrine cancer in 0–50% of the studies retrieved. Breast was the endocrine organ with a higher number of studies, but only in 29% of them a positive ES was reported, so most studies did not find an increased cancer risk.

OSPs exposure has shown a relevant increased risk for endocrine neoplasia in thyroid, prostate, and uterus (67–75% of the studies), although the number of studies retrieved was small (three or four studies). In this group, only bisphenols (BPA, BPAF, and BPE) exposure showed a significant association with ThC risk; no other EDCs within the OSPs group were shown to be relevant in thyroid neoplasia (Marotta et al., 2019; Zhou et al., 2017). BPA (a synthetic substance used in daily items) was reported as associated with PC development (Salamanca-Fernandez et al., 2021), similarly to other organic solvents (benzene, styrene, toluene, and xylene) (Blanc-Lapierre et al., 2018).

PFCs were not associated with increased endocrine neoplasia in most of the endocrine organs evaluated (Fig. 3). There were studies showing a positive ES only for breast (3 out of 6 studies) and ovary (1 out of 2 studies), so an association between PFCs exposure and increased risk of EC development cannot be suggested according to our results. In contrast, Omoike et al. (2021) have shown increased odds of developing BC after exposure to PFCs, in a dose-dependent manner (Omoike et al., 2021).

Phthalates are industrial chemicals used in a large variety of

consumer products and are usually necessary for the improvement of products quality, safety, and durability (Encarnacao et al., 2019; Giulivo et al., 2016). They are present in pharmaceuticals, plastics [*e.g.*, polyvinyl chloride (PVC)], cosmetics, and other personal care products, medical devices, household furnishing, and clothes. In the textile industry, phthalates are applied as additives, to produce coated textiles. Phthalates can easily migrate and release esters into the environment, since they are not covalently bound to their plastic matrix, leading to a widespread exposure (Schettler, 2006). The exposure to phthalates has been evaluated in few studies, however most of them showed a positive ES (Fig. 3). The two studies on phthalates exposure and ThC risk reported phthalates as carcinogenic to thyroid gland, although with *low level of evidence* for health effect (Supplementary Table B3) (Marotta et al., 2019; Miao et al., 2021). Exposure to certain phthalate metabolites may contribute to increased risks of ThC and benign nodules, affecting thyroid growth and function (Liu et al., 2020). Phthalates can impact prostate biology, but significant knowledge gaps remain regarding phthalates exposure and PC risk, as only scarce data are available at the cellular and molecular levels (Lacouture et al., 2022). Our search returned two studies about phthalates (Chang et al., 2018; Chuang et al., 2020) both reporting association with increased PC risk. However, one of them presented *low confidence in the body of evidence*. A positive association of phthalates with BC risk has been described in the Wan et al. (2021) systematic review (Wan et al., 2021), but in our review, only 50% of the studies reported a positive ES.

PM showed a positive ES in 47% of the studies. Breast stood out as the organ with the highest percentage of studies with positive ES (44%). A meta-analysis conducted by Zhang et al. (2019) did not find a significant association with PM exposure and BC incidence risk (Zhang et al., 2019b). For the other organs, the number of studies reported was very small to draw conclusions.

FRs are chemicals used in the industry as additives in combustible materials to prevent or retard fire ignition in household products such as furniture, electronics, clothes and construction products (Pantelaki and Voutsa, 2019). They easily release to the environment because they are not chemically bound to the products in which they were included; due to their lipophilic characteristics they persist in the environment and bioaccumulate in the food chain, but they mainly bioaccumulate in home environments (Hoffman et al., 2017a, 2017b; Pantelaki and Voutsa, 2019). To date, few studies have assessed the association between FRs and endocrine cancer, with conflicting results, potentially due to different congeners and different routes of exposure (Alsen et al., 2021). In our review, the positive ES on cancer risk was not relevant in most endocrine organs but was important in the thyroid gland (positive ES in four out of six studies, although the number of reported studies was small) (Fig. 3C), which may suggest a specific effect of those EDCs on thyroid cells.

Our search retrieved less than 10 studies for each of the last three EDC groups (dioxins, ApCs, and salts) (Fig. 2), so conclusions cannot be drawn.

The carcinogenic mechanisms that take place upon EDCs exposure are not fully understood. Direct gene alterations (genotoxicity) may occur, but several other possible mechanisms may also operate (Supplementary Table A1). Even the individual genetic susceptibility to some pesticides may be at stake (Alavanja et al., 2013).

We found a high heterogeneity of studies (case-controls, cohorts, nested case-control, nested-cohort, case-series, case-cohorts, cross-sectional, ecological), characterised by distinct study methodologies, with half of the studies relying on information from questionnaires/interviews.

It is relevant to note that few studies evaluated mixtures of compounds; this made it difficult to analyse the effect of a single EDC (Gerhardsson et al., 2021). In fact, the general population is exposed to a mixture of compounds, and their additive effect or chemical interaction may exacerbate adverse effects on human health (Wen et al., 2020). Considering this assumption, exposure to mixtures should be monitored and measured, preferably in cohort designs (Fernandez-Martinez et al., 2020).

Alternative manufacturing practices can be applied to mitigate exposure to EDCs. Additional costs to society will need to be weighed against the economic benefits of decreased disease and disability, as well as other societal effects (*e.g.*, ecosystem effects) (Kahn et al., 2020).

Our study has some limitations. Firstly, our search was conducted by applying free vocabulary in the search databases (Supplementary Table A3). Despite the careful inclusion and choice of keywords, some disadvantages may arise from the choice of this strategy, namely the possibility of the non-inclusion of some studies or wrong inclusion of others. This can happen due to the existence of possible homographs or synonyms that may not have been considered. Spelling variations may also be an issue as well as different choices in vocabulary (common vs. scientific designations). Secondly, different studies grouped chemicals differently, as was the case with PCBs (Trabert et al., 2015; Gammon et al., 2002; Lambertino et al., 2011; Recio-Vega et al., 2011), which sometimes made it difficult to include them in a specific group. Thirdly, some reports are based on cohorts with a small number of cases, sometimes resulting in impaired conclusions.

5. Conclusions

Thyroid was the endocrine organ with more studies showing a positive ES for cancer risk. Having in mind that the thyroid gland plays a major role in the metabolism, growth, and development of the human body, EDCs seem to be a crucial issue for this endocrine organ and its tumourigenesis.

Pesticides were the most frequently studied EDCs, probably due to the early recognition of its potential deleterious health effects, which is shown in our search retrieving many studies in the 1980–2000 period. However, the positive ES was below 50%, pointing to the need for more detailed and highly controlled studies, supported by biochemical analyses, as several studies were only based on questionnaires.

The innovative nature of our study comes from including only human studies of endocrine-related neoplasia and comparing EDC exposure effects among six endocrine organs.

We are facing a global problem whose resolution depends strongly on politics. European and American guidelines and laws on the use or disposal of chemicals practically do not exist in less developed countries. More studies and regulations must be applied to minimise the impact of these compounds in human health aiming to reduce the incidence of some EDCs-related cancers.

We hope this work raises awareness for the dangers of the invisible carcinogens present in our industries, our streets, our homes, and our food – "We are what we eat" – Ludwig Feuerbach (German philosopher), 1848. We believe that highly reliable studies will start to emerge.

Author contributions

Conceptualization, PS, LM-A; methodology, SM, ET, TBG, MAS; software, SM, ET, TBG, MAS; validation, SM, ET, TBG, MAS, PB, PS; formal analysis, SM, ET, TBG, MAS, PB; investigation, SM, ET, TBG, MAS; data curation, SM, ET, TBG; writing-original draft preparation, SM, ET, TBG; writing-review and editing all authors; visualization, SM, ET, TBG, MAS, PB, LM-A, PS; supervision, PS, LM-A; project administration, LM-A, P.S.; funding acquisition, SM, ET, TBG, MAS, LM-A, PS.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.114869.

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Supplementary Data

Endocrine-disrupting chemicals and endocrine neoplasia: a forty-year systematic review

Macedo A, Teixeira E, and Gaspar TB, *et al.* (2022)

Supplementary Figure A1 – Decision making diagram for evidence integration. 2
Supplementary Table A1 – Mechanisms of action of EDCs..... 3
Supplementary Table A2 – Relevance of the chosen tumour locations. 4
Supplementary Table A3 – Search keywords. 5
Supplementary Table A4 – Exclusion reasons by organ..... 9
Supplementary Table A5 – All records in Breast. 10
Supplementary Table A6 – All records in Prostate. 13
Supplementary Table A7 – All records in Thyroid. 15
Supplementary Table A8 – All records in Uterus..... 16
Supplementary Table A9 – All records in Testis. 17
Supplementary Table A10 – All records in Ovary. 18

Supplementary Figure A1 – Decision making diagram for evidence integration.

ES effect size. Diagram adapted from the OHAT Handbook (March 4, 2019) – OHAT Framework for Systematic Review and Evidence Integration [1].

Supplementary Table A1 – Mechanisms of action of EDCs.

MAs	Main effect	MA key characteristics	EDCs examples
MA1	Hormone receptor	Interaction with or activation of hormone receptors	DDT, PCBs
MA2		Antagonization of hormone receptors	DDT, Dieldrin, Lindane
MA3		Alteration of hormone receptor expression	BPA, DDT, DEHP, PCBs
MA4	Signalling pathway	Alteration of signal transduction in hormone-responsive cells	Atrazine, BPA, DEHP
MA5	Epigenetic alterations	Induction of epigenetic modifications in hormone-producing or hormone-responsive cells	BPA, DEHP, DES, Methoxychlor, PCBs
MA6	Hormone synthesis	Alteration of hormone synthesis	Atrazine, Methimazole, Perchlorate, Phthalates
MA7	Hormone transportation	Alteration of hormone transport across cell membranes	BPA, DDT, Imidazoline
MA8		Alteration of hormone distribution or circulating levels of hormones	BPA, DES, PBDEs
MA9	Hormone clearance	Alteration of hormone metabolism or clearance	HCB, Parabens, PCBs
MA10	Hormone target	Alteration of the fate of hormone-producing or hormone-responsive cells	Oxybenzone, PCBs, Tebuconazole, Triclosan

BDE bromodiphenyl ether, **BPA** bisphenol A, **DDT** dichloro-diphenyl-trichloroethane, **DEHP** di(2-ethylhexyl), phthalate, **DES** diethylstilbesterol, **EDC** endocrine disrupting chemical, **MA** mechanism of action, **PBDEs** polybrominated diphenyl ethers, **PCBs** polychlorinated byphenyls. Data retrieved from La Merrill *et al*. [2].

Supplementary Table A2 – Relevance of the chosen tumour locations.

Cancer	Incidence*	Rank	Mortality*	Rank
Breast	47.8	1	13.6	5
Prostate	30.7	4	7.7	9
Uterus	22.0[a]	7	9.1[b]	8
Thyroid	6.6	10	0.4	24
Ovary	6.6	18	4.2	14
Testis	1.8	27	0.2	32

*per 100.000, **a** cervix uteri (13.3), corpus uteri (8.7); **b** cervix uteri (7.3), corpus uteri (1.8). Data retracted from The Global Cancer Observatory (GCO) [3]

Supplementary Table A3 – Search keywords.

1. PubMed search strategy:
EDCs-related keywords in title or abstract were searched. To find papers on the relationship between cancer risk and EDCs, we conducted separate searches for each organ (search #3) and combined searches #1 and #3 with the "AND" descriptor, resulting in "search #4" (articles on EDCs and cancer). To exclude *in vitro* or animal reports and articles related to interventional procedures, we made search #2 and combined it with search #4 using the descriptor "NOT." We also excluded review articles (search #6) and filtered the studies to contain only papers with full text available in English and published since 1980.
Search #1 – Title/abstract
("endocrine disruptor" OR "endocrine disruption" OR "endocrine disruptors" OR "endocrine disrupting chemical" OR "endocrine-disrupting chemical" OR "endocrine disrupting chemicals" OR "endocrine-disrupting chemicals" OR EDC OR EDCs OR "environmental pollutant" OR "environmental pollutants" OR POP OR POPs OR "persistent organic pollutant" OR "persistent organic pollutants" OR "environmental exposure" OR "environmental contaminant" OR "environmental contaminants" OR "occupational exposure" OR PCB OR PCBs OR "polychlorinated biphenyls" OR BPA OR bisphenol OR metal OR metals OR pesticides OR pesticide OR "organic solvent" OR "organic solvents" OR phthalates OR phthalate OR "flame retardant" OR "flame retardants" OR "polybrominated diphenyl ether" OR "polybrominated diphenyl ethers" OR PBDEs OR PBDE OR "polybrominated biphenyl" OR "polybrominated biphenyls" OR PBB OR PBBs OR PCP OR PCPs OR "personal care product" OR "personal care products" OR dioxin OR dioxins OR "perfluorinated compound" OR "perfluorinated compounds" OR PFC OR PFCs OR "perfluoroalkyl chemicals" OR "perfluoroalkyl chemical" OR PFAs OR PFA OR PFOA OR PFOS OR "particulate matter")
Search #2 – Title/abstract
(cells OR "in vitro" OR "in-vitro" OR "cell line" OR "cell culture" OR nanoparticle OR rat OR rats OR mouse OR mice OR zebrafish OR chemotherapy OR radiotherapy OR x-ray OR radioiodine OR smoke OR smoking OR tobacco OR cigarette OR tomography OR endoscopy OR radiation OR mri OR ct OR "iodine-131" OR "iodine-125")
Search #3 – All fields
Breast ("breast cancer" OR "breast carcinoma" OR "breast neoplasia" OR "breast tumor" OR "breast tumour" OR "mammary cancer" OR "mammary carcinoma" OR "mammary neoplasia" OR "mammary tumor" OR "mammary tumour")
Thyroid ("thyroid cancer" OR "thyroid carcinoma" OR "thyroid neoplasia" OR "thyroid tumor" OR "thyroid tumour")
Breast ("breast cancer" OR "breast carcinoma" OR "breast neoplasia" OR "breast tumor" OR "breast tumour" OR "mammary cancer" OR "mammary carcinoma" OR "mammary neoplasia" OR "mammary tumor" OR "mammary tumour")
Thyroid ("thyroid cancer" OR "thyroid carcinoma" OR "thyroid neoplasia" OR "thyroid tumor" OR "thyroid tumour")
Prostate ("prostate cancer" OR "prostate carcinoma" OR "prostate neoplasia" OR "prostate tumor" OR "prostate tumour")
Testis ("testicular cancer" OR "testicular carcinoma" OR "testicular neoplasia" OR "testicular tumor" OR "testicular tumour" OR "testicle cancer" OR "testicle carcinoma" OR "testicle neoplasia" OR "testicle tumor" OR "testicle tumour" OR "testis cancer" OR "testis carcinoma" OR "testis neoplasia" OR "testis tumor" OR "testis tumour" OR "testes cancer" OR "testes carcinoma" OR "testes neoplasia" OR "testes tumor" OR "testes tumour" OR "germ cell cancer" OR "germ cell carcinoma" OR "germ cell neoplasia" OR "germ cell tumor" OR "germ cell tumour")

Ovary (“ovary cancer” OR “ovary carcinoma” OR “ovary neoplasia” OR “ovary tumor” OR “ovary tumour” OR “ovarian cancer” OR “ovarian carcinoma” OR “ovarian neoplasia” OR “ovarian tumor” OR “ovarian tumour”)
Uterus (“endometrium cancer” OR “endometrium carcinoma” OR “endometrium neoplasia” OR “endometrium tumor” OR “endometrium tumour” OR “endometrial cancer” OR “endometrial carcinoma” OR “endometrial neoplasia” OR “endometrial tumor” OR “endometrial tumour” OR “uterine cancer” OR “uterine carcinoma” OR “uterine neoplasia” OR “uterine tumor” OR “uterine tumour” OR “uterine cancer” OR “uterine carcinoma” OR “uterine neoplasia” OR “uterine tumor” OR “uterine tumour” OR “uterus cancer” OR “uterus carcinoma” OR “uterus neoplasia” OR “uterus tumor” OR “uterus tumour” OR “uterus cancer” OR “uterus carcinoma” OR “uterus neoplasia” OR “uterus tumor” OR “uterus tumour”)
Search #4
(#1) AND (#3)
Search #5
(#4) NOT (#2)
Search #6
#5 NOT (review [Publication type])
Filters: Language: ENGLISH Year of publication: YEAR (reports before 1980 were excluded)
2. Embase search strategy
The steps for Embase search were like those for PubMed, using the same keywords and filters in sequential and combined searches. Embase search differed only in filtering by also requiring the selection of “ARTICLE” and requiring the period of publication (1980-2020).
3. Scopus search strategy
Scopus database presents different requirements than PubMed or Embase. The search was carried out separately for each organ, resorting to the same keywords and descriptors used in the previous searches. EDCs and cancers were combined and animal/in vitro studies were excluded, as well as interventional articles. To the results obtained, we applied the filters to include only articles in English published after 1980 and exclude review articles.
Breast TITLE-ABS-KEY (“endocrine disruptor” OR “endocrine disruption” OR “endocrine disrupting chemical” OR edc OR “environmental pollutant” OR pop OR “persistent organic pollutant” OR “environmental exposure” OR “occupational exposure” OR “endocrine disruptors” OR “endocrine-disrupting chemical” OR “endocrine disrupting chemicals” OR “endocrine-disrupting chemicals” OR edcs OR “environmental pollutants” OR pops OR “persistent organic pollutants” OR “environmental contaminant” OR “environmental contaminants”) AND TITLE-ABS-KEY (“breast cancer” OR “breast carcinoma” OR “breast neoplasia” OR “breast tumor” OR “breast tumour” OR “mammary cancer” OR “mammary carcinoma” OR “mammary neoplasia” OR ”mammary tumor” OR ”mammary tumour”) AND NOT TITLE-ABS-KEY (cells OR “in vitro” OR “in-vitro” OR “cell line” OR “cell culture” OR nanoparticle OR rat OR rats OR mouse OR mice OR zebrafish OR chemotherapy OR radiotherapy OR x-ray OR radioiodine OR smoke OR smoking OR tobacco OR cigarette OR tomography OR endoscopy OR radiation OR mri OR ct OR “iodine-131” OR “iodine-125”) AND (LIMIT-TO (DOCTYPE, “ar”)) AND (LIMIT-TO (LANGUAGE, “English”)) AND (EXCLUDE (PUBYEAR, 1979) OR EXCLUDE (PUBYEAR, 1978) OR EXCLUDE (PUBYEAR, 1976) OR EXCLUDE (PUBYEAR, 1975) OR EXCLUDE (PUBYEAR , 1972))
Thyroid TITLE-ABS-KEY (“endocrine disruptor” OR “endocrine disruption” OR “endocrine disrupting chemical” OR edc OR ”environmental pollutant” OR pop OR “persistent organic pollutant” OR “environmental exposure” OR “occupational exposure” OR “endocrine disruptors” OR “endocrine-disrupting chemical” OR ”endocrine disrupting chemicals” OR “endocrine-disrupting chemicals” OR edcs OR “environmental pollutants” OR pops OR “persistent organic pollutants” OR “environmental contaminant” OR “environmental contaminants”) AND TITLE-ABS-KEY (“thyroid cancer” OR “thyroid

carcinoma" OR "thyroid neoplasia" OR "thyroid tumor" OR "thyroid tumour") AND NOT TITLE-ABS-KEY (cells OR "in vitro" OR "in-vitro" OR "cell line" OR "cell culture" OR nanoparticle OR rat OR rats OR mouse OR mice OR zebrafish OR chemotherapy OR radiotherapy OR x-ray OR radioiodine OR smoke OR smoking OR tobacco OR cigarette OR tomography OR endoscopy OR radiation OR mri OR ct OR "iodine-131" OR "iodine-125") AND (LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (LANGUAGE, "English")) AND (EXCLUDE (PUBYEAR, 1974))
Prostate TITLE-ABS-KEY ("endocrine disruptor" OR "endocrine disruption" OR "endocrine disrupting chemical" OR edc OR "environmental pollutant" OR pop OR "persistent organic pollutant" OR "environmental exposure" OR "occupational exposure" OR "endocrine disruptors" OR "endocrine-disrupting chemical" OR "endocrine disrupting chemicals" OR "endocrine-disrupting chemicals" OR edcs OR "environmental pollutants" OR pops OR "persistent organic pollutants" OR "environmental contaminant" OR "environmental contaminants") AND TITLE-ABS-KEY ("prostate cancer" OR "prostate carcinoma" OR "prostate neoplasia" OR "prostate tumor" OR "prostate tumour" OR "prostate carcinoma" OR "testicle neoplasia" OR "testis cancer" OR "testis carcinoma" OR "testis neoplasia" OR "testis tumor" OR "testis tumour" OR "testes cancer" OR "testes carcinoma" OR "testes neoplasia" OR "testes tumor" OR "testes tumour" OR "germ cell tumor" OR "germ cell tumour" OR "germ cell cancer") AND NOT TITLE-ABS-KEY (cells OR "in vitro" OR "in-vitro" OR "cell line" OR "cell culture" OR "nanoparticle" OR rat OR rats OR mouse OR mice OR zebrafish OR chemotherapy OR radiotherapy OR "x-ray" OR "radioiodine" OR "smoke" OR smoking OR tobacco OR cigarette OR "tomography" OR "endoscopy" OR "radiation" OR mri OR ct OR "iodine-131" OR "iodine-125") AND (LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (LANGUAGE, "English")) AND (EXCLUDE (PUBYEAR, 1978) OR EXCLUDE (PUBYEAR, 1977) OR EXCLUDE (PUBYEAR, 1976) OR EXCLUDE (PUBYEAR, 1975) OR EXCLUDE (PUBYEAR, 1972) OR EXCLUDE (PUBYEAR, 1970) OR EXCLUDE (PUBYEAR, 1969))
Testis TITLE-ABS-KEY ("endocrine disruptor" OR "endocrine disruption" OR "endocrine disrupting chemical" OR edc OR "environmental pollutant" OR pop OR "persistent organic pollutant" OR "environmental exposure" OR "occupational exposure" OR "endocrine disruptors" OR "endocrine-disrupting chemical" OR "endocrine disrupting chemicals" OR "endocrine-disrupting chemicals" OR edcs OR "environmental pollutants" OR pops OR "persistent organic pollutants" OR "environmental contaminant" OR "environmental contaminants") AND TITLE-ABS-KEY ("testicular cancer" OR "testicular carcinoma" OR "testicular neoplasia" OR "testicular tumor" OR "testicular tumour" OR "testicle cancer" OR "testicle carcinoma" OR "testicle neoplasia" OR "testicle tumor" OR "testicle tumour" OR "testis cancer" OR "testis carcinoma" OR "testis neoplasia" OR "testis tumor" OR "testis tumour" OR "testes cancer" OR "testes carcinoma" OR "testes neoplasia" OR "testes tumor" OR "testes tumour" OR "germ cell cancer" OR "germ cell carcinoma" OR "germ cell neoplasia" OR "germ cell tumor" OR "germ cell tumour") AND NOT TITLE-ABS-KEY (cells OR "in vitro" OR "in-vitro" OR "cell line" OR "cell culture" OR nanoparticle OR rat OR rats OR mouse OR mice OR zebrafish OR chemotherapy OR radiotherapy OR x-ray OR radioiodine OR smoke OR smoking OR tobacco OR cigarette OR tomography OR endoscopy OR radiation OR mri OR ct OR "iodine-131" OR "iodine-125") AND (LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (LANGUAGE, "English")) AND (EXCLUDE (PUBYEAR, 1976) OR EXCLUDE (PUBYEAR, 1975))
Ovary TITLE-ABS-KEY ("endocrine disruptor" OR "endocrine disruption" OR "endocrine disrupting chemical" OR edc OR "environmental pollutant" OR pop OR "persistent organic pollutant" OR "environmental exposure" OR "occupational exposure" OR "endocrine disruptors" OR "endocrine-disrupting chemical" OR "endocrine disrupting chemicals" OR "endocrine-disrupting chemicals" OR edcs OR "environmental pollutants" OR pops OR "persistent organic pollutants" OR "environmental contaminant" OR "environmental contaminants") AND TITLE-ABS-KEY ("ovary cancer" OR "ovary carcinoma" OR "ovary neoplasia" OR "ovary tumor" OR "ovary tumour" OR "ovarian cancer" OR "ovarian carcinoma" OR "ovarian neoplasia" OR "ovarian tumor" OR "ovarian tumour") AND NOT TITLE-ABS-KEY (cells OR "in vitro" OR "in-vitro" OR "cell line" OR "cell culture" OR nanoparticle OR rat OR rats OR mouse OR zebrafish OR chemotherapy OR radiotherapy OR x-ray OR radioiodine OR smoke OR smoking OR tobacco OR cigarette OR tomography OR endoscopy OR radiation OR mri OR ct OR "iodine-131" OR "iodine-125") AND (LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (LANGUAGE, "English")) AND (EXCLUDE (PUBYEAR, 1975))
Uterus TITLE-ABS-KEY ("endocrine disruptor" OR "endocrine disruption" OR "endocrine disrupting chemical" OR edc OR "environmental pollutant" OR pop OR "persistent organic pollutant" OR

“environmental exposure” OR “occupational exposure” OR “endocrine disruptors” OR “endocrine-disrupting chemical” OR “endocrine disrupting chemicals” OR “endocrine-disrupting chemicals” OR edcs OR “environmental pollutants” OR pops OR “persistent organic pollutants” OR “environmental contaminant” OR “environmental contaminants”) AND TITLE-ABS-KEY (“endometrium cancer” OR “endometrium carcinoma” OR “endometrium neoplasia” OR “endometrium tumor” OR “endometrium tumour” OR “endometrial cancer” OR “endometrial carcinoma” OR “endometrial neoplasia” OR “endometrial tumor” OR “endometrial tumour” OR “uterine cancer” OR “uterine carcinoma” OR “uterine neoplasia” OR “uterine tumor” OR “uterine tumour” OR “uterine cancer” OR “uterine carcinoma” OR “uterine neoplasia” OR “uterine tumor” OR “uterine tumour” OR “uterus cancer” OR “uterus carcinoma” OR “uterus neoplasia” OR “uterus tumor” OR “uterus tumour” OR “uterus cancer” OR “uterus carcinoma” OR “uterus neoplasia” OR “uterus tumor” OR “uterus tumour”) AND NOT TITLE-ABS-KEY (cells OR “in vitro” OR “in-vitro” OR “cell line” OR “cell culture” OR nanoparticle OR rat OR rats OR mouse OR mice OR zebrafish OR chemotherapy OR radiotherapy OR x-ray OR radioiodine OR smoke OR smoking OR tobacco OR cigarette OR tomography OR endoscopy OR radiation OR mri OR ct OR “iodine-131” OR “iodine-125”) AND (LIMIT-TO (DOCTYPE, “ar”)) AND (LIMIT-TO (LANGUAGE, “English”)) AND (EXCLUDE (PUBYEAR, 1975) OR EXCLUDE (PUBYEAR, 1972) OR EXCLUDE (PUBYEAR,1970))

Supplementary Table A4 – Exclusion reasons by organ.

Exclusion criteria	Breast	Prostate	Thyroid	Uterus	Testis	Ovary	Totals
Not available [a]	7	6	1	0	1	2	17
Not English	2	1	2	0	2	1	8
Non-original	231	124	40	21	64	31	511
Not human [b]	26	9	7	9	17	7	75
Not EDC [c]	269	104	83	40	34	61	591
EDC not specified [d]	44	16	3	2	4	2	71
Natural EDC or drug [e]	7	0	0	8	2	4	21
Transgenerational	4	1	4	1	16	0	26
Not neoplasia	111	30	52	7	15	16	231
Human-made accident [f]	7	6	10	2	2	2	29
Mortality accessement	84	84	12	7	15	24	226
Methodology [h]	104	75	30	9	30	13	261

Notes: a unavailable even after contacting authors, **b** (*e.g.*, animals, cells, *in silico* models), **c** (*e.g.*, asbestos, talc), **d** (*e.g.*, personal care products); includes occupational/jobs, **e** (*e.g.*, DES, oestrogens, contraceptives), **f** (*e.g.*, World Trade Centre terrorist attack, Chernobyl accident, Seveso accident), **g** methodology, i.e., absence of effect size measurements, or other kinds as RD, IR, IRR, SIR, SRR, WSR), and also includes recurrences/metastases.

Supplementary Table A5 – All records in Breast.

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Breast									
Aschengrau A., et al. [4]	1996	a	USA	1983-1986	C.-Control	-	Moderate	P. Low	Moderate
Ashley-Martin J., et al. [5]	2012	a	Canada	1999-2002	C.-Control	-	Moderate	P. Low	Low
Beane Freeman L.E., et al. [6]	2011	a	USA	1993-2007	Cohort	-	Moderate	D. High	Low
Brody J.G., et al. [7]	2004	a	USA	1988-1995	C.-Control	-	Low	P. High	V. Low
Charlier C., et al. [8]	2004	a	Belgium	2001-2002	C.-Control	Blood	Low	P. Low	Low
Cohn B.A., et al. [9]	2007	a	USA	1959-1998	N. C.-Control	Blood	Low	D. Low	Low
Cohn B.A., et al. [10]	2019	a	USA	1945-2010	N. C.-Control	Blood	Low	P. Low	Low
Duell E.J., et al. [11]	2000	a	USA	1993-1996	C.-Control	-	Moderate	D. High	Low
El-Zaemey S., et al. [12]	2013	a	Australia	2009-2011	C.-Control	-	Moderate	P. Low	High
El-Zaemey S., et al. [13]	2014	a	Australia	2009-2011	C.-Control	-	Moderate	P. Low	Moderate
Eldakroory S.A., et al. [14]	2017	a	Egypt	2013-2014	C.-Sectional	Other	Low	P. Low	Moderate
Engel L.S., et al. [15]	2017	a	USA	1993-2011	Cohort	-	Moderate	D. High	Low
Espejo-Herrera N., et al. [16]	2016	a	Spain	2008-2013	C.-Control	-	Moderate	P. Low	Moderate
Farooq U., et al. [17]	2010	a	USA	1994-1996	C.-Control	-	Moderate	P. High	Low
Hopenhayn-Rich C., et al. [18]	2002	a	USA	1993-1997	Ecologic	Other	V. Low	P. Low	V. Low
Ibarluzea J.M., et al. [19]	2004	a	Spain	1996-1998	C.-Control	Fat tissue	Moderate	D. Low	Moderate
Khanjani N., et al. [20]	2006	a	Australia	1983-2002	Ecologic	Other	V. Low	P. High	V. Low
Lerro C.C., et al. [21]	2015	a	USA	1993-2011	Cohort	-	Moderate	D. High	Moderate
Lopez-Carrillo L., et al. [22]	1997	a	Mexico	1994-1996	C.-Control	Blood	Low	P. Low	Low
McElroy J.A., et al. [23]	2007	a	USA	1995-1997	C.-Control	-	Moderate	P. High	Low
Mills P.K., et al. [24]	2019	a	USA	2008-2009	C.-Control	-	Moderate	P. Low	Moderate
Niehoff N.M., et al. [25]	2016	a	USA, Puerto Rico	2003-2009	Cohort	-	Moderate	D. High	Low
Olaya-Contreras P., et al. [26]	1998	a	Colombia	1995-1996	C.-Control	Blood	Moderate	D. Low	Moderate
Parron T., et al. [27]	2014	a	Spain	1998-2005	C.-Control	-	Low	P. Low	Low
Reynolds P., et al. [28]	2004	a	USA	1993-1999	Cohort	-	Moderate	P. Low	Moderate
Romieu I., et al. [29]	2000	a	Mexico	1990-1995	C.-Control	Blood	Moderate	D. Low	High
Salerno C., et al. [30]	2016	a	Italy	2002-2009	C.-Control	-	Moderate	D. High	Low
Schecter A., et al. [31]	1997	a	Vietnam	1994	C.-Control	Blood	Moderate	P. Low	Moderate
Tang M., et al. [32]	2014	a	China	2005-2007	C.-Control	Blood	Moderate	D. Low	High
Teitelbaum S.L., et al. [33]	2007	a	USA	1996-1997	C.-Control	-	Moderate	P. Low	Moderate
Van 'T Veer P., et al. [34]	1997	a	Europe #1	1991-1992	C.-Control	Fat tissue	Low	P. Low	Moderate
White A.J., et al. [35]	2013	a	USA	1996-1998	C.-Control	Blood	Moderate	D. Low	Moderate
Xu X., et al. [36]	2010	a	USA	1999-2008	C.-Sectional	Blood	Moderate	P. Low	Moderate
Zota A.R., et al. [37]	2010	a	USA	1988-2000	C.-Control	-	Moderate	P. Low	Moderate
Aronson K.J., et al. [38]	2000	a, b	Canada	1995-1997	C.-Control	Other	Low	D. Low	Moderate
Arrebola J.P., et al. [39]	2015	a, b	Tunisia	2012	C.-Control	Blood	Low	P. Low	Low
Demers A., et al. [40]	2000	a, b	Canada	1994-1997	C.-Control	Blood	Low	P. Low	Moderate
Gammon M.D., et al. [41]	2002	a, b	USA	1996-1997	C.-Control	Blood	Moderate	D. Low	High
Gatto N.M., et al. [42]	2007	a, b	USA	1995-1998	C.-Control	Blood	Low	D. Low	Low
Hoyer A.P., et al. [43]	2000	a, b	Denmark	1976-1992	N. C.-Control	Blood	Low	D. Low	Low
Hoyer A.P., et al. [44]	2001	a, b	Denmark	1976-1997	N. C.-Control	Blood	Low	D. Low	Moderate
Krieger N., et al. [45]	1994	a, b	USA	1964-1990	N. C.-Control	Blood	Moderate	D. Low	Low
Laden F., et al. [46]	2001	a, b	USA	1989-1994	N. C.-Control	Blood	Low	D. Low	Low
Lopez-Carrillo L., et al. [47]	2002	a, b	Mexico	1994-1996	C.-Control	Blood	Low	P. Low	V. Low
McCready D., et al. [48]	2004	a, b	Canada	1995-1997	C.-Control	Other	Moderate	P. Low	Moderate
Millikan R., et al. [49]	2000	a, b	USA	1993-1996	C.-Control	Blood	Moderate	D. Low	High

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Breast (continued)									
Moysich K.B., et al. [50]	1998	a, b	USA	1986-1991	C.-Control	Blood	Moderate	D. Low	High
Moysich K.B., et al. [51]	1999	a, b	USA	1986-1991	C.-Control	Blood	Low	D. Low	Moderate
Pavuk M., et al. [52]	2003	a, b	Slovakia	1997-1999	C.-Control	Blood	Low	P. Low	Moderate
Raaschou-Nielsen O., et al. [53]	2005	a, b	Denmark	1993-1997	N. C.-Control	Fat tissue	Moderate	D. Low	High
Stellman S.D., et al. [54]	2000	a, b	USA	1994-1996	C.-Control	Fat tissue	Low	P. Low	Low
Ward E.M., et al. [55]	2000	a, b	Norway	1973-1993	C.-Control	Blood	Moderate	P. Low	Low
Wolff M.S., et al. [56]	2000	a, b	USA	1985-1991	Cohort	Blood	Moderate	D. Low	Moderate
Woolcott C.G., et al. [57]	2001	a, b	Canada	1995-1997	C.-Control	Fat tissue	Low	D. Low	Moderate
Zheng T., et al. [58]	2000	a, b	USA	1995–1997	C.-Control	Blood	Moderate	D. Low	Moderate
Holmes A.K., et al. [59]	2014	a, b, g, i	USA	1999-2002	C.-Control	Blood	Low	P. Low	Moderate
Acheampong T., et al. [60]	2018	a, c, d, e	Singapore	1993-2014	Cohort	-	Moderate	P. Low	Moderate
Ekenga C.C., et al. [61]	2015	a, c, d, e	USA	2003-2009	Cohort	-	Moderate	D. High	Low
Engel L.S., et al. [62]	2005	a, d	USA	1993-2000	Cohort	-	Moderate	D. High	Moderate
Mills P.K. & Yang R. [63]	2005	a, e	USA	1988-2001	N. C.-Control	-	Moderate	P. Low	High
O'Leary E.S., et al. [64]	2004	a, e	USA	1980-1992	N. C.-Control	-	Moderate	P. Low	High
Bencko V., et al. [65]	2009	b	Slovakia	1987-1996	Ecologic	-	V. Low	D. High	V. Low
Charlier C.J., et al. [66]	2004	b	Belgium	-	C.-Control	Blood	Low	P. Low	V. Low
Cohn B.A., et al. [67]	2012	b	USA	1959-1967	N. C.-Control	Blood	Low	D. Low	Low
Donat-Vargas C., et al. [68]	2016	b	Sweden	1997-2012	Cohort	-	Moderate	D. High	Low
Holford T.R., et al. [69]	2000	b	USA	1994-1997	C.-Control	Fat tissue	Low	P. Low	Moderate
Laden F., et al. [70]	2002	b	USA	1976-1994	N. C.-Control	Blood	Low	D. Low	Moderate
Li Y., et al. [71]	2005	b	USA	1993-1996	C.-Control	Blood	Moderate	D. Low	High
Recio-Vega R., et al. [72]	2011	b	Mexico	-	C.-Control	Blood	Moderate	P. Low	High
Rusiecki J.A., et al. [73]	2004	b	USA	1994-1997	C.-Control	Blood	Moderate	P. Low	High
Zhang Y., et al. [74]	2004	b	USA	1994-2002	C.-Control	Blood	Low	P. Low	Moderate
Morgan M., et al. [75]	2017	b, e, g	USA	1999-2010	C.-Sectional	Blood	Moderate	P. Low	Moderate
Amadou A., et al. [76]	2020	c	France	1990-2008	N. C.-Control	-	Low	P. Low	Low
Chen L.-J., et al. [77]	2012	c	China	2009-2010	C.-Control	Urine	Moderate	D. Low	High
Gallagher C.M., et al. [78]	2010	c	USA	2008-2009	N. C.-Control	Urine	Moderate	P. Low	Moderate
Garland M., et al. [79]	1996	c	USA	1976-1986	N. C.-Control	Other	Low	P. Low	Low
Gaudet M.M., et al. [80]	2019	c	Italy	1993-1998	C.-Control	Blood	Low	D. Low	Low
Kresovich J.K., et al. [81]	2019	c	USA	2005-2008	C.-Series	-	Moderate	P. Low	Moderate
López-Carrillo L., et al. [82]	2014	c	Mexico	2007-2011	C.-Control	Urine	Moderate	D. Low	High
Lopez-Carrillo L., et al. [83]	2020	c	Mexico	2007-2011	C.-Control	Urine	Moderate	D. Low	High
Marciniak W., et al. [84]	2020	c	Poland	2010-2017	Cohort	Blood	Moderate	P. Low	High
Nagata C., et al. [85]	2013	c	Japan	2000-2002	C.-Control	Urine	Low	P. Low	Moderate
Peng L., et al. [86]	2015	c	China	-	C.-Control	Blood	Low	D. Low	Moderate
Thompson D., et al. [87]	2005	c	USA	1941-1996	N. C.-Control	-	Moderate	P. Low	Low
Wei Y. & Zhu J. [88]	2019	c	USA	2003-2012	C.-Sectional	Blood	Moderate	P. Low	Moderate
White A.J., et al. [89]	2019	c	USA	2003-2015	Cohort	-	Moderate	D. High	Low
Hart J.E., et al. [90]	2018	c, d, e, g, h	USA	1989-2011	Cohort	-	Moderate	D. High	Low
Strumylaite L., et al. [91]	2019	c, j	Lithuania	2007-2011	C.-Control	Urine	Low	P. Low	Moderate
Bonner M.R., et al. [92]	2005	d	USA	1993-2001	C.-Control	-	Low	P. Low	Low
Debono N.L., et al. [93]	2020	d	Canada	1983-2016	Cohort	-	Moderate	P. High	V. Low
Hansen J. [94]	2000	d	USA	1970-1989	C.-Control	-	Moderate	P. Low	High
Lee D.G., et al. [95]	2019	d	Canada	2005-2010	C.-Control	-	Moderate	P. Low	Moderate
Mordukhovich I., et al. [96]	2016	d	USA	1996-1997	C.-Control	Blood	Moderate	P. Low	High
Mordukhovich I., et al. [97]	2016	d	USA	1996-1997	C.-Control	-	Moderate	P. Low	Moderate

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Breast (continued)									
Pedersen J.E., et al. [98]	2020	d	Denmark	1964-2016	C.-Control	-	Moderate	P. Low	Moderate
Rai R., et al. [99]	2016	d	Australia	2009-2011	C.-Control	-	Moderate	P. Low	Moderate
Laouali N, et al. [100]	2018	d, e	Europe #2	1995-1997	C.-Control	-	Moderate	P. Low	High
Petralia S.A., et al. [101]	1999	d, e	USA	1986-1991	C.-Control	-	Moderate	P. Low	High
Ekenga C.C., et al. [102]	2014	e	USA	2003-2009	Cohort	-	Moderate	D. High	Low
Gallagher L.G., et al. [103]	2011	e	USA	1983-1993	C.-Control	-	Moderate	P. High	Low
Glass D.C., et al. [104]	2015	e	Australia	2009-2011	C.-Control	-	Moderate	P. Low	Moderate
Parada H. Jr, et al. [105]	2019	e	USA	1996-1997	C.-Control	Urine	Low	D. Low	Low
Pedersen J.E., et al. [106]	2021	e	Denmark	1964-2016	C.-Control	-	Moderate	P. Low	Low
Peplonska B, et al. [107]	2010	e	Poland	2000-2003	C.-Control	-	Moderate	P. Low	High
Ruckart P.Z., et al. [108]	2015	e	USA	1995-2013	C.-Control	-	Low	P. Low	Low
Ghisari M., et al. [109]	2017	f	Denmark	1996-2002	N. C.-Control	Blood	Low	D. Low	Moderate
Hurley S., et al. [110]	2018	f	USA	1995-2015	N. C.-Control	Blood	Low	D. Low	Moderate
Mancini F.R., et al. [111]	2020	f	France	1990-2013	N. C.-Control	Blood	Moderate	P. Low	High
Omoike O.E., et al. [112]	2021	f	USA	2005-2012	C.-Sectional	Blood	Moderate	P. Low	Moderate
Tsai M.-S., et al. [113]	2020	f	Taiwan	2013-2015	C.-Control	Blood	Low	D. Low	Low
Vieira V.M., et al. [114]	2013	f	USA	1996-2005	Cohort	Blood	Moderate	P. Low	Moderate
Lopez-Carrillo L., et al. [115]	2010	g	Mexico	2007-2008	C.-Control	Urine	Low	D. Low	Moderate
Merida-Ortega A., et al. [116]	2016	g	Mexico	2007-2008	C.-Control	Urine	Low	D. Low	Low
Parada H., et al. [117]	2018	g	USA	1996-2010	C.-Control	Urine	Low	D. Low	Low
Aschengrau A, et al. [118]	1998	g, k	USA	1983-1986	C.-Control	-	Moderate	P. Low	Moderate
Amaral A., et al. [119]	2006	h	Portugal	1991-2001	C.-Control	-	Low	D. High	Low
Bai L., et al. [120]	2020	h	Canada	2001-2015	Cohort	-	Moderate	P. High	V. Low
Crouse D.L., et al. [121]	2010	h	Canada	1996-1997	C.-Control	-	Low	P. Low	V. Low
Goldberg M.S., et al. [122]	2017	h	Canada	2008-2011	C.-Control	-	Moderate	P. Low	Moderate
Hart J.E., et al. [123]	2016	h	USA	1993-2011	Cohort	-	Moderate	D. High	Low
Hystad P., et al. [124]	2015	h	Canada	1975-1994	C.-Control	-	Moderate	P. Low	Low
Shekarrizfard M., et al. [125]	2015	h	Canada	1996-1997	C.-Control	-	Low	P. High	V. Low
White A.J., et al. [126]	2019	h	USA, Puerto Rico	2003-2016	Cohort	-	Moderate	P. High	V. Low
Hurley S., et al. [127]	2011	i	USA	1995-1998	C.-Control	Fat tissue	Low	P. Low	Low
Hurley S., et al. [128]	2019	i	USA	1995-2015	N. C.-Control	Blood	Low	D. Low	Low
Mancini F.R., et al. [129]	2020	i	France	1990-2013	N. C.-Control	Blood	Moderate	P. Low	Moderate
Danjou A.M.N., et al. [130]	2015	j	France	1990-2008	Cohort	-	Moderate	D. High	Low
Danjou A.M.N., et al. [131]	2019	j	France	1990-2008	N. C.-Control	-	Low	P. Low	Moderate
Hardell L., et al. [132]	1996	j	Sweden	1993-1995	C.-Control	Fat tissue	Low	P. Low	V. Low
Viel J.-F., et al. [133]	2008	j	France	1996-2002	C.-Control	-	Moderate	P. Low	High
VoPham T., et al. [134]	2020	j	USA	1989-2013	Cohort	-	Moderate	D. High	Low
Peremiquel-Trillas P., et al. [135]	2019	k	Spain	2008-2013	C.-Control	-	Moderate	P. Low	Moderate

a pesticides, **b** polychlorinated biphenyls (PCBs), **c** heavy metals (HMs), **d** petroleum-based products (PBPs), **e** organic solvents & phenols (OSPs), **f** perfluorinated compounds (PFCs), **g** phthalates, **h** particulate matter (PM), **i** flame retardants (FRs), **j** dioxins, **k** alkylphenolic compounds (ApCs). **C.-Control** case control, **C.-Sectional** cross-sectional, **C.-Series** case-series, **N. C.-Control** nested case-control. **D. High** definitely high, **D. Low** definitely low, **P. High** probably high, **P. Low** probably low, **V. High** very high, **V. Low** very low. **#1** various (Germany, Netherlands, Northern Ireland, Switzerland and Spain, **#2** various (Denmark, France, Germany, Italy, Latvia, Portugal, Spain and Sweden)

Supplementary Table A6 – All records in Prostate.

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Prostate									
Barry K.H., et al. [136]	2011	a	USA	1993-2005	N. C.-Control	Other	Moderate	P. Low	High
Barry K.H., et al. [137]	2012	a	USA	1993-2007	Cohort	-	Moderate	P. Low	Moderate
Beane Freeman L.E., et al. [6]	2011	a	USA	1993-2007	Cohort	-	Moderate	D. High	Low
Bonner M.R., et al. [138]	2010	a	USA	1993-2005	Cohort	-	Moderate	D. High	Low
Christensen C.H., et al. [139]	2010	a	USA	1993-2005	Cohort	-	Moderate	D. High	Low
Cockburn M., et al. [140]	2011	a	USA	2005-2006	C.-Control	-	Moderate	P. Low	High
Dieye M., et al. [141]	2014	a	France	1981-2005	Cohort	-	Moderate	D. High	Low
Giri V.N., et al. [142]	2004	a	USA	2000-2001	C.-Control	-	Moderate	P. Low	Low
Greenburg D.L., et al. [143]	2008	a	USA	1993-2004	Cohort	-	Moderate	D. High	Low
Hessel P.A., et al. [144]	2004	a	USA	1997-1999	N. C.-Control	-	Moderate	P. Low	Low
Hou L., et al. [145]	2006	a	USA	1993-2002	Cohort	-	Moderate	D. High	Low
Koutros S., et al. [146]	2010	a	USA	1993-2004	N. C.-Control	Other	Moderate	P. Low	High
Koutros S., et al. [147]	2013	a	USA	1999-2003	N. C.-Control	Other	Moderate	P. Low	High
Koutros S., et al. [148]	2013	a	USA	1993-2007	Cohort	-	Moderate	P. Low	High
Lerro C.C., et al. [149]	2015	a	USA	1993-2011	Cohort	-	Moderate	D. High	Low
Lynch S.M., et al. [150]	2006	a	USA	1993-2002	Cohort	-	Moderate	D. High	Low
Lynch S.M., et al. [151]	2009	a	USA	1993-2004	Cohort	-	Moderate	D. High	Moderate
Mahajan R., et al. [152]	2006	a	USA	1993-2002	Cohort	-	Moderate	D. High	Low
Mahajan R., et al. [153]	2006	a	USA	1993-2002	Cohort	-	Moderate	D. High	Low
Mahajan R., et al. [154]	2007	a	USA	1993-2003	Cohort	-	Moderate	D. High	Low
Meyer T.E., et al. [155]	2007	a	USA	1999-2001	C.-Control	-	Moderate	P. Low	Moderate
Mills P.K. & Yang R. [156]	2003	a	USA	1987-1999	N. C.-Control	-	Moderate	P. Low	High
Mozzachio A.M., et al. [157]	2008	a	USA	1993-2004	Cohort	-	Moderate	D. High	Low
Multigner L., et al. [158]	2010	a	Guadaloupe	2004-2007 #1	C.-Control	Blood	Moderate	P. Low	Moderate
Parron T., et al. [27]	2014	a	Spain	1998-2005	C.-Control	-	Low	P. Low	Moderate
Pearce N.E., et al. [159]	1987	a	New Zeland	1979	C.-Control	-	Moderate	P. High	V. Low
Rusiecki J.A., et al. [160]	2006	a	USA	1993-2002	Cohort	-	Moderate	D. High	Low
Rusiecki J.A., et al. [161]	2009	a	USA	1993-2004	Cohort	-	Moderate	D. High	Low
Salerno C., et al. [30]	2016	a	Italy	2002-2009	C.-Control	-	Moderate	D. High	Low
Sawada N., et al. [162]	2010	a	Japan	1990-1995	C.-Control	Blood	Low	D. Low	V. Low
van Bemmel D.M., et al. [163]	2008	a	USA	1993-2004	Cohort	-	Moderate	D. High	Low
Xu X., et al. [36]	2010	a	USA	1999-2005	C.-Sectional	Blood	Moderate	P. Low	High
Yi S.-W., et al. [164]	2013	a	Vietnam	2004	Cohort	-	Moderate	P. High	V. Low
Aronson K.J., et al. [165]	2010	a, b	Canada	1997-1999	C.-Control	Blood	Low	D. Low	Low
Emeville E., et al. [166]	2015	a, b	Guadeloupe	-	C.-Control	Blood	Low	P. Low	Moderate
Lim J.-E., et al. [167]	2017	a, b	South Korea	1994-2013	C.-Cohort	Blood	Moderate	P. Low	High
Hardell L., et al. [168]	2006	a, b, i	Sweden	1997-1999	C.-Control	Fat tissue	Low	P. Low	Moderate
Pi N., et al. [169]	2016	a, b, i	Singapore	2007-2010	C.-Control	Blood	Moderate	P. Low	High
Boers D., et al. [170]	2005	a, c, d	Netherlands	1986-1995	Cohort	-	Moderate	D. High	Low
van der Gulden J.W., et al. [171]	1995	a, c, d	Netherlands	1988-1990	C.-Control	-	Moderate	P. High	Moderate
Koutros S., et al. [172]	2011	a, d, e	USA	1993-2004	N. C.-Control	Other	Moderate	P. Low	High
Ruder A.M., et al. [173]	2017	b	USA	2007	Cohort	-	Moderate	P. Low	Moderate
Armstrong B.G. & Kazantzis G. [174]	1985	c	UK	1940-1970	C.-Control	-	Moderate	P. Low	Moderate
Neslund-Dudas C., et al. [175]	2014	c	USA	2001-2004	C.-Control	-	Moderate	P. High	Moderate

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Prostate (continued)									
Nyqvist F., et al. [176]	2017	c	Sweden	1979-2010	Cohort	-	Moderate	D. High	V. Low
Platz E.A., et al. [177]	2002	c	USA	1989-1996	N. C.-Control	Blood	Low	D. Low	Low
Roh T., et al. [178]	2017	c	USA	1994-2013	Cohort	Other	Moderate	P. Low	Low
Román M.D., et al. [179]	2018	c	Argentina	2008-2015	C.-Control	Other	Moderate	P. Low	Moderate
Rousseau M.-C., et al. [180]	2007	c	Canada	1979-1985	C.-Control	-	Moderate	P. Low	Moderate
Vinceti M., et al. [181]	2007	c	Italy	-	C.-Control	Other	Moderate	D. Low	Low
Chang W.-H., et al. [182]	2018	c, g	Taiwan	-	C.-Control	Blood	Low	P. Low	V. Low
Debono N.L., et al. [93]	2020	d	Canada	1983-2016	Cohort	-	Moderate	P. High	V. Low
Nadon L., et al. [183]	1995	d	Canada	1979-1986	C.-Control	-	Moderate	P. High	Low
Rybicki B.A., et al. [184]	2006	d	USA	2001-2004	C.-Control	Blood	Moderate	P. Low	Moderate
Weichenthal S., et al. [185]	2017	d	Canada	2005-2009	C.-Control	-	Moderate	P. Low	Low
Blanc-Lapierre A., et al. [186]	2018	e	Canada	2005-2009	C.-Control	-	Moderate	P. Low	High
Christensen K.Y., et al. [187]	2013	e	Canada	1979-1985	C.-Control	-	Moderate	P. High	Moderate
Tse L.A., et al. [188]	2018	e	China	2011-2016	C.-Control	-	Moderate	D. High	Low
Eriksen K.T., et al. [189]	2009	f	Denmark	1993-2006	C.-Control	Blood	Low	P. Low	Low
Grice M.M., et al. [190]	2007	f	USA	1961-1997	Cohort	-	Moderate	P. High	Low
Omoike O.E., et al. [112]	2021	f	USA	2005-2012	C.-Sectional	Blood	Moderate	P. Low	Moderate
Steenland K., et al. [191]	2015	f	USA	2008-2011	Cohort	-	Moderate	P. High	V. Low
Vieira V.M., et al. [114]	2013	f	USA	1996-2005	Cohort	Blood	Moderate	P. Low	Moderate
Chuang S.C., et al. [192]	2020	g	Taiwan	1991-2015	N. C.-Control	Urine	Low	D. Low	Moderate
Parent M.-E., et al. [193]	2013	h	Canada	2005-2008	C.-Control	-	Moderate	P. Low	Low
Shekarrizfard M., et al. [125]	2015	h	Canada	1996-1997	C.-Control	-	Low	P. Low	V. Low
Peremiquel-Trillas P., et al. [135]	2019	k	Spain	2008-2013	C.-Control	-	Moderate	P. Low	Moderate

a pesticides, **b** polychlorinated biphenyls (PCBs), **c** heavy metals (HMs), **d** petroleum-based products (PBPs), **e** organic solvents & phenols (OSPs), **f** perfluorinated compounds (PFCs), **g** phthalates, **h** particulate matter (PM), **i** flame retardants (FRs), **j** dioxins, **k** alkylphenolic compounds (ApCs). **C.-Control** case control, **C.-Sectional** cross-sectional, **N. C.-Control** nested case-control. **D. High** definitely high, **D. Low** definitely low, **P. High** probably high, **P. Low** probably low, **V. High** very high, **V. Low** very low. **#1** 2004-2007 (cases) and 2005-2006 (controls)

Supplementary Table A7 – All records in Thyroid.

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Thyroid									
Beane Freeman L.E., et al. [6]	2011	a	USA	1993-2007	Cohort	-	Moderate	D. High	Moderate
Lerro C.C., et al. [21]	2015	a	USA	1993-2011	Cohort	-	Moderate	D. High	Moderate
Salerno C., et al. [30]	2016	a	Italy	2002-2009	C.-Control	-	Moderate	D. High	Low
Zeng F., et al. [194]	2017	a	USA	2010-2011	C.-Control	-	Moderate	P. Low	High
Deziel N.C., et al. [195]	2021	a, b	USA	2010-2011	C.-Control	Blood	Low	D. Low	V. Low
Lerro C.C., et al. [196]	2018	a, b	Norway	1972-2008	N. C.-Control	Blood	Low	D. Low	Low
Han X., et al. [197]	2019	a, b, i	China	2016-2017	C.-Control	Blood	Low	D. Low	Low
Lerro C.C., et al. [198]	2021	a, d	USA	1993–1997	Cohort	-	Moderate	P. High	Low
Bencko V., et al. [65]	2009	b	Slovakia	1987-1996	Ecologic	-	V. Low	D. High	V. Low
Park E., et al. [199]	2021	c	South Korea	2003-2011	Cohort	Urine	Moderate	P. Low	High
Zhang C., et al. [200]	2019	c	China	2016-2018	C.-Control	Urine	Low	D. Low	Moderate
Lope V., et al. [201]	2009	c, d, e, h	Sweden	1971-1989	Cohort	-	Moderate	D. High	Low
Zhou Z., et al. [202]	2017	e	China	2013	C.-Control	Blood	Low	P. Low	Low
Marotta V., et al. [203]	2019	e, g	Italy	2017	C.-Sectional	Blood	Low	P. High	Low
Vieira V.M., et al. [114]	2013	f	USA	1996-2006	Cohort	Blood	Moderate	P. Low	Moderate
Miao H., et al. [204]	2020	g	China	2017	C.-Control	Urine	Low	P. Low	Low
Aschebrook-Kilfoy B., et al. [205]	2015	i	USA	1992-2009	N. C.-Control	Blood	Moderate	D. Low	Moderate
Deziel N.C., et al. [206]	2019	i	USA	2010-2011	C.-Control	Blood	Low	D. Low	Moderate
Hoffman K., et al. [207]	2017	i	USA	2014-2016	C.-Control	Blood	Moderate	P. Low	Moderate
Huang H., et al. [208]	2020	i	USA	2000-2013	N. C.-Control	Blood	Low	D. Low	Moderate
Li F.X., et al. [209]	2001	l	USA	1997-1998	Ecologic	-	V. Low	D. High	V. Low

a pesticides, **b** polychlorinated biphenyls (PCBs), **c** heavy metals (HMs), **d** petroleum-based products (PBPs), **e** organic solvents & phenols (OSPs), **f** perfluorinated compounds (PFCs), **g** phthalates, **h** particulate matter (PM), **i** flame retardants (FRs), **j** dioxins, **k** alkylphenolic compounds (ApCs), **l** salts. **C.-Control** case control, **C.-Sectional** cross-sectional, **N. C.-Control** nested case-control. **D. High** definitely high, **D. Low** definitely low, **P. High** probably high, **P. Low** probably low, **V. High** very high, **V. Low** very low.

Supplementary Table A8 – All records in Uterus.

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Uterus									
Lerro C.C., et al. [21]	2015	a	USA	1993-2011	Cohort	-	Moderate	D. High	Low
Parron T., et al. [27]	2014	a	Spain	1998-2005	C.-Control	-	Low	P. Low	Low
Salerno C., et al. [30]	2016	a	Italy	2002-2009	C.-Control	-	Moderate	D. High	Low
Weiderpass E., et al. [210]	2000	a, b	Sweden	1996-1997	C.-Control	Blood	Moderate	D. Low	Moderate
Hardell L., et al. [211]	2004	a, b, i	Sweden	1997-1998	C.-Control	Fat tissue	Low	P. Low	V. Low
Trabert B., et al. [212]	2015	a, b, i, f	USA	2007-2009	N. C.-Control	Fat tissue	Low	P. Low	V. Low
Weiderpass E., et al. [213]	2001	a, c, d, e, h	Finland	1971-1995	Cohort	-	Moderate	P. Low	Moderate
Shen Y., et al. [214]	2013	a, d	China	2010-2012	C.-Control	-	Moderate	P. High	Moderate
Donat-Vargas C., et al. [68]	2016	b	Sweden	1997-2012	Cohort	-	Moderate	D. High	Low
Lambertino A., et al. [215]	2011	b	USA	2001-2005	Cohort	Blood	Moderate	P. Low	High
Betenia N., et al. [216]	2012	c	USA	1985-2004	Cohort	-	Moderate	P. High	Low
Johnstone E.B., et al. [217]	2014	c	USA	-	Cohort	Blood	Moderate	P. Low	Low
McElroy J.A., et al. [218]	2017	c	USA	2010-2012	C.-Control	Urine	Low	D. Low	V. Low
Wernli K.J., et al. [219]	2008	c, e, h	China	1989-1991	N. C.-Cohort	-	Moderate	P. High	Moderate
Rashidi B.H., et al. [220]	2017	e	Iran	2013-2014	C.-Control	Urine	Low	D. Low	V. Low
Omoike O.E., et al. [112]	2021	f	USA	2005-2012	C.-Sectional	Blood	Moderate	P. Low	Moderate
Vieira V.M., et al. [114]	2013	f	USA	1996-2007	Cohort	Blood	Moderate	P. Low	Moderate
Kim Y.A., et al. [221]	2016	g	South Korea	2013-2015	C.-Control	Urine	Low	P. Low	Low
Wen H.J., et al. [222]	2020	k	Taiwan	2011-2014	C.-Control	Urine	Low	P. Low	Moderate

a pesticides, **b** polychlorinated biphenyls (PCBs), **c** heavy metals (HMs), **d** petroleum-based products (PBPs), **e** organic solvents & phenols (OSPs), **f** perfluorinated compounds (PFCs), **g** phthalates, **h** particulate matter (PM), **i** flame retardants (FRs), **j** dioxins, **k** alkylphenolic compounds (ApCs). **C.-Control** case control, **C.-Sectional** cross-sectional, **N. C.-Cohort** nested case-cohort, **N. C.-Control** nested case-control. **D. High** definitely high, **D. Low** definitely low, **P. High** probably high, **P. Low** probably low, **V. High** very high, **V. Low** very low.

Supplementary Table A9 – All records in Testis.

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Testis (continued)									
Bullman T.A., et al. [223]	1994	a	USA	1982-1991	C.-Control	-	Moderate	D. High	Moderate
Giannandrea F., et al. [224]	2011	a	Italy	2006-2008	C.-Control	Blood	Low	D. Low	Moderate
McGlynn K.A., et al. [225]	2008	a	USA	2002-2005	C.-Control	Blood	Low	D. Low	Low
Parron T., et al. [27]	2014	a	Spain	1998-2005	C.-Control	-	Low	P. Low	Low
Walschaerts M., et al. [226]	2007	a	France	2002-2005	C.-Control	-	Moderate	P. High	Low
Chia V.M., et al. [227]	2010	a, b	USA	2002-2005	C.-Control	Blood	Low	D. Low	Moderate
Paoli D., et al. [228]	2015	a, b, g, k	Italy	-	C.-Control	Blood	Moderate	D. Low	Moderate
Hardell L., et al. [229]	1998	a, d, e	Sweden	1989-1992	C.-Control	-	Moderate	P. High	Moderate
Bencko V., et al. [65]	2009	b	Slovakia	1987-1996	Ecologic	-	V. Low	D. High	V. Low
McGlynn K.A., et al. [230]	2009	b	USA	2002-2005	C.-Control	Blood	Low	D. Low	Moderate
Behrens T., et al. [231]	2012	c, d	Germany	1989-2006	N. C.-Control	-	Moderate	P. Low	Moderate
Hardell L., et al. [232]	1997	d, e, g	Sweden	1989-1992	C.-Control	-	Moderate	P. High	Moderate
Vieira V.M., et al. [114]	2013	f	USA	1996-2005	Cohort	Blood	Moderate	P. Low	Moderate
Hardell L., et al. [233]	2004	g	Sweden	1993-1997	C.-Control	-	Moderate	P. Low	Moderate
Westberg H.B.T., et al. [234]	2005	g	Sweden	1993–1997	C.-Control	-	Moderate	P. High	Low

a pesticides, **b** polychlorinated biphenyls (PCBs), **c** heavy metals (HMs), **d** petroleum-based products (PBPs), **e** organic solvents & phenols (OSPs), **f** perfluorinated compounds (PFCs), **g** phthalates, **k** alkylphenolic compounds (ApCs). **C.-Control** case control, **C.-Sectional** cross-sectional, **N. C.-Cohort** nested case-cohort, **N. C.-Control** nested case-control. **D. High** definitely high, **D. Low** definitely low, **P. High** probably high, **P. Low** probably low, **V. High** very high, **V. Low** very low.

Supplementary Table A10 – All records in Ovary.

Authors	Year	EDCs	Location (country)	Study period	Study design	Laboratory test	Initial confidence	Risk of bias	Confidence in the b. e.
Ovary									
Hopenhayn-Rich C., et al. [18]	2002	a	USA	1993-1997	Ecologic	Other	V. Low	P. Low	V. Low
Inoue-Choi M., et al. [235]	2016	a	USA	1986-2010	Cohort	-	Moderate	P. Low	Moderate
Lerro C.C., et al. [21]	2015	a	USA	1993-2011	Cohort	-	Moderate	D. High	Moderate
Parron T., et al. [27]	2014	a	Spain	1998-2005	C.-Control	-	Low	P. Low	Low
Salerno C., et al. [30]	2016	a	Italy	2002-2009	C.-Control	-	Moderate	D. High	Low
Young H.A., et al. [236]	2005	a	USA	2000-2001	C.-Control	-	Moderate	P. Low	Moderate
Ploteau S., et al. [237]	2017	a, b, i, j	France	2013-2015	C.-Control	Blood	Moderate	D. Low	Moderate
Bencko V., et al. [65]	2009	b	Slovakia	1987-1996	Ecologic	-	V. Low	D. High	V. Low
Donat-Vargas C., et al. [68]	2016	b	Sweden	1997-2012	Cohort	-	Moderate	D. High	Low
Wernli K.J., et al. [238]	2008	c, e, h	China	1989-1998	N. C.-Cohort	-	Moderate	P. High	Moderate
Guo J., et al. [239]	2004	d	Finland	1945-1995	Cohort	-	Moderate	D. High	Moderate
Shu X.O., et al. [240]	1989	d, e	China	1984-1986	C.-Control	-	Moderate	P. Low	High
Omoike O.E., et al. [112]	2021	f	USA	2005-2012	C.-Sectional	Blood	Moderate	P. Low	Moderate
Vieira V.M., et al. [114]	2013	f	USA	1996-2005	Cohort	Blood	Moderate	P. Low	Moderate

a pesticides, **b** polychlorinated biphenyls (PCBs), **c** heavy metals (HMs), **d** petroleum-based products (PBPs), **e** organic solvents & phenols (OSPs), **f** perfluorinated compounds (PFCs), **h** particulate matter (PM), **i** flame retardants (FRs), **j** dioxins. **C.-Control** case control, **C.-Sectional** cross-sectional, **N. C.-Cohort** nested case-cohort, **N. C.-Control** nested case-control. **D. High** definitely high, **D. Low** definitely low, **P. High** probably high, **P. Low** probably low, **V. High** very high, **V. Low** very low.

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V. Relevant dose of environmental contaminant, tributyltin, promotes histomorphological changes in the thyroid gland of male rats

In continuation of our efforts to comprehensively explore the impact of endocrine-disrupting chemicals (EDCs) on thyroid health, in collaboration with a Brazilian research group, we had the opportunity to extend our investigations to the endocrine disruptor, Tributyltin (TBT), a prevalent contaminant from antifouling paints in marine vessels, by means of an environmentally relevant exposure, to a mammalian model, the rat. This work contributed with insights for our understanding of thyroid-related disorders, hopefully guiding public health measures and future research on the implications of endocrine disruptors exposure for thyroid health.

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Relevant dose of the environmental contaminant, tributyltin, promotes histomorphological changes in the thyroid gland of male rats

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ABSTRACT

Organotin compounds, such as tributyltin (TBT), are common environmental contaminants and suspected endocrine-disrupting chemicals. Tributyltin is found in antifouling paints, widely used in ships and other vessels. The present study evaluated whether a 15-day treatment with TBT at a dose of 100 ng/kg/day could induce histomorphological changes in the thyroid gland of rats. TBT promoted relevant alterations in the thyroid architecture, being the most relevant histological findings the presence of increased number of small-size follicles in the treated group. In qualitative analyses, colloid vacuolization, papillary budging structures, cystic degeneration and chronic thyroiditis, were observed. Moreover, histomorphometric analysis showed statistically significant changes in the follicular architecture of TBT-treated rats, mainly a decrease in the follicle area (colloid) and an increased epithelial height that resulted in an increased epithelial height/colloid ratio. Augmented collagen deposition was also seen in the thyroids of treated groups. In immunohistochemical (IHC) analyses, the localization of NIS protein was described and a significant increased proliferation index (evaluated by Ki67 positive cells) in the treated group was reported. As an indirect measurement of oxidative stress, mitochondrial protein SDHA was also analyzed by IHC analysis. Although the cytoplasmic expression of SDHA was observed in both groups, the staining intensity score was higher in TBT-treated group. Our results suggest that besides causing histomorphological changes, environmental relevant dose of TBT treatment can also induce oxidative alterations.

1. Introduction

Organotin compounds (OT), as tributyltin (TBT), are prevalent contaminants that have been widely used as biocides, agriculture fungicides, wood preservatives and antifouling paints for marine vessels (Antizar-Ladislao, 2008; Castro et al., 2012). Studies evaluating the possible effects of the TBT environmental contamination in marine organisms started to emerge in the beginning of the 1970s, when toxic effects were reported in different animal models. Hence, studies were driven to better understand the actual impact of OT pollution on health and its environmental risk (Alzieu et al., 1991; Swennen et al., 2010). Despite the restrictions on the use of organotin compounds since the 1980s, high concentrations are still being found in coastal areas of harbors and shipyards, including the Brazilian coast (Santos et al., 2002; Delgado-Filho et al., 2011; Graceli et al., 2013; Bertuloso et al., 2015; Podratz et al., 2015).

TBT rapidly dissociates in water, forming different derivates, e.g. monobutyltin (MBT), dibutyltin (DBT) and tin. TBT half-life can vary depending on the place where it accumulates, reaching up to years in sediment (ECHA, 2008). Living organisms can easily assimilate all TBT

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derivates.

The exposure of humans to OTs can occur through consumption of contaminated fish, shellfish, water, use of biocidal products and atmospheric deposition (Godoi et al., 2003; Antizar-Ladislao, 2008; Silva et al., 2014). Antizar-Ladislao (2008) reviewed that humans can be indirectly exposed through consumption of contaminated animals in which TBT levels ranged from 0.47 to 1610 ng/g accordingly to the geographical location and type of the seafood analyzed.

It has been shown that TBT affects energetic homeostasis and hormones in mammals, such as leptin levels, and might be involved in body weight regulation. In 2018, Freitas-Lima et al. observed that serum leptin levels increased together with the body weight in rats treated with 100 ng/kg of TBT (Freitas-Lima et al., 2018). On the other hand, Bo et al. (2016) documented that the exposure to TBT (0.025 mg/kg/day) induced leptin serum levels reduction and did not promote body weight gain in mice. Moreover, TBT alters gene expression of thyrotropin-releasing hormone (TRH) and/or melanocortin 4 receptor (MC4r) which plays a key role in the food supply (Decherf et al., 2010). Additionally, it has been demonstrated that TBT interacts with RXR/PPAR receptor isoforms involved in the regulation of hypothalamic TRH (Laflamme et al., 2002; Kouidhi et al., 2010). In mice exposed to a single dose of TBT (10 mg/kg), the compound seemed to act on the hypothalamic arcuate nucleus by activating c-fos in both fed and fasted states, suggesting an imbalance in neuroendocrine mechanisms of controlling food intake (Bo et al., 2011). Huang et al. (2018) reported the fact that oxidative stress may have an effect on glucose homeostasis and in pancreatic beta cell apoptosis in low doses of TBT in mice. Coutinho et al. (2016) demonstrated that TBT exposure promotes renal dysfunction through inflammation and oxidative stress in female rats.

The possible effects of TBT on oxidative stress have also been explored in previous studies. Oxidative stress is defined as the imbalance between cellular oxidant and antioxidant systems (Giorgio et al., 2007; Jones, 2008). The redox imbalance has been associated with histological alterations such as cytoplasmic vacuolization in liver, apparently swollen glomeruli in kidneys and loss of mucosal epithelial lining in lungs (Mitra et al., 2014). These structural changes were confirmed by increased semiquantitative scores of tissue damage in the group of adult rats that were exposed to TBT for one month (1 and 5 mg/kg) (Mitra et al., 2014).

The action of endocrine disruptions (EDs) spans many endocrine axes, including the hypothalamic-pituitary-thyroid (HPT) axis. Recent reports demonstrated that EDs are capable to affect the HPT axis in TBT-treated female rats by altering TRH mRNA levels, as pituitary thyroid-stimulating hormone (TSH), iodothyronine deiodinases 1 (Dio1) and 2 (Dio2) mRNA levels remained unchanged (Andrade et al., 2018). EDs can additionally disrupt the HPT axis by altering plasma levels of T3, T4 and TSH, thus affecting thyroid axis homeostasis (Pandey and Mohanty, 2015; Andrade et al., 2018).

Our group has shown that TBT can be considered a thyroid disruptor in mammals at environmental relevant concentrations (Rodrigues-Pereira et al., 2015; Andrade et al., 2018). Environmentally relevant dose of TBT (daily dose of 1000 ng/kg, 15 days) was able to change thyroid morphology and serum T4 levels of male rats. This exposure also increased the activity and expression of dual oxidase (DUOX) and induced the production of hydrogen peroxide (H_2O_2). The H_2O_2 is important for thyroid hormonal biosynthesis, but since H_2O_2 is a reactive oxygen species (ROS), its increase could lead to an imbalance of redox homeostasis, thus predisposing the gland to disease (Rodrigues-Pereira et al., 2015). Up to date, there are only few works describing the histopathological findings in thyroid of animal models exposed to TBT in the literature (Zhang et al., 2009; Coutinho et al., 2016; El Dine et al., 2017; Yan et al., 2018; Andrade et al., 2018). We demonstrated that female rats treated with 200 and 1000 ng/kg of TBT for 40 days exhibited an abnormal morphology of the thyroid gland, characterized by size differences in thyroid follicles with epithelial stratification and vacuolization, besides the colloidal eosinophilia and vascular congestion (Andrade et al., 2018). El Dine et al. (2017) also reported a significant reduction of the mean colloid area of the thyroid follicles, colloid vacuolization, swelling and vacuolization of thyroid follicular cells, and anucleated thyrocytes in rat thyroids exposed to 5 mg/kg of TBT during 30 days.

More recently, the aforementioned effects were also reported at lower levels of TBT (100 ng/kg) in female rats (Podratz et al., 2012; Merlo et al., 2016; Coutinho et al., 2016; Sena et al., 2017; de Araújo et al., 2018). Since thyroid gland is known to be highly susceptible to the effects of EDs, we decided to evaluate the putative histomorphological and oxidative stress outcome in this organ through subacute exposure of 100 ng/kg of TBT, a low dose within the wide range of environmental detected concentrations. Moreover, we want to not only show the changes in the architecture of the thyroid gland but also try to elucidate which molecules could be involved in this process by performing, for the first time, the immunohistochemically evaluation of Ki67, NIS and SDHA, in rat thyroid's.

2. Materials and methods

2.1. Animals and procedures

In the present study, we used a total of 36 adult male Wistar rats, weighing 230–250 g, eight weeks old, and exposed them to TBT or vehicle during a 15-day period. All animals were housed in pairs under controlled conditions of light/dark (12/12 h) and temperature (22 °C). The study was revised and approved by the Ethics Committee on the Use of Animals in Research of the Federal University of Rio de Janeiro (CEUA-UFRJ) (Code: DAHEICB082). The procedures were carried out in compliance with the International Guiding Principles for Biomedical Research Involving Animals of the Council for International Organizations of Medical Sciences (Geneva, Switzerland) and the guiding principles for care and use of animals from the American Veterinary Medical Association.

TBT chloride (96% of purity, code T50202-100G) was purchased from Sigma-Aldrich S.A., San Diego, USA. Animals were randomly assigned into two groups: TBT-treated group (100 ng TBT/kg, diluted in 0.4% ethanol) (n = 20) and control group (vehicle only, 0.4% ethanol) (n = 16). To carry out this study, three independent experiments were performed (total = 36 animals): experiment 1 = 16 animals (8 treated, 8 controls), experiment 2 = 10 animals (6 treated, 4 controls), experiment 3 = 10 animals (6 treated, 4 controls). The TBT dose was chosen based on previous studies (Godoi et al., 2003; Sant'Anna et al., 2014; Merlo et al., 2016; Coutinho et al., 2016; Sena et al., 2017; de Araújo et al., 2018) and the route of administration was oral gavage, which was performed daily, at the morning period. The food was daily weighted and replaced in order to always have available a total of 50 g/animal of the commercial chow diet (Teklad Global 14% Protein Rodent Maintenance Diet, Autoclavable, 214S, Envigo).

The body weight was recorded at the start and at the end of the treatment and the food intake per animal was documented daily. Behavioral and other possible clinical signs were also monitored every day.

2.2. Euthanasia and organ collection

Rats were humanely euthanized by exsanguination, under deep anesthesia with isoflurane, followed by intracardiac injection of sodic pentobarbital (270 mg/kg) to confirm death. Both thyroid glands were collected and immediately fixed in 10% neutral buffered formalin.

2.3. Histopathological evaluation

Thyroid glands were processed and included in paraffin blocks. Both thyroid lobes were included together in the same block and evaluated as a unit. Sectioning was done at 4 μm.

Table 1
Definitions of the histopathological parameters in rat thyroid gland evaluation.

Zonal variation	Existence of larger follicles with squamous to low cuboidal epithelium full of colloid in the periphery of the gland, accompanied by smaller follicles with cuboidal epithelium in the gland.
Thyrocytes cytoplasmic vacuolization	Random microvacuoles of different sizes inside of thyrocyte \cytoplasm.
Scalloping and random colloid vacuolization	Vacuolization of intraluminal colloid mainly localized along the border of the follicle lumen is defined as "scalloping colloid vacuolization", whereas random vacuolization of intraluminal colloid is refer as "random colloid vacuolization".
Papillary budging	Presence of small proliferations of thyrocytes into the follicle lumen.
Cystic degeneration	Presence of histiocytes accompanied with regenerative-degenerative changes of the thyrocytes.
Chronic thyroiditis foci	Focal lymphocytic infiltrate with a minimal follicular damage.

The thyroid slides stained for hematoxylin and eosin (H&E) were evaluated by a pathologist unaware of the given treatment. The following parameters were considered: (1) loss of zonal variation, (2) thyrocytes cytoplasmic vacuolization, (3) scalloping and random colloid vacuolization, (4) papillary budging, (5) cystic degeneration and (6) chronic thyroiditis. All parameters were evaluated as absence of the alteration (less than 10% altered), low-grade lesion (10–50% altered) and-high-grade lesion (more than 50% altered), with the exception of thyrocytes vacuolization that was differently evaluated due to the physiological presence of vacuolization throughout the rat thyroid (low/normal vacuolization– less than 30%, moderate vacuolization level– 30 to 60%, and high vacuolization level– more than 60% of the total thyroid tissue).

The definitions of the aforementioned histopathological parameters can be checked below, in Table 1.

2.4. Histomorphometric analysis

The histomorphometric analysis of the thyroid glands was performed on Periodic Acid Schiff (PAS) stained slides. The images were obtained using Carl Zeiss Axio Observer microscope equipped with Zen 2 software. The analysis was done in 2 images/slide/animal at 200x of magnification, using the ImageJ digital software (National Institutes of Health, USA). The parameters evaluated were the following: (1) epithelial height, (2) epithelial area, (3) follicular area, (4) colloid area, (5) epithelial/colloid ratio and (6) mean number of follicles per lobe (the number of follicles/image/group was manually counted). The formulas were applied as previously reported (Capen, 1998; Wade et al., 2002; Bianco et al., 2014; Rodrigues-Pereira et al., 2015; Andrade et al., 2018). All the histomorphological parameters were evaluated particularly in the central area of the thyroid lobe. Thyroid periphery in rats exhibits capsule, major connective septa and vessels, which creates complexity and subjectivity for the evaluated parameters. For these reasons, these peripheric areas were excluded from the analysis. The results obtained were expressed in percentage of marked area. All analyses were blindly performed regarding the treatment state. PAS staining was used to circumscribe the thyroid basal and apical membrane of the follicular epithelium to enable the histomorphometric analysis using ImageJ software.

2.5. Collagen fibers quantification

Thyroid sections from control and treated groups were stained with Masson's trichrome for the measurement of collagen fibers deposition, as an indicator of fibrosis.

For this quantification, we analyzed 4–5 images/animal at 200x magnification, which were also restricted to the central area of the thyroid lobe in order to avoid the marked areas of the capsule and major connective septa. These analyzes were performed in the ImageJ software. The results obtained were expressed as percentage of marked area. All analyses were performed in a blindly manner regarding the treatment state.

2.6. Immunohistochemistry in thyroid gland

Immunohistochemistry for sodium-iodide symporter (NIS) was performed as previously described by our group (Tavares et al., 2018). Briefly, sections were subjected to heat-induced antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% of H_2O_2 and nonspecific binding with Large Volume Ultra V Block reagent (TA-125-PBQ, Thermo Scientific). Sections were then incubated overnight at 4 °C with anti-NIS pAb 795 IgG (20 μg/ml) (kindly supplied by Dr. Selmi-Ruby) (Trouttet-Masson et al., 2004). A biotinylated goat polyvalent secondary antibody was used (Thermo Scientific, TP-125-BN). Detection was performed with a ready-to-use horseradish peroxidase streptavidin detection system (Vector, SA-5704). NIS quantification was done by a pathologist blinded to the treatment state and both lobes for all the animals were analyzed (200x magnification). Each lobe was divided in several areas and each area was considered as positive or negative for NIS staining regarding the presence or absence of positive foci. In order to obtain a percentage of staining, we applied the reason of positive areas in the total of areas observed per lobe and we divided into three groups of different grades of positivity (< 10% positivity, 10–40% positivity and > 40% positivity). For this analysis, only membranous NIS expression was considered.

For the proliferation marker Ki67, antigen retrieval was performed with citrate buffer pH 6.0 (Thermo Scientific, TA-050-CBX). Endogenous peroxidase activity was blocked with 3% of H_2O_2 and a blockage with normal swine serum diluted at 1:5 (Dako, X0901) was performed. The dilution used for the rabbit monoclonal primary antibody anti-Ki67 (Sigma SAB, 5500134) was 1:100. Sections were incubated overnight at 4 °C. A biotinylated swine anti-rabbit secondary antibody diluted at 1:100 (Dako, E0353) was used. Detection was done by an avidin-biotin complex ABC Kit (Vectastain PK-6100).

Six images/slide/animal were analyzed (200x magnification). To obtain the percentage of staining, we applied the ratio of cells marked by total cells per area.

For succinate dehydrogenase complex, subunit A (SDHA) detection, the sections were subjected to heat-induced antigen retrieval EDTA buffer pH 9.0 (RE7119-CE, Novocastra). Endogenous peroxidase activity was blocked with Ultra Vision Hydrogen peroxide block (TA-060-H202Q, Thermo scientific) and nonspecific binding with the Ultra Vision Protein Block reagent (TA-060-PBQ, Thermo Scientific). Sections were then incubated for 1 h at room temperature with the mouse monoclonal antibody anti-SDHA (MS204, Abcam), diluted at 1:1250. SDHA intensity in the cytoplasm of the thyrocytes was scored from 1 to 3 (1 – low, 2 – mild, and 3 – strong intensity).

For all the antibodies, chromogenic detection was performed with 3,3′-diaminobenzidine (Dako, K3468) reaction and counterstained with Mayers' hematoxylin.

2.7. Statistical analysis

Data obtained regarding body weight, food intake, thyroid morphometry, collagen fibers quantification and immunohistochemistry results were expressed as mean ± standard deviation of three independent experiments and statistical comparisons were carried out by

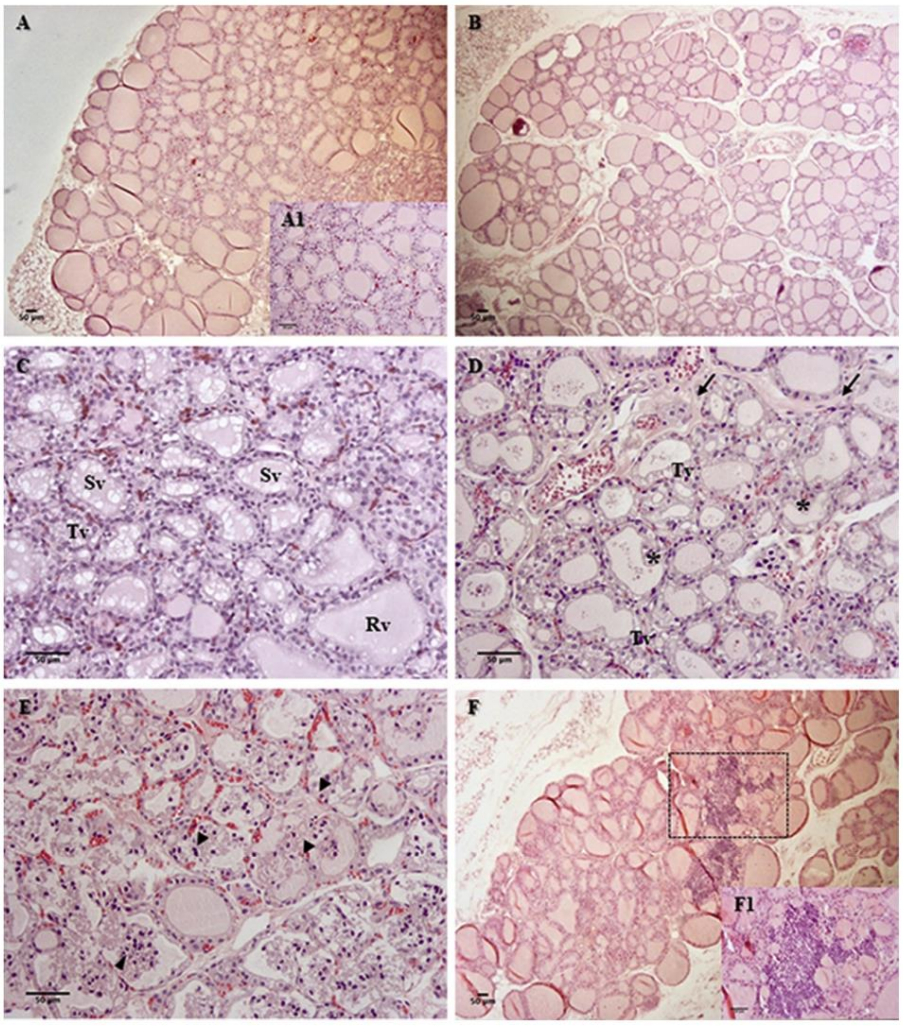

Fig. 1. Histopathological analysis. **A** – normal histology of Wistar-Han rat thyroid (control group) (200x), with preserved zonal variation **(A1)** (40x). **B** – loss of zonal variation (treated group) (40x). **C** –presence of scalloping (**Sv**) and random (**Rv**) colloid vacuolization, thyrocytes cytoplasmic vacuolization (**Tv**) (treated group) (200x). **D** – papillary budging (✻), fibrosis (**arrows**) and thyrocytes cytoplasmic vacuolization (**Tv**) (200x). **E** – cystic degeneration (**triangles**) (200x). **F** – chronic thyroiditis focus, with large lymphocytes infiltrate (40x), with higher magnification (**F1**, 200x).

chi-square test, unpaired *t*-test and Fisher test. All statistical analyses were performed using GraphPad Prism 6.0 and SPSS Statistics 25. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Body weight and food intake

During the 15 days of the TBT experiment, animals were monitored on a daily basis regarding food intake and weight. No significant differences were detected in both parameters between the control and the TBT group (data not shown).

3.2. Histopathological evaluation

The histopathological analysis of the control group demonstrated thyroid follicles composed of cuboidal thyrocytes that palisades the follicle lumen full of eosinophilic material/colloid in the background of well-vascularized stroma (Fig. 1A). In the control group we observed a preservation of zonal variation (Table 1; Fig. 1A1), that contrasts with a trend towards disruption of this pattern, termed loss of zonal variation, meaning that the size of the follicles is similar in all regions of the gland

Table 2
Frequency distribution table according to scoring (histopathological evaluation).

	Scoring level			p-values
Parameter	0	1	2	
Loss of zonation				
Control (n = 16)	62.5 (n = 10)	25.0 (n = 4)	12.5 (n = 2)	0.985[a]
Treated (n = 17)	64.7 (n = 11)	29.4 (n = 5)	5.9 (n = 1)	0.596[b]
				0.500[c]
				0.500[d]
Papillary budging				
Control (n = 16)	68.8 (n = 11)	12.5 (n = 2)	18.8 (n = 3)	0.299[a]
Treated (n = 17)	64.7 (n = 11)	35.3 (n = 6)	0	0.212[b]
				0.158[c]
				0.061[d]
Thyrocytes vacuolization				
Control (n = 16)	6.3 (n = 1)	50.0 (n = 8)	43.8 (n = 7)	0.367[a]
Treated (n = 17)	5.9 (n = 1)	23.5 (n = 4)	70.6 (n = 12)	0.604[b]
				0.629[c]
				0.106[d]
Random colloid vacuolization				
Control (n = 16)	43.8 (n = 7)	37.5 (n = 6)	18.8 (n = 3)	0.553[a]
Treated (n = 17)	29.4 (n = 5)	64.7 (n = 11)	5.9 (n = 1)	0.198[b]
				0.511[c]
				0.189[d]
Scalloping colloid vacuolization				
Control (n = 16)	93.8 (n = 15)	6.3 (n = 1)	0	0.985[a]
Treated (n = 17)	94.1 (n = 16)	0	5.9 (n = 1)	0.500[b]
				0.531[c]
				0.500[d]
Central epithelial atrophy				
Control (n = 14)	28.6 (n = 4)	57.1 (n = 8)	14.3 (n = 2)	0.686[a]
Treated (n = 17)	52.9 (n = 9)	41.2 (n = 7)	5.9 (n = 1)	0.207[b]
				0.304[c]
				0.588[d]
Central cystic degeneration				
Control (n = 14)	35.7 (n = 5)	28.6 (n = 4)	35.7 (n = 5)	0.541[a]
Treated (n = 17)	52.9 (n = 9)	35.3 (n = 6)	11.8 (n = 2)	0.582[b]
				0.140[c]
				0.218[d]
Oncocytic appearance				
Control (n = 16)	68.8 (n = 11)	31.2 (n = 5)		0.603[b]
Treated (n = 17)	70.6 (n = 12)	29.4 (n = 5)		
Thyroiditis				
Control (n = 16)	100 (n = 16)	0		0.258[b]
Treated (n = 17)	88.2 (n = 15)	11.8 (n = 2)		

[a] p-value obtained from chi-square test incorporating Yates' correction for continuity (http://www.quantpsy.org/chisq/chisq.htm).
[b] p-value obtained from a Fisher exact test between scores 0 and 1.
[c] p-value obtained from a Fisher exact test between scores 0 and 2.
[d] p-value obtained from a Fisher exact test between scores 1 and 2.

(Fig. 1B).

We observed that the treated group showed a tendency to thyrocyte cytoplasmic vacuolization (Fig. 1C and D). Yet, the presence of scalloping and random colloid vacuolization (Fig. 1C), papillary budging structures (Fig. 1D) and cystic degeneration (Fig. 1E) was verified both in control and treated groups.

The presence of interstitial fibrosis (Fig. 1D) was more evident in treated rats. Finally, chronic thyroiditis was only present in two TBT-treated rats, with large lymphocytes infiltrate (Fig. 1F).

A summary of the histopathological features in each group and the corresponding statistical evaluation is present in Table 2.

3.3. Histomorphometric evaluation

We found a significant decrease in the follicle area in the treatment group in comparison to control group ($p < 0.0001$) (Fig. 2A), shadowed by a correspondent decrease in the colloid area (not statistically significant, $p = 0.234$) (Fig. 2B). We also detected a significant increase in the epithelial height ($p = 0.0323$) (Fig. 2D) in the treated group, that results in increased epithelial/colloid ratio ($p = 0.0303$) (Fig. 2E), despite the epithelial area has remained similar among groups (Fig. 2C). Furthermore, the TBT-treated group presented a greater increase in the mean number of follicles in comparison with controls ($p < 0.0001$) (Fig. 2F).

3.4. Collagen deposition

The interfollicular connective stroma of the treated rats exhibited a significant higher deposition of connective tissue material in comparison with the control group ($p = 0.0117$) (Fig. 3A and B), supporting the result found in the histopathological evaluation. Collagen fibers quantification was made in the central area of the thyroid and the percentage of marked area was determined (Fig. 3C).

3.5. Expression of Ki67 in thyroid follicular epithelial cells

To evaluate cell proliferation, immunohistochemical staining for the proliferative marker Ki67 was performed. Representative images of Ki67 expression are present in Fig. 4. We observed a significant higher percentage of Ki67 positive cells in the treated group ($p = 0.022$), in comparison with controls.

3.6. Expression of NIS in thyroid follicular epithelial cells

The distribution of NIS in follicular thyroid cells of rat is depicted in Fig. 5A and B. Thyrocytes typically exhibited plasma membrane immunoreactivity in both groups (Fig. 5A and B) with a focal pattern. As described for human thyroid, NIS membrane immunoreactivity was heterogeneous, whit positive thyrocytes side-to-side to negative thyrocytes, as observed in Fig. 5. Cytoplasmic expression was also noted in both groups with a diffuse immunoreactive signal on perinuclear region. Hence, we observed higher NIS expression in the treated group (27%) in comparison with controls (21%) but no statistical significance was found (Fig. 5C).

3.7. Expression of SDHA in the thyroid gland

SDHA expression was cytoplasmic and granular displaying a higher staining intensity in the adipose tissue and muscle (positive controls, Fig. 6A and B, respectively). The same expression pattern of SDHA was found in the thyroid for all cases, controls and treated (Fig. 6F). Albeit not statistically significant, 40% of TBT-treated animals presented high intensity in staining (score 3) for SDHA, whereas only 25% of the controls presented the same score.

4. Discussion

Over the last decades, a dramatic increase in hormonal disorders has been reported, presumably linked, at least in part, to a growing exposure to endocrine disrupting chemicals (EDCs) (Braun et al., 2009; Hatch et al., 2010). Several studies using different experimental models have shown that EDCs are responsible for a wide range of deleterious effects on human health (U.S. EPA, 2003). The current study focused on the consequences of subacute exposure (15 days) to low dose of TBT (100 ng/kg/day) in the thyroid histopathology of male rats. TBT is an endocrine disruptor, known to disrupt thyroid gland in different species like rats, amphibians and fish (Zhang et al., 2009; Shi et al., 2012; El Dine et al., 2017). Here we demonstrate that TBT is able to alter thyroid histomorphology of male rats at 100 ng/kg/day.

TBT levels already reported to be found in the environment were 10 and 79 ng/g in fish (Shue et al., 2014), 1,57 and —to 50 μg/kg in male mice (Xu et al., 2019). Moreover, the World Health Organization (WHO) considers tolerable the intake of 15 μg of TBT per day and the European Food Health Authority 250 ng/kg. According to studies

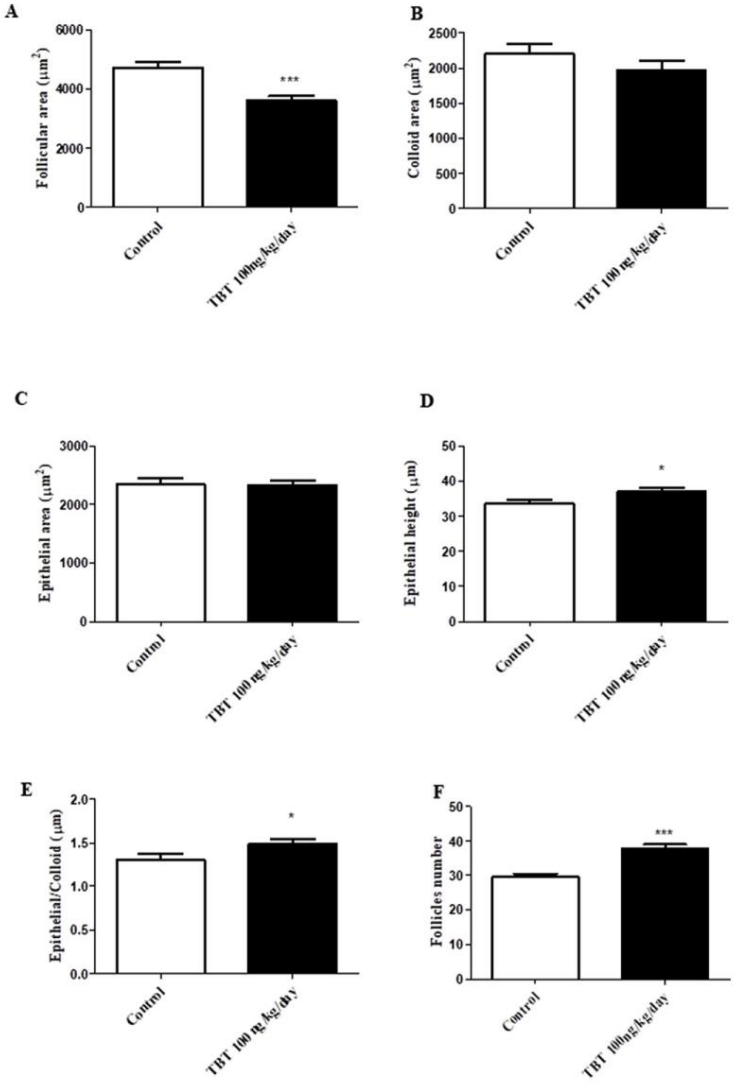

Fig. 2. Histomorphometric analysis. Control (white column, n = 16) and TBT 100 ng/kg/day (black column, n = 20); **A** – follicular area; **B** – colloid area; **C** – epithelial area; **D** – epithelial height; **E** – epithelial/colloid area ratio; **F** – follicles numbers. Unpaired *t*-test ****p < 0.0001 vs control; *p < 0.05 vs control.

conducted in Salvador and Guanabara Bay (Rio de Janeiro), Brazil, if a person weighting 70 kg consumes 150 g of mussel every day, he/she will be theoretically at risk (Fernadez et al., 2005; Artifon et al., 2016). Therefore, the dose used in our study is in the range of the doses estimated for human exposure and within the range of TBT found in body tissues of marine organisms. Therefore, we used a subacute exposure with a low dose of TBT.

Our results show increased fibrosis and a change in the architecture (zonal variation) of the gland in TBT-treated group. These effects were accompanied by a tendency to an increase in the thyrocytes vacuolization. This disorganization in the thyroid gland can contribute to loss of thyroid homeostasis (Rodrigues-Pereira et al., 2015; Rajab et al., 2017). Similar findings have also been previously reported in other mammals and vertebrates. For example, the thyroid gland of *Sebasticus marmuratus* showed a decrease in follicle numbers accompanied by a reduction in the concentration of 10 and 100 ng/L TBT. The same result was seen in the amphibian thyroid gland of the species *Xenopus laevis* at a concentration of 200 ng/L TBT over a 19-day exposure (Zhang et al.,

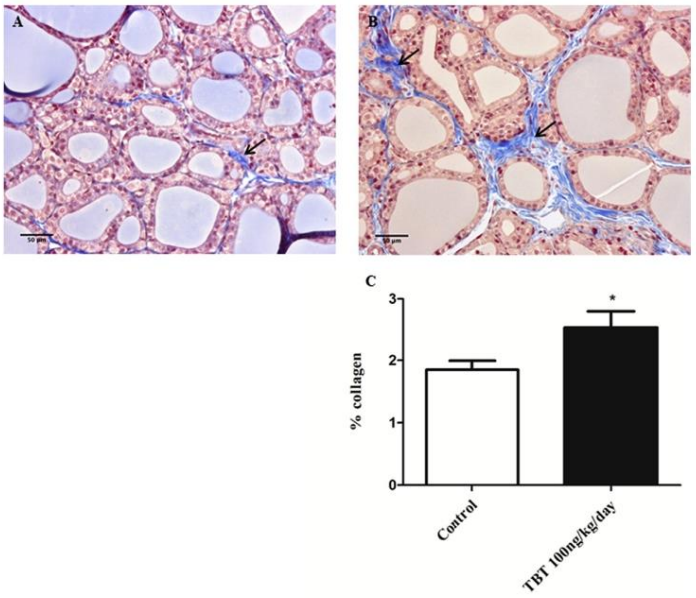

Fig. 3. Analysis of collagen deposition (Masson's trichrome-stained slides). **A** – control (n = 16); **B** – TBT 100 ng/kg/day (n = 20), presence of fibrosis (**arrows**). Unpaired *t*-test *p < 0.05 vs control.

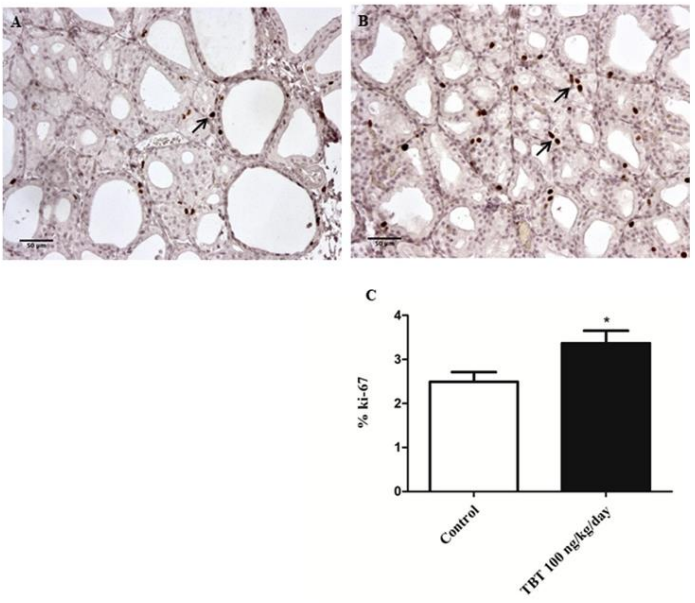

Fig. 4. Ki67 immunohistochemistry analysis. **A** – control (n = 15) and **B** – TBT 100 ng/kg/day (n = 19), Ki67-positive cells are indicated by the arrows. Unpaired *t*-test p < 0.05* vs control. Magnification 200x.

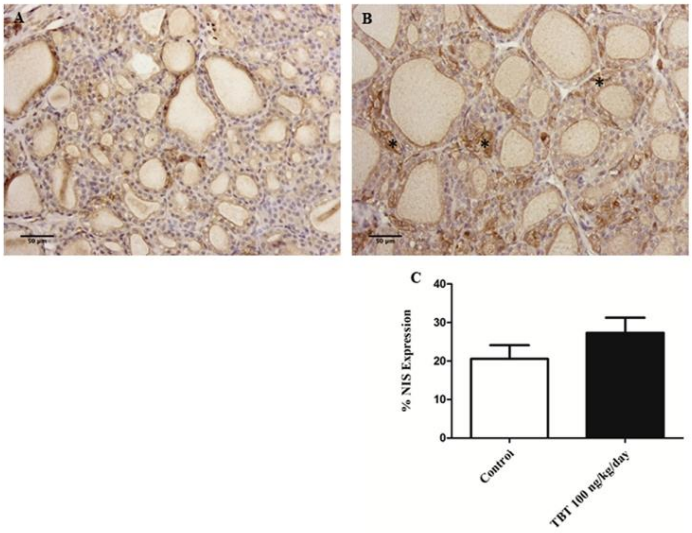

Fig. 5. NIS immunohistochemistry analysis. **A** – control (n = 15) and **B** – TBT 100 ng/kg/day (n = 18). Membranous NIS expression is indicated by the arrows Unpaired *t*-test $p > 0.05$ vs control. Magnification 200x.

2009; Shi et al., 2012). In zebrafish, using another endocrine disruptor, at 10 weeks of exposure, perchlorate at both concentrations (10 and 100 mg/L) caused thyroidal hypertrophy and colloid depletion (Mukhi and Patiño, 2007), changes that are associated with thyroid disease.

TBT was able to induce histomorphological alterations in the thyroid namely an increase in the number of small-sized follicles ($p < 0.001$), reduction of the follicular area ($p < 0.0001$) with increased epithelial height ($p = 0.0323$) and increased epithelium/colloid ratio ($p = 0.0303$). These findings suggest that TBT induced follicular proliferation of the thyroid.

Follicular epithelial height is associated with thyroid function in mammals: progression from squamous and low cuboidal to cuboidal and columnar epithelium is associated with functionally active follicles (Bacha and Bacha, 2012). Our results suggest that 100 ng of TBT/kg/15 days is able to induce follicular proliferation along with thyrocyte cytoplasmic vacuolization, and could reflect an increase in thyroid function. To analyse the degree of proliferation in the thyroid gland, we performed Ki67 immunohistochemistry and the proliferation index was found to be significantly higher in TBT-treated rats than in control animals.

Most findings are in accordance with a previous work from our group that reported a hypertrophic and hyperplastic epithelium and increased frequency of small follicles in animals exposed to higher doses of TBT (1000 ng/kg) for 40 days (Andrade et al., 2018). However, Andrade and collaborators also observed, a significant increase in vacuolization in colloid (Andrade et al., 2018), which was not observed in the present study, possibly due to differences in time of exposure (subacute) and dose of TBT.

Previous studies showed that TBT has the ability to alter thyroid hormonal biosynthesis, affecting its morphology and the HPT axis (Rodrigues-Pereira et al., 2015; Santos-Silva et al., 2018; Andrade et al., 2018). These alterations are accompanied by changes of TSH, THs serum and receptor hormone (TRH) and DIO1 mRNA levels of the thyroid (Rodrigues-Pereira et al., 2015; Santos-Silva et al., 2018; Andrade et al., 2018). THs are produced by the thyroid in response to TSH stimulation in a process that involves the active transport of iodide into follicular cells, mediated by the Na^+/I^-symporter (NIS) (Yen, 2001). The functional NIS protein is found in the basolateral plasma membrane of the thyroid follicular cells and its expression in the normal thyroid is relatively low and heterogeneous (Tavares et al., 2018). In the present study we performed IHC analysis on NIS protein and, to the best of our knowledge this is the first immunohistochemical evaluation of NIS in rat thyroid gland. Just like in humans (Tavares et al., 2018), we observed, both in controls and TBT-treated groups, an intense staining in the basolateral membrane of the thyrocytes but with a heterogeneous tissue distribution. We did not observe a significant difference in NIS expression in the TBT-treated group in comparison with controls ($p > 0.05$) after a 15 days treatment, probably due to the short time of exposure to TBT. It was previously shown that TBT is capable of altering both thyroid gland architecture, particularly follicular cells, and the HPT axis which are important for thyroid hormone biosynthesis (Andrade et al., 2018). We also know that proteins, such as thyroglobulin and thyroperoxidase, and molecules, such as iodine, are key for the integrity and metabolic homeostasis of the gland, and that TBT can interfere with them causing metabolic imbalance (Decherf et al., 2010; Rodrigues-Pereira et al., 2015). Our hypothesis for the absence of significant changes in NIS expression in our study is that our exposure time is too short for major molecular changes that could impact NIS expression.

We observed several morphologic alterations in the thyroids of TBT-treated animals that could be indicative of oxidative damage. It is known that ROS can be produced during normal thyrocyte function and can increase after exposure to environmental contaminants like TBT (Ishihara et al., 2012). As demonstrated by Li and collaborators, carp (Cyprinus carpio) after 7 day exposure to TBT showed increased ROS levels due to a significant inhibition of antioxidant enzyme activity as well as Na/K ATPase activity (Lee et al., 2016). Increased oxidative stress due to the overproduction of reactive oxygen species (ROS) may be related to increased collagen synthesis (Tsukamoto et al., 1995; Poli and Parola, 1997) and/or intense interfollicular collagen deposition, namely fibrosis, as well as vacuolization of follicular cells (Wade et al., 2002). Interestingly, increased collagen amount (fibrosis) was observed

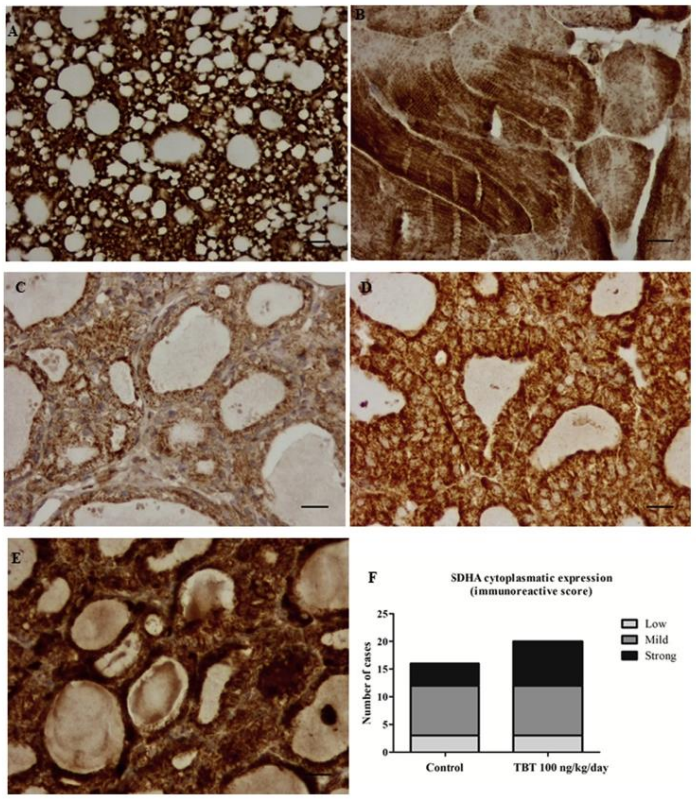

Fig. 6. SDHA immunohistochemistry analysis. Control (n = 16) and TBT 100 ng/kg/day (n = 20). **A** – positive control (adipose tissue); **B** – positive control (muscle). C-E representative images from different animals showing the three patterns of SDHA staining observed; **C** – score 1, low intensity, **D** – score 2, mild intensity, **E** – score 3, strong intensity. **F** – Comparison of the positive immunostaining in both groups. Fisher test $p > 0.05$ vs control. Magnification 400x.

in the interfollicular connective stroma in the TBT-treated group. Moreover, previous work from our group demonstrated that male rats treated for 15 days with TBT at 1000 ng/kg showed increased expression of thyroid DUOX calcium-dependent H_2O_2 generating activity protein. Therefore, the increase collagen deposition into the gland could be due to a higher expression and/or activity of the NADPH oxidases in the presence of TBT (Rodrigues-Pereira et al., 2015). Similar results were reported by others (Ximenes et al., 2017; Rodrigues et al., 2014). Curiously, thyrocytes with large, slightly granular and eosinophilic cytoplasms were noticed in the treated and control groups that resembled oncocytes/oxyphilic cells (mitochondrion-rich cells). The increase of mitochondrial mass due to effect of oxidative stress was already described in different models (Wallace, 1999; Lee et al., 2002). It has also been shown that TBT has a significant effect on mitochondrial biogenesis, inhibiting pro-fusion proteins, namely mitofusin 1 (Mfn1), leading to mitochondria fragmentation (Yamada et al., 2016). To confirm this phenotype, and the higher number of mitochondria, SDHA immunostaining was used. Despite the stronger cytoplasmic staining in the TBT-treated group, there was no statistical significance between the two groups. This could be due to the short-term exposure assay (15 days), that could not be enough for the fully development of the oncocytic phenotype detected at the histopathologic level.

Ultimately, the presence of foci of chronic thyroiditis that we have detected in two TBT treated animals, might have been triggered by the induced stress of TBT in the treated rats. To our knowledge, no other study reported such alteration in TBT treated animals, but our figures are too small to allow definitive assumptions.

In the present study we performed a morphologic and immunohistochemical characterization of short-term TBT-treated rats at an environmental relevant dose. The most relevant findings concern the thyroid morphologic changes suggestive of oxidative stress. Additional studies are necessary to disclose the putative association between TBT exposition and oxidative stress.

This study underlines the TBT effects in histomorphological and functional levels of thyroid gland even under short-term and low doses exposure of TBT. These results may suggest that daily human TBT exposure allowed by WHO should be reconsidered.

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Declaration of competing interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartially of this manuscript.

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VI. TP53 is required for transformation of BRAF^{V600E}-expressing thyroid cells in zebrafish

Prior to my engagement in the project and in the course of previous investigation in my group into the role of the B-type Raf kinase (BRAF) V600E mutation, usually frequent in papillary thyroid carcinomas (PTCs), it was initiated the generation and characterization of a transgenic zebrafish, with targeted expression of BRAF^{V600E} mutation specifically in thyroid cells under the control of the thyroglobulin promoter (*tg*).

This animal model appeared to us as an interesting tool for the investigation of the effects of endocrine disruptors in thyroid, but further characterization of the animal model was needed. My focused contribution to the study involved a thorough characterization at the 3 months post-fertilization time point, elucidating critical insights into the early steps of BRAF^{V600E} tumourigenesis, within the context of p53 mutation. The analysis of young animals revealed abnormal thyroid morphogenesis and hyperplasia upon BRAF^{V600E} induction, while as adults, they manifested goiter without clear evidence of neoplasia. This model will be used in the ongoing studies reported in Article VII.

TP53 is required for transformation of BRAF^{V600E}-expressing thyroid cells in zebrafish

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ABSTRACT

The B-type Raf kinase (BRAF) V600E mutation is the most common genetic alteration in papillary thyroid carcinomas (PTCs) and is found in 29%-83% of the cases.

$BRAF^{V600E}$ mutations are often present in more advanced stages of the disease such as in anaplastic carcinomas arising from PTCs. $BRAF^{V600E}$ kinase activates the mitogen-activated protein kinases (MAPK) pathway, promoting cellular processes such as proliferation, survival, motility, and invasion.

We generated a transgenic zebrafish model to study the role of $BRAF^{V600E}$ in thyroid cancer pathogenesis. We targeted $BRAF^{V600E}$ expression to thyroid cells under the control of the zebrafish thyroglobulin promoter (*tg*). Expression of $BRAF^{V600E}$ of young animals resulted in abnormal thyroid morphogenesis and hyperplasia. As adults however, they develop goiter but no clear neoplasia. Additionally, growth impairment was observed from 3 months onwards.

To overcome a possible p53-imposed block to the progression of cancer, we targeted $BRAF^{V600E}$ expression to thyroid cells of $tp53^{M214K}$ mutant zebrafish. Surprisingly, $BRAF^{V600E}$-expressing $tp53^{M214K}$ fish developed normally without thyroid abnormalities until adulthood. Loss of p53 function may constitute a late event in thyroid cancer, while its function is still required in early steps of $BRAF^{V600E}$ tumourigenesis.

Introduction

BRAF serine-threonine kinase belongs to the RAF family together with A-Raf Proto-Oncogene, Serine/Threonine Kinase (ARAF) and C-Raf Proto-Oncogene, Serine/Threonine Kinase (CRAF). BRAF is an intracellular effector mediating the response to external growth factors through the Mitogen-Activated Protein Kinases (MAPK) pathway that controls cell proliferation and differentiation (McKay & Morrison, 2007). Amongst all the RAF proteins, BRAF has the highest basal kinase activity, and it is the most potent activator of the MAPK signalling pathway (Marais et al., 1997).

Up to 90% of the *BRAF* gene mutations consist of a single base change converting valine to glutamic acid at position 600 (V600E) (Frasca et al., 2008; Garnett & Marais, 2004). This mutation constitutes an activating mutation in the kinase domain. In sporadic papillary thyroid carcinoma (PTC), *BRAF* gene mutations are found in 29%-83% of the cases and are often alternative to *RAS* mutations, *RET* (*RET*/PTC) and *NTRK1* rearrangements (Frasca et al., 2008; Soares et al., 2003; Xing, 2005).

The early evidence that $BRAF^{V600E}$ is required for cell proliferation, transformation and tumourigenesis of follicular thyroid cells was demonstrated in a xenograft mice model (Knauf et al., 2005; Liu et al., 2007). These studies supported the role of BRAF mutations in tumour initiation. Targeted expression of the $BRAF^{V600E}$ in thyroid cells was induced in transgenic FVB/N mice using a bovine thyroglobulin promoter. This model showed that BRAF induced thyroid dysfunction, which was compensated by increased levels of thyroid-stimulating hormone (TSH), and goiter development. Also, multifocal tumours involving both lobes of the thyroid gland with mixed papillary and follicular growth pattern were observed in 12 and 22-week-old mice. Tumours from one of the transgenic lines progressed into poorly differentiated carcinomas (Knauf et al., 2005).

Mutations in*TP53* are not frequent in well differentiated thyroid cancer, but have been well documented in anaplastic carcinomas (Reddi et al., 2015). Although frequently present in less differentiated carcinomas, *TP53* mutations are very rare in well-differentiated thyroid cancers (Agrawal et al., 2014). This is suggestive of a later role for *TP53* in cancer progression to dedifferentiated and aggressive stages. Consistently, thyroid cancer models in mice have shown that acquired mutations drive tumour progression. Using Cre-inducible thyroid $BRAF^{V600E}$ expression in a conditional *TP53* mouse model, a study showed that loss of *TP53* function enables progression to aggressive anaplastic thyroid cancer (McFadden et al., 2014). Nevertheless, other events are likely to be involved in anaplastic conversion.

In 2017, Anelli et al., described a transgenic model with $BRAF^{V600E}$ expression in zebrafish thyrocytes that resulted in significant disruption of follicles, thyroid hormone production, and increase in cell proliferation (Anelli et al., 2017). The model developed carcinoma of the thyroid by one year of age with key features of human papillary thyroid carcinoma (Anelli et al., 2017).

In this work, we describe the generation of a transgenic zebrafish line, tg(*tg*:mCh-T2A-$BRAF^{V600E}$), that expresses $BRAF^{V600E}$ specifically in thyroid cells. The $BRAF^{V600E}$-expressing fish developed hyperplasia followed by colloid goiter, but not cancer. Knowing the critical role of p53 loss in tumour progression (McFadden et al., 2014; Patton et al., 2005), we generated a novel zebrafish line expressing $BRAF^{V600E}$ specifically in thyroid cells of tp53M214K mutant zebrafish. Surprisingly, the zebrafish line preserved the thyroid architecture, providing new insights of the dynamics of $BRAF^{V600E}$ and *tp53* in zebrafish thyroid.

Material and Methods

Ethics statement

All Zebrafish work was conducted according to National Guidelines and approved by the Ethical Committee of the Instituto Gulbenkian de Ciência (IGC), Instituto de Investigação e Inovação em Ciência (i3S) and the Portuguese Veterinary Authority - Direcção Geral de Alimentação e Veterinária (DGAV).

Zebrafish husbandry and embryo culture

Matting pairs of desired genotypes were crossed, and the resulting offspring (0-7 days post-fertilization, dpf) were kept in embryo medium at 28ºC on a 14 hour light/10 hour dark cycle. If the offspring was let to develop further (more than 7dpf), animals were kept in a recirculating system at 28ºC, on a 14-hour light/10-hour dark cycle according to (Westerfield, 2007). Fish were bleached up to 24 hours post-fertilization (hpf) to remove possible contaminations and reared in standard densities (10-12 fish/liter of water).

Generation of transgenic lines

A BAC genomic clone DKEY-97I18 BAC (GenBank CR855311.15) containing the first exon of the zebrafish thyroglobulin (tg) gene was obtained from SourceBioScience. An

insert corresponding to the region from -2041bp to -1bp (relative to the tg ATG start codon) was amplified and cloned into a p5E-MCS plasmid provided in the Tol2Kit (Kwan et al., 2007). The fluorophore mCherry was chosen as a reporter because a cmlc2:EGFP transgenesis marker was to be included in the plasmid backbone. The cmlc2:EGFP cassette comprises a ~900-bp enhancer-promoter from the cardiac myosin light chain (cmlc2) gene which drives cytoplasmic EGFP expression specifically in the developing heart (Auman et al., 2007). This cassette is particularly useful to screen embryos and fish. mCherry-T2A-BRAF^{V600E} was amplified and cloned into a pME-entry clone from previous constructs generated in the lab (Faustino et al., 2012). To build the final constructs, we used the Tol2 Gateway cloning system and assembled [tg promoter, mCherry and polyA] or [tg promoter, mCherry-T2A-BRAF^{V600E} and polyA] into a pDestTol2CG2 containing the cmlc2:EGFP transgenesis marker. For the purpose of this paper, named Tol2-tg:mCh and Tol2-tg:mCh-T2A-BRAF^{V600E}, respectively. The constructs were finally verified using Sanger sequencing.

Transgenesis

WT and *tp53*^{M214K} (Berghmans et al., 2005) embryos were injected with 30ng/µL of plasmid and 25 ng/µL of capped transposase mRNA at one-cell stage. Microinjections were performed using a SZX10 Stereo Microscope (Olympus) and PV820 Pneumatic PicoPump (World Precision Instruments, Inc.). Mosaic embryos were screened for fluorescent markers (mCherry and/or GFP). The following transgenic lines were created: tg(*tg*:mCh;*cmlc2*:GFP), tg(*tg*:mCh-T2A-BRAFV600E;*cmlc2*:GFP), *tp53M214K* tg(*tg*:mCh;*cmlc2*:GFP) and *tp53M214K* tg(*tg*:mCh-T2A-BRAFV600E;*cmlc2*:GFP).

Genotyping

tp53M214K mutation was genotyped using primers to amplify the loss-of-function (LOF) point mutation on exon 7 of the zebrafish *tp53* gene as previously described (Berghmans et al., 2005): forward primer 5'-CTGTTTTTGCAGCTTGGTGC-3' and reverse primer 5'-CAGTTTTATGCTCAGGCACG-3'. The different genotypes were identified based on the RFLP assay (Restriction Fragment Length Polymorphism) (Botstein et al., 1980). *tp53*^{M214K} mutations gives rise to a second MboII restriction within the amplified fragment.

Histology

7dpf larvae were euthanized by submersion in ice water (5 parts of ice/1 part of water, 0-4ºC) for at least twenty minutes to ensure death by hypoxia. Fish ≥ 8dpf were euthanized by an overdose of MS222 (200mg/L, SIGMA). Larvae between 7 and 14dpf were fixed in 10% neutral buffered formalin for one day, and fish with ≥ 15dpf were fixed for two days. After, animals were decalcified in 0.5M EDTA (SIGMA) for two days, before paraffin embedding and sectioning. Three-micrometer longitudinal sections were stained with haematoxylin and eosin (HE) for histopathological analysis. Microphotographs were acquired in a Leica DM LB2 upright microscope equipped with an IDS color CCD camera (with 10x 0.25NA and 40x 0.75NA objectives), using the Eye Cockpit software (Imaging Development Systems GmbH).

Thyroid extraction

After euthanized by an overdose of MS222, the thyroid follicles of 3 mpf zebrafish were collected under a fluorescence stereomicroscope (MZ10F, Leica Microsystems) by dissecting the fluorescent tissue on the pharyngeal area, between the first gill arch and the bulbus arteriosus, along the ventral aorta.

Western blot analysis

Thyroid tissue or whole embryos were placed in RIPA Buffer (50 mM Tris, pH 8.0; 150 mM NaCl, 0.1% SDS; 1% NP40 and 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktail according to manufacturer's instructions and homogenized with a sterile pestle and the homogenates were incubated 30min on ice. Protein homogenates were centrifuged (10 min at 4°C, 13.000rpm) and the supernatant was collected. A 2x Sample Buffer (2x Laemelli Buffer, with 10% mercaptoethanol) was added, in equal amounts to the collected supernatant. The samples were heated for 5 min at 95ºC and stored at -20ºC if not used immediately. Samples were loaded on a 10% SDS-PAGE gel and subjected to electrophoresis, after which they were transferred to nitrocellulose Membranes, 0.45 µm (GE Healthcare, USA).

The primary antibodies used for proteins analysis were: α-Tubulin mouse monoclonal antibody (mAb) (T5168, Sigma), Phospho-p44/42 MAPK (Erk1/2) rabbit mAb (#4370, Cell Signalling Technology), Total p44/42 MAPK (Erk1/2) mAb (#9102, Cell Signalling Technology), Phospho-Akt (Ser473) rabbit mAb (#4060 (Cell Signalling Technology) and

total AKT Akt (pan) (11E7) rabbit mAb (#4685, Cell Signalling Technology). For the analysis of phosphorylated proteins, the quantification was performed by normalizing the phosphorylated signal to the total protein signal, ensuring accurate ratios in the subsequent analysis. Then target protein quantification was normalized with endogenous tubulin. Protein expression was analysed in Odyssey ® CLx Infrared 577 Imaging System (LI-COR Biotech, USA) and read at 800 and 680 nm absorbance using fluorescent Secondary Antibodies IRDye® 800CW Donkey Anti-Rabbit, IRDye®, 800CW Goat Anti-Mouse, and IRDye® 680RD Goat anti-Rabbit and IRDye® 680RD Donkey anti-Goat (LI-COR Biotech, USA), respectively.

RNA extraction and Reverse Transcription

Total RNA from zebrafish thyroid tissue was extracted using GRS Total RNA Kit – Tissue (GK09.0100, GrisP, Portugal). Protocol was performed according to the manufacturer instructions. For cDNA conversion, 1 µg of total RNA from the thyroid tissue frozen samples was treated with DNase and reverse transcribed using the RevertAid RT Reverse transcription Kit (REF: K1691, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

Real-Time Polymerase Chain Reaction (RT-PCR)

The mRNA expression of tg, pax8 and slc5a5 was evaluated by RT-PCR. The PCR reaction was performed with Taqman® Universal PCR Master Mix (REF: 4324018, Applied Biosystems, Foster City, CA, USA). The probes used for the genes of interest were TaqMan gene expression assays: tg (Dr03986645_m1), pax8 (Dr03986637_g1), slc5a5 (Dr03118167_m1) and actin (Dr03432610_m1), the later used as endogenous control for normalization of the mRNA expression. The RT-PCR method was as follows: an initial step at 95C for 10 min, followed by 50 cycles of 15 s at 95 C and 1 min at 60C. Duplicates and non-template controls (NTC) were performed for all the samples. Samples were considered positive when genes were amplified at maximum at cycle threshold (Ct) 37. Quantification of mRNA expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Body Mass Index and Hyperplasia quantification

Body Mass Index (BMI): was calculated by dividing fresh body weight (kg) of each

individual by the square of its longest body axes - length (m^2).

Morphometric studies: 3-micrometer thick longitudinal sections spanning the whole thyroid were collected. Sections were stained with haematoxylin and eosin. Photos were collected and then evaluated using ImageJ software (version 1.53t, National Institutes of Health, USA).

Hyperplasia quantification: was performed by delineating the area around each follicle, counting the number of follicles per unit area, and subsequently calculating the mean area per follicle.

Goiter quantification: was defined by a highly trained pathologist (MSS) as 1) presence of enlarged follicles of 2) variable shapes, and with 3) excessive colloid accumulation. All sections of each thyroid were examined blinded to experimental groups, and assessed for goiter-like phenotypes.

Statistical analysis

Statistical analysis was performed in GraphPad Prism8 (GraphPad Software, Inc.). Mann–Whitney's and Student's *t*-test were used for comparisons. A critical value for significance of $p< 0.05$ was used throughout the study.

Results and Discussion

Activated BRAF promotes thyroid hyperplasia in zebrafish

In order to determine the effect of $BRAF^{V600E}$ on zebrafish thyroid, we generated a construct in which the zebrafish thyroid-specific thyroglobulin promoter (*tg* promoter) drove expression of human $BRAF^{V600E}$ (Figure 3A). One-cell stage embryos were microinjected with *tg*-mCherry-T2A-$BRAF^{V600E}$ (Figure 3A) or *tg*-mCherry constructs. The T2A sequence was used to generate a polycistronic gene in which mCherry would not be fused to $BRAF^{V600E}$. To study the biology of $BRAF^{V600E}$ expression in thyroid cells, we screened for founders and generated stable lines of *tg*-mCherry-T2A-$BRAF^{V600E}$ transgenic fish (hereafter designated as *tg-*$BRAF^{V600E}$) having *tg*-mCherry as controls. Both these lines displayed clear thyroid cell fluorescence (Figure 3B).

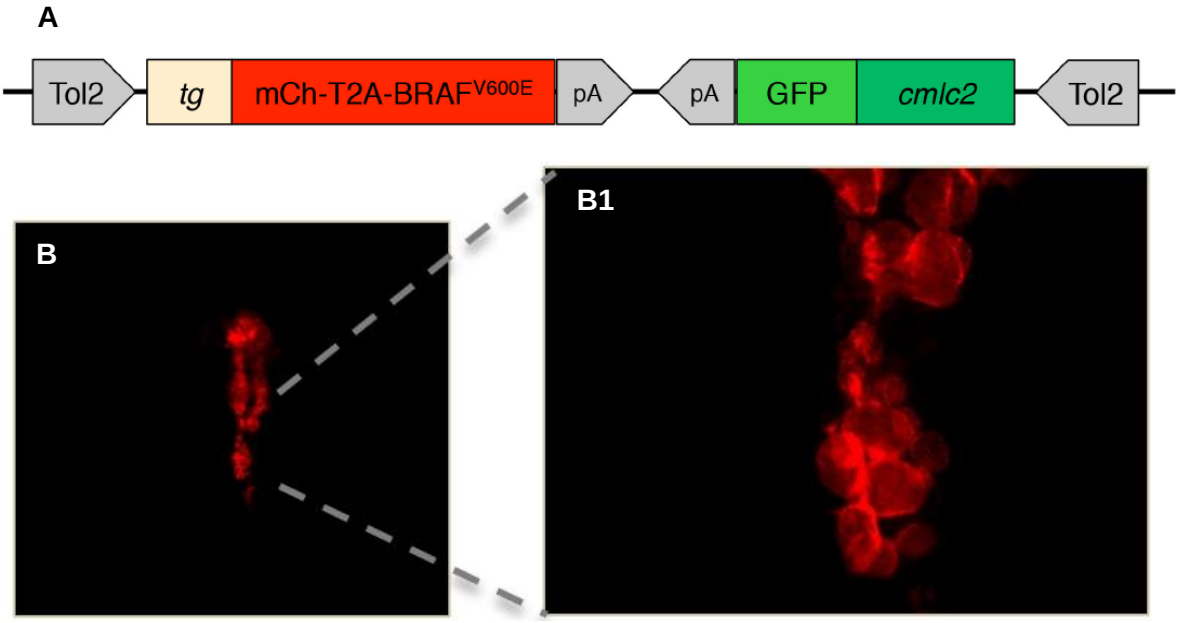

Figure 3 – (A) Schematic representation of the construct used to generate thyroid-expressing human BRAF^{V600E} zebrafish. Briefly, a thyroid-specific thyroglobulin promoter (tg promoter) was placed upstream of the human BRAF^{V600E} coding sequence, followed by a polyA with a downstream GFP fusion with a heart marker (cmlc2), used for positive confirmation of successful transgenesis; (B) Representative fluorescence stereoscopic images of transgenic zebrafish thyroid follicles expressing mCherry fluorescence in lower magnification (24x) and (B1) higher magnification (120x).

Stable expression of BRAF^{V600E} in thyroid of *tg-BRAF^{V600E}* zebrafish led to abnormal tissue morphogenesis. This was clearly evident from 7 days *post* fertilization (dpf) onwards (Figure 4). During early larval stage (7dfp) unique follicles were predominant and lined by overlapping layers of epithelium consisting of basophilic cuboidal cells. These did not detach to form more follicles, as observed in controls, showing impaired thyroid morphogenesis (Figure 4). By 28dpf, the thyroid became organized into a follicular-like structure in the connective tissue (situated at the lower jaw of the animal). In the *BRAF^{V600E}* zebrafish this was also observed, but the structure still remained attached, with overlapping layers of epithelial tissue, and presented less colloid accumulation (Figure 4).

At 3 months of age, thyroid examination revealed that colloid-filled control follicles were predominantly distributed within the connective tissue along the aorta and lower jaw, oval in shape and surrounded by a monolayer epithelium (Figure 4). In contrast, *BRAF^{V600E}*-expressing zebrafish presented follicles of different sizes and shapes, with patterns of follicular hyperplasia. To confirm this, we quantified the number of follicles, and the area of each follicle in each section of both *tg-BRAF^{V600E}* and control (Figure 5).

We observed that animals expressing $BRAF^{V600E}$ in thyroid cells had significantly increased thyroid area ($p<0.0001$) (Figure 5A) but the follicles were significantly less ($p<0.001$) when compared to the control animals (Figure 5B). This culminated in a significantly higher follicle ratio ($p<0.0001$) for the *tg-$BRAF^{V600E}$* animals (Figure 5C), i.e., larger follicles, but fewer. Nevertheless, the follicles appeared well differentiated and were lined by basophilic cuboidal to columnar epithelial cells. The impairment of thyroid organization observed during larval stages, developed into thyroid hyperplasia in $BRAF^{V600E}$ – expressing fish.

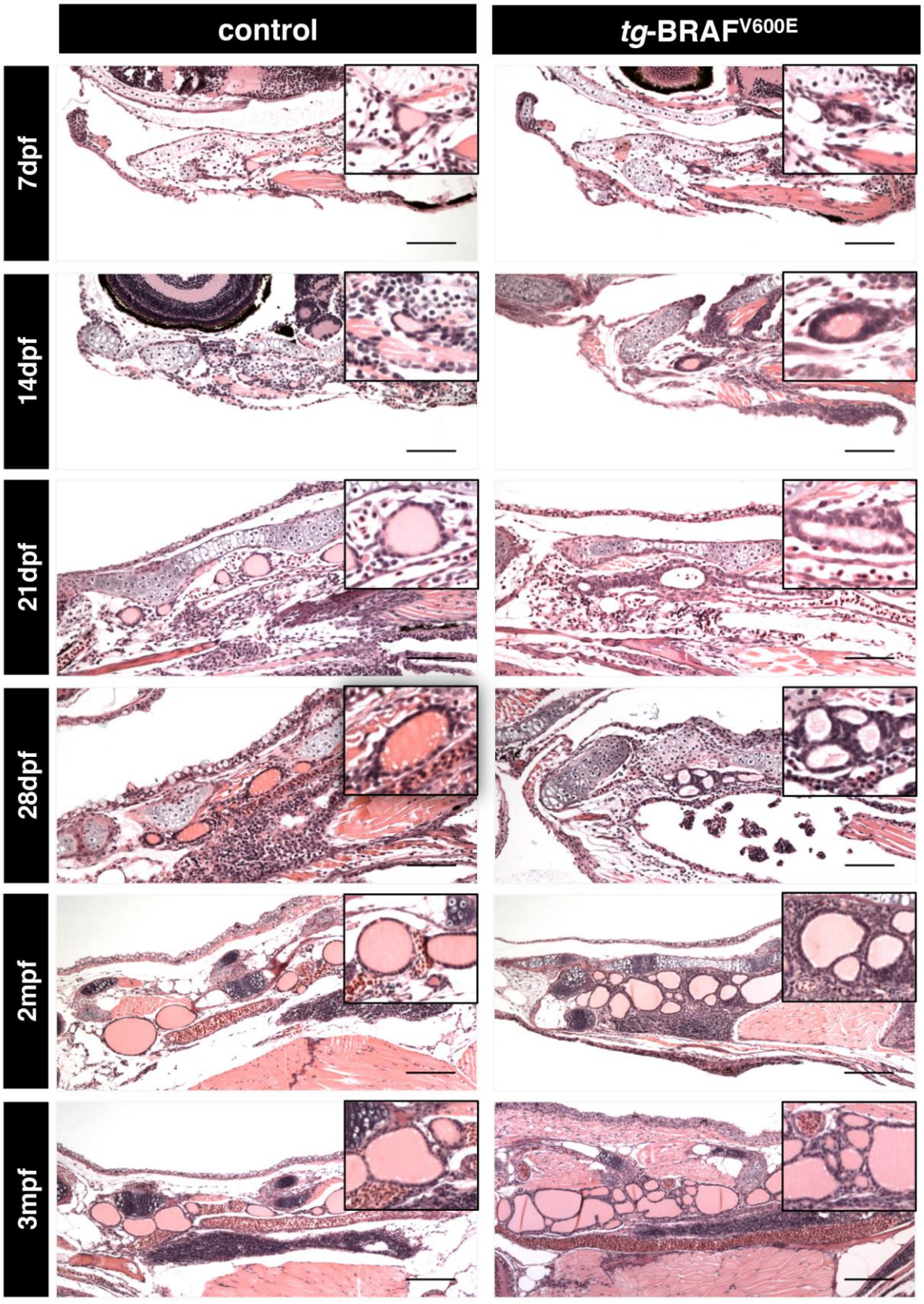

Figure 4 – Representative thyroid histology sections of control and tg-BRAF^{V600E} animals at different timepoints.

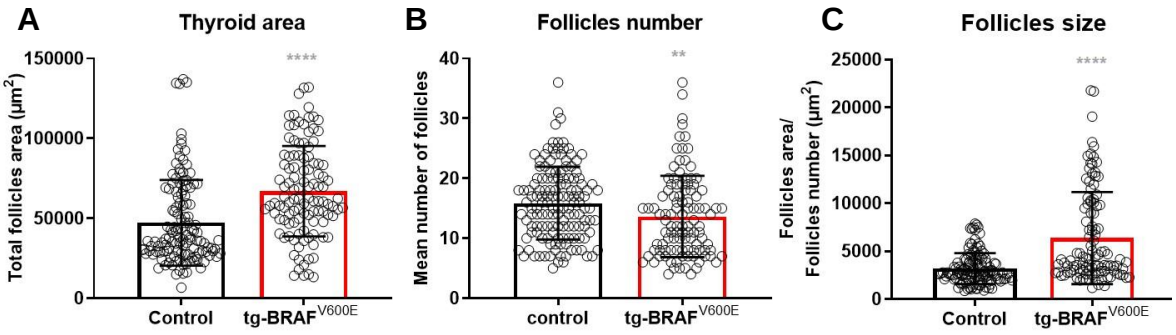

Figure 5 – Morphometric quantification of thyroid hyperplasia of tg-BRAF^{V600E} (n=8) and control fish (n=11); (A) Mean thyroid area (p<0.0001); (B) Mean follicles number (p<0.001) and (C) Mean follicle size (p<0.0001), at 3 mpf.

We continued to closely monitor the thyroids of *tg-BRAF^{V600E}* fish, as we expected the observed hyperplasia to develop into neoplasia in later stages of life. By 12 months of age, most fish developed features resembling colloid goiter with the presence of enlarged follicles filled with colloid (Figure 6).

However, there was no development of clear thyroid neoplasia in all the fish throughout this study. Thus, our results show that the most common human BRAF mutation is capable of inducing alterations in thyroid morphogenesis in zebrafish, namely hyperplasia and goiter, yet, in our model, it was not sufficient for neoplasia development up until 12 months of age.

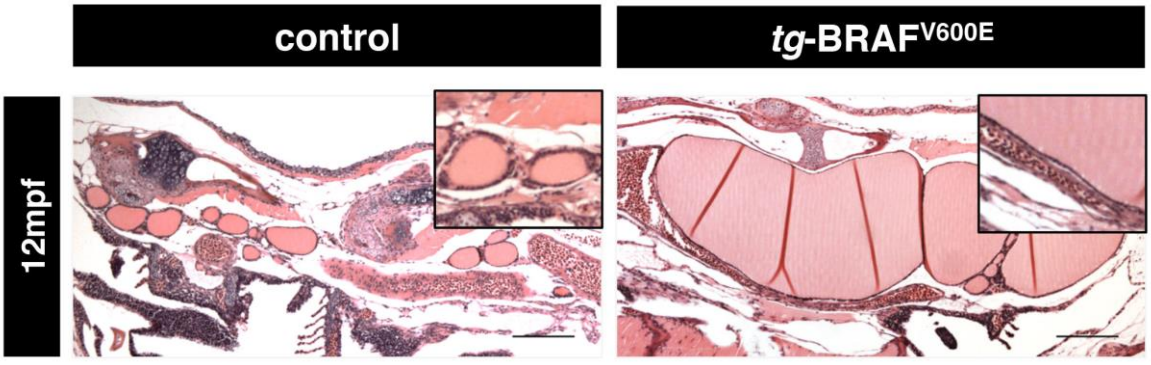

Figure 6 - Representative thyroid histology sections of control and tg-BRAF^{V600E} animals at 12 mpf. Note the enlarged follicles, resembling goiter, in the tg-BRAF^{V600E} animals.

BRAF^{V600E} expression in thyroid results in decreased body mass

Concomitant to thyroid hyperplasia, we observed a dramatic decrease in body mass of 3 mpf *tg-BRAF^{V600E}* zebrafish, when compared to controls ($p<0.0001$) (Figure 7A). Consistently, reduction in the growth of BRAF^{V600E}-expressing fish was maintained through 12mpf when compared to controls (Figure 7B). No further macroscopic phenotypic alterations were evident in these fish. Our observation of lower body weight in *tg-BRAF^{V600E}* zebrafish suggests that the thyroid of these animals may have failed to produce sufficient thyroid hormone (TH) throughout development.

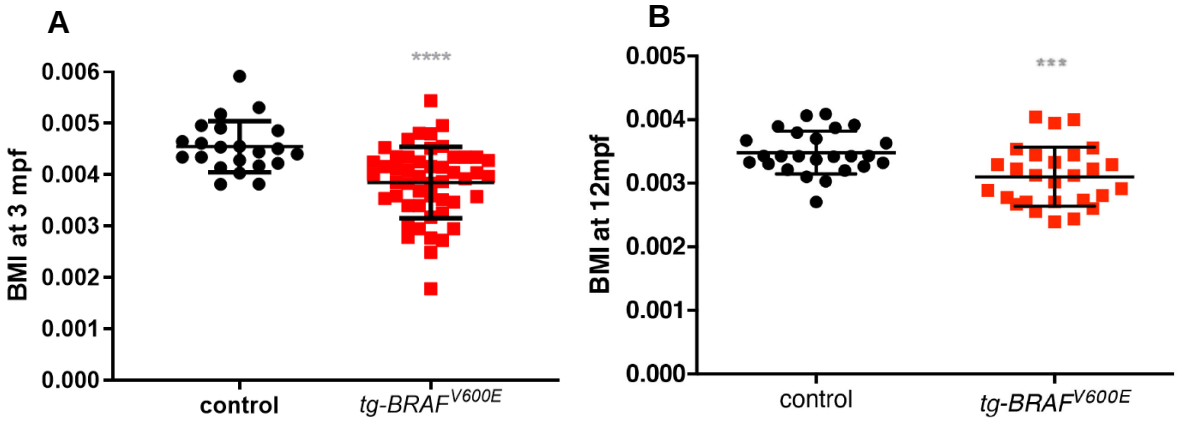

*Figure 7 - Body Mass Index (BMI) measured from (A) controls (n=22) and tg- BRAF^{V600E} (n=52) at 3 mpf; and (B) controls (n=25) and tgBRAF^{V600E} (n=28) at 12 mpf. Unpaired T-test comparison (**** - p<0.0001) and (*** - p<0.001), respectively.*

Dysregulated NIS Expression in tg-BRAF^{V600E} fish

In order to elucidate the molecular mechanisms underlying the observed thyroid dysfunction and growth impairment in our *tg-BRAF^{V600E}* zebrafish model, we performed quantification of key genes involved in thyroid differentiation. Given the essential role of thyroglobulin (tg), nis (slc5a5), and pax8 in thyroid function, we performed RT-PCR to assess their expression levels specifically in the thyroid of these animals (Figure 8). We observed, that *tg-BRAF^{V600E}* animals at 3 mpf expressed significantly more nis mRNA ($p<0.05$) than the controls (Figure 8C), and, although presenting higher mRNA expression, no significant differences were detected ($p>0.05$) in the tg and pax8 thyroid differentiation markers (Figures 8A, B). An increase in nis expression may be an adaptive response to an increased demand for thyroid hormone or iodide. Thyroid

hyperplasia at this timepoint, together with the low BMI and increased nis expression, could result from a compensatory mechanism to enhance iodide uptake for thyroid hormone synthesis. An imbalance in thyroid hormone synthesis could explain the observed low body mass, as thyroid hormones play a crucial role in regulating metabolism and overall growth in the first steps of development.

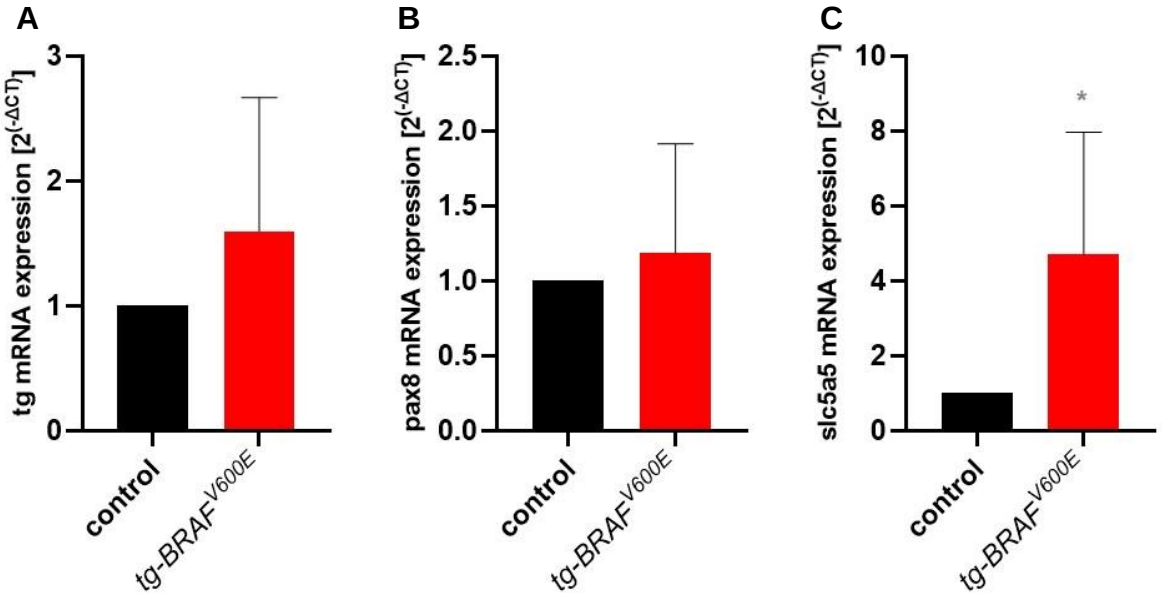

Figure 8 - mRNA expression of tg (p>0.05), pax8 (p>0.05) and slc5a5 (p<0.05) genes in thyroid of tg-BRAFV600E (n=16) in comparison with controls (n=5) at 3 mpf.

BRAF^{V600E} expression does not activate MAPK and PI3K pathways in thyroid cells

Given that hyperplasia was observed at 3 months of age, we investigated if BRAF activation was responsible for follicular cell proliferation in *tg-BRAF^{V600E}* fish. No differences in the protein expression between *tg-BRAF^{V600E}* fish and controls were observed (p>0.05) at 3mpf (Figure 9). *BRAF^{V600E}* did not seem to overactivate the MAPK and PI3K signalling pathways, as it was expected.

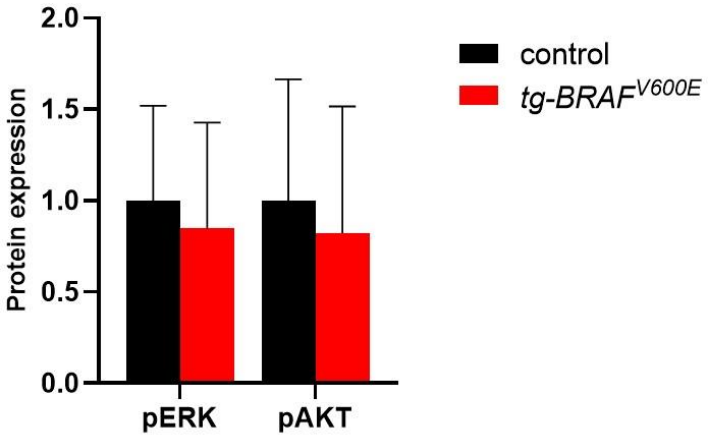

Figure 9 - Protein expression of ERK and AKT proteins in thyroid tissue of tg-BRAFV600E (n=19) and control (n=17) at 3 mpf. No significant differences were observed (p>0.05).

Disruption of the p53 pathway prevents thyroid hyperplasia in BRAF^{V600E}-expressing cells

Loss of *TP53* function, in addition to activating mutations in the *BRAF* gene, is a common signature of human carcinomas that progress from PTCs. To test whether deficiency in the p53-pathway is key for thyroid neoplasia, we generated a stable *tg-BRAF^{V600E}* in a *tp53^{M214K}* homozygous mutant background (*tg-BRAF^{V600E} tp53^{M214K}*) (Berghmans et al., 2005). p53^{M214K} protein is deficient in DNA binding (Berghmans et al., 2005; Olivier et al., 2002) and, consequently, *tp53^{M214K}* mutants are unable to mount apoptotic responses to γ-irradiation and it was described that they will develop malignant neural tumours beginning at 8.5 months of age (Berghmans et al., 2005; Storer & Zon, 2010).

Expressing *BRAF^{V600E}* in *tp53^{M214K}* mutant thyroid cells did not lead to an abnormal thyroid morphogenesis (Figure 10). The follicles of the tg-*BRAF^{V600E} tp53^{M214K}* were round or oval and lined by a single-layered cuboidal epithelium and the centre of these follicles was filled with colloid (Figure 10). During adult stages, all *tg-BRAF^{V600E} tp53^{M214K}* zebrafish showed normal thyroid morphology and presented neither hyperplasia nor colloid goiter (Figure 10). No differences were detected in the morphometric analysis for number of thyroid follicles and thyroid mean area quantification (p>0.05) (Figure 11). Likewise, BMI differences were absent throughout all stages of life, including at 3mpf and 12mpf (Figure 12). These results suggest that *TP53* function seems to be required to develop the observed defects induced by BRAF activation in zebrafish thyroid.

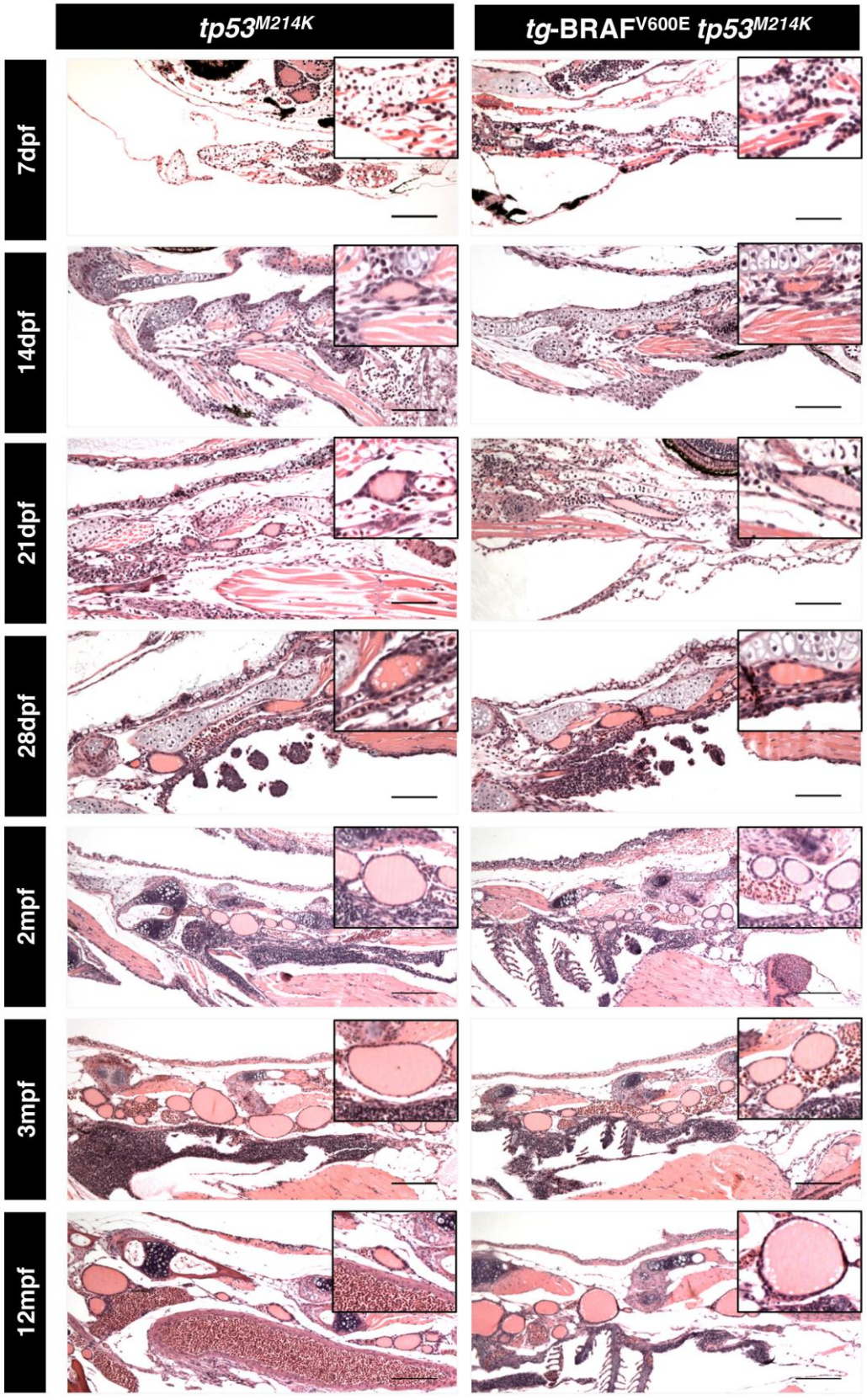

Figure 10 – Representative thyroid histology sections of control ($tp53^{M214K}$) and tg-BRAFV600E $tp53^{M214K}$ animals at different timepoints.

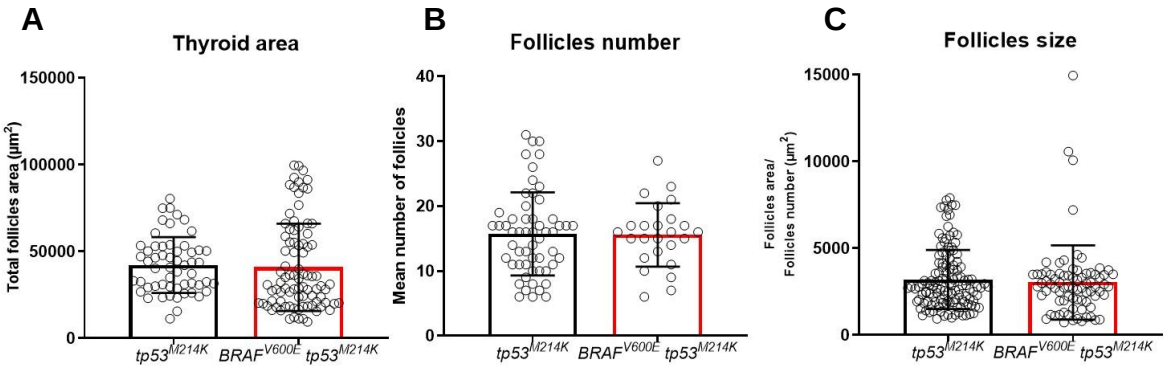

Figure 11 - Morphometric quantification of thyroid follicles of tg-BRAFV600E tp53^{M214K} (n=6) and control tp53^{M214K} fish (n=3); (A) Mean thyroid area; (B) Mean follicles number and (C) Mean follicle size, at 3 mpf. No statistically significant differences were detected (p>0.05).

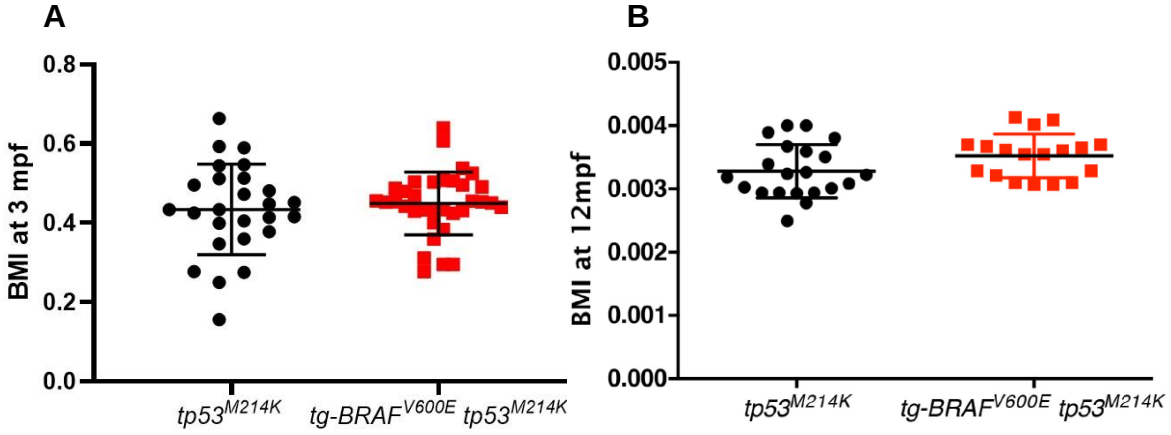

Figure 12 - Body Mass Index (BMI) measured from (A) tp53^{M214K} (n=26) and tg-mCherry BRAF^{V600E} tp53^{M214K} (n=34) at 3 mpf; and (B) tp53^{M214K} (n=25) and tgBRAF^{V600E} (n=28) at 12 mpf. Unpaired T-test comparison (p>0.05).

Thyroglobulin decreased expression in BRAF^{V600E} tp53^{M214K} fish

TP53 loss reverted the NIS increased expression that was observed in the *tg-BRAF^{V600E}* fish (figure 13C). On the other hand, a significant decrease ($p<0.05$) in tg mRNA expression was observed in the *BRAF^{V600E} tp53^{M214K} fish* when compared to the *tp53^{M214K}* control fish (Figure 13A). The pax8 expression remained unaltered in these models (Figure 13B).

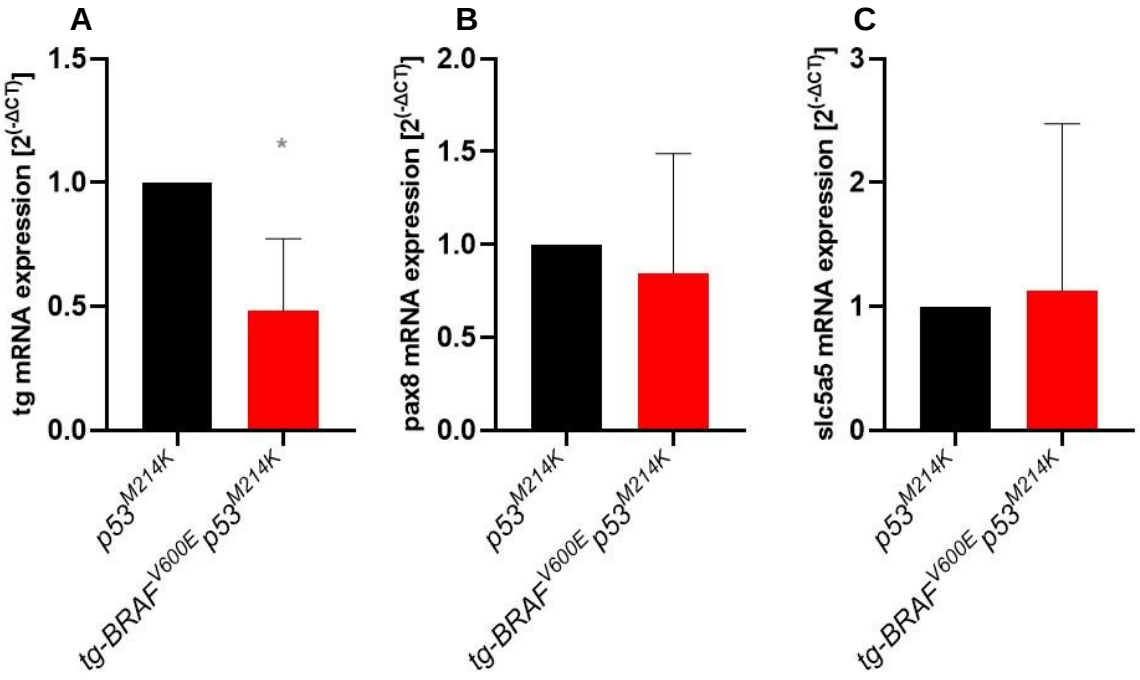

Figure 13 - mRNA expression of tg ($p<0.05$), pax8 ($p>0.05$) and slc5a5 ($p>0.05$) genes in thyroid of tg-BRAF^{V600E} tp53^{M214K} (n=5) in comparison with tp53^{M214K} controls (n=5) at 3 mpf.

p53 was not sufficient for MAPK and PI3K activation in BRAF^{V600E}-expressing thyroid cells

Upon loss of *TP53*, expression of BRAF^{V600E} was no longer sufficient to produce the hyperplasia phenotype (Figures 10, 11). In order to understand this difference, we produced protein extracts from 3-month-old *tp53^{M214K}* mutant thyroid cells expressing *BRAF^{V600E}* and compared them to the control *tp53^{M214K}* animals. Hence, the MAPK and PI3K pathways were not upregulated in this background, since no differences in protein expression of pERK and pAKT, respectively, were observed between the models as observed in the presence of the WT *TP53* (Figure 14).

Likewise, absence of WT *TP53* function was expected to lead to the inability to activate senescence and/or apoptosis in these animals. Apparently, p53M214K protein was unable to induce a programmed cell death program in tg-BRAFV600E fish.

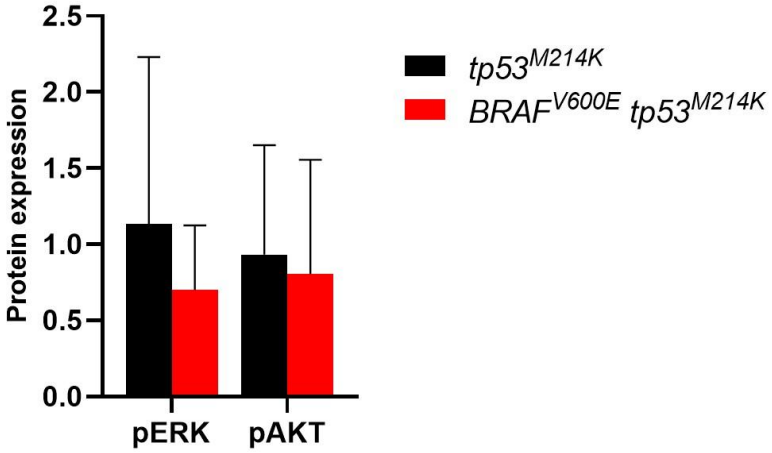

Figure 14 - Protein expression of ERK and AKT proteins in thyroid tissue of tg-BRAF^{V600E} (n=20) and tp53^{M214K}control fish (n=17). No significant differences were observed (p>0.05

Concluding Remarks

Our data shows that, even though expression of BRAF^{V600E} can cause transformation of thyroid cells, additional mutations are likely to be required for cancer progression. Molecularly, loss of p53 function is a well-characterized genetic signature of thyroid tumour progression (Bai & Zhu, 2006; Morita et al., 2008; Soares et al., 2003). However, *tg-BRAF^{V600E} tp53^{M214K}* zebrafish neither give rise to discernible neoplasia nor indeed hyperplasia, as observed for *tg-BRAF^{V600E}* fish. Our results show that *tp53^{M214K}* mutation prevents tumourigenesis initiation in our model. It is possible that WT *TP53* function may be required in early stages of tumourigenesis, such that hyperplasia can only occur in response to p53-dependent pathways. This would explain why loss of *TP53* function might constitute a later event in thyroid cancer progression. Conditional p53 mutants will allow us to reveal *tp53* mutations at both early and late stages of thyroid transformation thus revealing an anticipated requirement for p53 function in thyroid cancer.

In summary, thyroid-specific expression of BRAF^{V600E} in zebrafish results in abnormal thyroid morphogenesis, which develops into hyperplasia and, in adulthood, colloid goiter. These phenotypes support an important role for *BRAF^{V600E}* in thyroid cells and anticipate our *tg-BRAF^{V600E}* transgenic line as an exciting new model to understand *BRAF^{V600E}*-dependent thyroid disease. However, not only did mutated *tp53^{M214K}* not cooperate with *tg-BRAF^{V600E}* to promote tumourigenesis, but also prevented abnormal thyroid morphogenesis. Our study raises the possibility that the normal function of *TP53* may have positive and negative roles in thyroid tumour formation. It may be that early

loss of p53 function in thyroid cells may avoid cell transformation by preventing cell senescence and inflammatory responses, for example. Thus, it may be only later that loss of the ‘guardian of the genome’ may unclench all its known deleterious effects. These two models will be useful tools for the study of thyroid cancer pathogenesis, namely, to disclose which signals are ultimately promoting and/or suppressing neoplasia.

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VII. Effect of the endocrine disruptor, Bisphenol A, in zebrafish embryos with thyroid cells targeted with $BRAF^{V600E}$-expression

After the characterization of the zebrafish transgenic model, expressing $BRAF^{V600E}$ mutation in the thyroid cells we wanted to pursue our aim of understanding how environmental factors, such as endocrine disruptors, can affect the thyroid health. In this case, we wanted to investigate EDCs effects in a context of predisposition for thyroid disease, such as the presence of a $BRAF^{V600E}$ mutation. To follow that, we performed preliminary ecotoxicological assays to Bisphenol A (BPA) in $BRAF^{V600E}$ transgenic zebrafish embryos for 21 days and observed the impact in the thyroid function.

Effect of the endocrine disruptor, Bisphenol A, in zebrafish embryos with thyroid cells targeted with BRAFV600E-expression

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Key Words – EDCs, BPA, Transgenic, Zebrafish, Thyroid, $BRAF^{V600E}$

Abstract

Endocrine-disrupting chemicals (EDCs) pose a significant threat to human and environmental health, with Bisphenol A (BPA) being an ubiquitous and recognized member of this class. Known for its widespread use in the production of plastics, BPA has been linked to disruption of thyroid function. In this study, we employed a previous characterized transgenic zebrafish model expressing the $BRAF^{V600E}$ mutation, with a known predisposition for thyroid disease, to explore the potential adverse effects of BPA on thyroid health. The primary objective was to investigate whether BPA exposure could anticipate or promote thyroid tumourigenesis in this genetic context.

We performed preliminary assays and exposed zebrafish transgenic embryos (controls and *tg*-$BRAF^{V600E}$) to BPA concentrations, ranging from 0.01 mg/L to 1 mg/L, over a 21-day exposure period. Thyroid differentiation markers, including thyroid-stimulating hormone (tsh), thyroglobulin (tg), pax8, and sodium/iodide symporter (slc5a5), were assessed using RT-PCR to unravel subcellular effects on thyroid function.

Preliminary results revealed no significant alterations in thyroid differentiation markers between the *tg*-$BRAF^{V600E}$ and control fish, but in tsh mRNA expression, discrepant effects were observed for control fish and *tg*-$BRAF^{V600E}$ fish after BPA exposure, indicating a potential modulatory role of the mutation. Moreover, lower doses of BPA demonstrated a more pronounced impact on the fish thyroid compared to higher doses, displaying the characteristic non-monotonic dose-response characteristic of EDCs.

In conclusion, although variation in thyroid markers could be detected in the preliminary study, further experiments are needed in order to provide insights in to the EDCs, genetic predisposition, and thyroid health.

Introduction

Endocrine disruptors are chemicals that interfere with the normal functioning of the endocrine system, which regulates hormone production and signalling in humans and other organisms. Bisphenol A (BPA), a synthetic compound widely used in the production of plastics, is recognized as an endocrine disruptor and potentially carcinogenic (Seachrist et al., 2016). Of particular concern is BPA's impact on the thyroid gland, an essential organ of the endocrine system responsible for regulating metabolism and growth. *In vivo* research has indicated that BPA can disrupt thyroid hormone homeostasis, leading to imbalances that may adversely affect thyroid function (Fernandez et al., 2018; Heimeier & Shi, 2010; Volz et al., 2024).

In the context of thyroid disruption, BPA has been identified as a thyroid endocrine disruptor due to its ability to interact with thyroid receptors, such as thyroid stimulating hormone receptor (TSHr) and influence the synthesis and secretion of thyroid hormones (Kim & Park, 2019). BPA effects in thyroid are of considerable significance given the crucial role of thyroid hormones in developmental processes, metabolism, and overall homeostasis.

To address these concerns and gain insights into the effects of BPA on thyroid function and tumourigenesis, this study focuses on a transgenic model system, previously generated by our group, a zebrafish with $BRAF^{V600E}$-expression in thyroid cells. Zebrafish serve as an invaluable model for studying the effects of endocrine disruptors due to their genetic and physiological similarities to humans, as well as their transparent embryos, and rapid development in the first stages of life, allowing for real-time observation (Patisaul et al., 2018). This specific model becomes even more compelling as the $BRAF^{V600E}$ mutation is known to predispose for thyroid cancer (Anelli et al., 2017; Vuong et al., 2017), and, specifically in this model, the mutation lead to an abnormal thyroid morphogenesis and hyperplasia by 3 months *post*-fertilization (mpf).

We intent to understand the consequences of endocrine disruptor exposure, such as BPA, in a context of a genetic predisposition to thyroid disease. This work provides a unique opportunity to understand the potential synergistic effects (environmental and genetics) that may promote thyroid tumourigenesis.

Material and Methods

Chemicals

The chemical tested in this study Bisphenol A (CAS number 80-05-7, Sigma-Aldrich) and all stock solutions were prepared in dimethylsulfoxide (DMSO) (CAS number 67-68-5, Panreac AppliChem, ITW reagents). For the first preliminary assays, 4 dilutions were performed to refine the tests: 0.01, 0.1, 1, and 10 mg/L of BPA. All stock solutions maintained a maximum final concentration of DMSO of 0.01%.

Zebrafish husbandry

Matting pairs of the desired genotypes were crossed, and the resulting offspring (0-7 day post-fertilization, dpf) were kept in embryo medium at 28ºC on a 14 hour light/10 hour dark cycle. If the offspring was let to develop further (more than 7dpf), animals were kept in a recirculating system at 28ºC, on a 14-hour light/10-hour dark cycle according to (Westerfield, 2007). Fish were bleached up to 24 hours post-fertilization (hpf) to remove possible contaminations and reared in standard densities (10-12 fish/liter of water).

Ecotoxicological bioassays

We start doing a first bioassay for testing concentrations ranging from 0.01 to 10 mg/L of BPA, using Wild type (WT) embryos. For that, thirty fertilized eggs of WT zebrafish, within 2 hours *post* fertilization (hpf), were distributed per glass plate/ per concentration, and each group concentration had 3 replicates per treatment. For the 21-day exposure of tg-BRAF^{V600E} animals, embryos underwent screening to confirm the presence of mCherry and EGFP fluorescence reporters, as detailed in Article VI. Briefly, the mCherry reporter fluorophore is regulated by the thyroglobulin (*tg*) promoter, leading to specific expression in the fish's thyroid, becoming visible at 72 hpf. Additionally, the animal features a cmlc2:EGFP transgenesis marker, with the enhancer-promoter from the cardiac myosin light chain (cmlc2) gene. This marker drives cytoplasmic EGFP expression exclusively in the heart, becoming visible at 48 hpf. After the screening, 30 embryos per glass plate, harbouring mCherry reporter (control) and both mCherry and EGFP reporter (tg-BRAF^{V600E}) were first exposed to BPA at 72 hpf until 6 dpf. From 7 to 24 dpf, the animals were moved to glass beakers (totalizing 21 days of exposure). Each treatment group had 2 replicates. In both assays, the embryos were incubated at 28.0 °C ± 1, at the same light/dark photoperiod as adults. The medium was renewed daily in order to maintain oxygen levels, ensuring the compounds' presence, and at the desired concentration.

Endpoints

Observations were conducted every 24 h for both assays, during the established periods (7 and 21 days). Several endpoints were recorded everyday: abnormal cell growth, embryo development, embryo abnormalities (eyes, head, yolk-sac, tail, pericardial edema, hemorrhages, and muscular involuntary contractions), behaviour alterations, heart rate, mortality, and percentage of hatched embryos at 48h and 72h, i.e., percentage of animals that successfully break through the chorion and hatch into free-swimming larvae at a given timepoint. These endpoints followed the rational of previous protocols (Macedo et al., 2017). The heart rate was measured at 72 hpf in three embryos per replicate for 15 s and converted to beats per minute (bpm).

RNA extraction and Reverse Transcription

Total RNA was obtained from pooled (n=5) zebrafish larvae of 24 dpf of each experimental condition was extracted using GRS Total RNA Kit – Tissue (GK09.0100), from GrisP, Portugal. Protocol was performed according to the manufacturer instructions. For cDNA conversion, 1µg of total RNA from the frozen samples was treated with DNase and reverse transcribed using the RevertAid RT Reverse transcription Kit (REF: K1691), from Thermo Fisher Scientific, Waltham, MA, USA, following the manufacturer's instructions.

Real-Time Polymerase Chain Reaction (RT-PCR)

The mRNA expression of tsh, tg, pax8 and slc5a5 was evaluated by RT-PCR. The PCR reaction was performed with Taqman® Universal PCR Master Mix (REF: 4324018, Applied Biosystems, Foster City, CA, USA). The probes used for the genes of interest were TaqMan gene expression assays: tsh (Dr03150633_m1), tg (Dr03986645_m1), pax8 (Dr03986637_g1), slc5a5 (Dr03118167_m1) and actin (Dr03432610_m1) used as endogenous control for normalization of the mRNA expression. The RT-PCR method was as follows: an initial step at 95C for 10 min, followed by 50 cycles of 15 s at 95 C and 1 min at 60C. Duplicates and non-template controls (NTC) were performed for all the samples. Samples were considered positive when genes were amplified at maximum at cycle threshold (Ct) 37. Quantification of mRNA expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical analysis was performed in GraphPad Prism8 (GraphPad Software, Inc.). One-Way ANOVA was used for group comparisons; and Krustal–Wallis with multiple comparison test was performed when homogeneity and normality were not achieved. A critical value for significance of $p< 0.05$ was used throughout the study.

Results and Discussion

BPA concentration test – 7 days exposure assay

In order to determine appropriate concentrations for the subsequent BPA exposure assay, a preliminary assessment was conducted in WT embryos. Various concentrations of BPA were tested in this initial assay to evaluate the response of zebrafish and, importantly, to identify and exclude any potentially lethal doses. This preliminary investigation allowed us to indentify the NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) in the zebrafish and establish the range of concentrations to use in the BPA assay with $BRAF^{V600E}$-transgenic zebrafish.

After a 7-day exposure assay, we observed that zebrafish tolerated all concentrations of BPA, except for the 10 mg/L that was the LOEC. In comparison with the solvent control, 10 mg/L of BPA reduced significantly the hatching percentage and the heart rate at 72 hpf ($p<0.05$) (Figure 15 A, B), at the end of the assay all embryos had an abnormal behaviour, with barely no movement (Figure 15 C) and pericardiac edema (Figure 15 D) ($p<0.05$), almost 25% of the embryos had tail abnormalities ($p<0.05$) (Figure 15 E) and an increased, although not significant, mortality.

BPA preliminary assay – 21 days exposure assay

Considering the insights gained from the preliminary 7-day exposure assay, we conducted a more extended investigation with a focus on lower concentrations of Bisphenol A (BPA) to simulate longer-term exposures, and environmental relevant concentrations. In this subsequent assay, we selected three concentrations—1 mg/L, 0.1 mg/L, and 0.01 mg/L and aimed to evaluate the chronic impact of BPA on the transgenic $BRAF^{V600E}$-transgenic zebrafish embryos over a 21-day period.

With the presence of the $BRAF^{V600E}$ mutation in these fish, already known for its predisposition to thyroid disease (as detailed in Article VI), our objective was to unravel the impact on thyroid differentiation markers within the larvae. Specifically, we investigated key

markers including thyroid-stimulating hormone (tsh), thyroglobulin (tg), pax8, and sodium/iodide symporter (nis or slc5a5) (Figure 16).

We were only able to reproduce this assay on time, only 2 replicates per group treatment (4 sub-replicates) were considered for statistic evaluation ($p>0.05$). We were able to disclose that in general, lower doses of BPA induced an increased expression of thyroid differentiation markers, when compared to the solvent control (but non-significant, $p>0.05$) (Figure 16). Interestingly, this data was more evident in the control fish, not harbouring the BRAF mutation, specifically in the tsh marker, since tsh mRNA doubled the expression of the solvent control ($p>0.05$). On the other hand, for the *tg-BRAF^{V600E} fish* tsh mRNA expression, a decrease was noted in comparison with the solvent control, but not significant ($p>0.05$).

We were also able to observe that, BPA 0.1 mg/L, although not being the highest concentration tested, was apparently the concentration causing more disturbance in the thyroid markers mRNA expression, resembling the inverted u-shape dose-response, usually observed in endocrine disruptors (Lagarde et al., 2015).

The conventional toxicological standard assays typically assume a monotonic dose-response relationship, wherein the effects of a compound increase or decrease monotonically with dose, leading to the establishment of no observed adverse effect levels (Lagarde et al., 2015). Non-monotonic dose-response (NMDR) relationships are now acknowledged as potential toxicological realities, especially in the case of EDCs (Lagarde et al., 2015). The alterations observed in thyroid differentiation markers at lower BPA concentrations highlight the complexity of the biological response to environmental contaminants, which emphasizes the need for more studies and better biological models to be used on EDCs research.

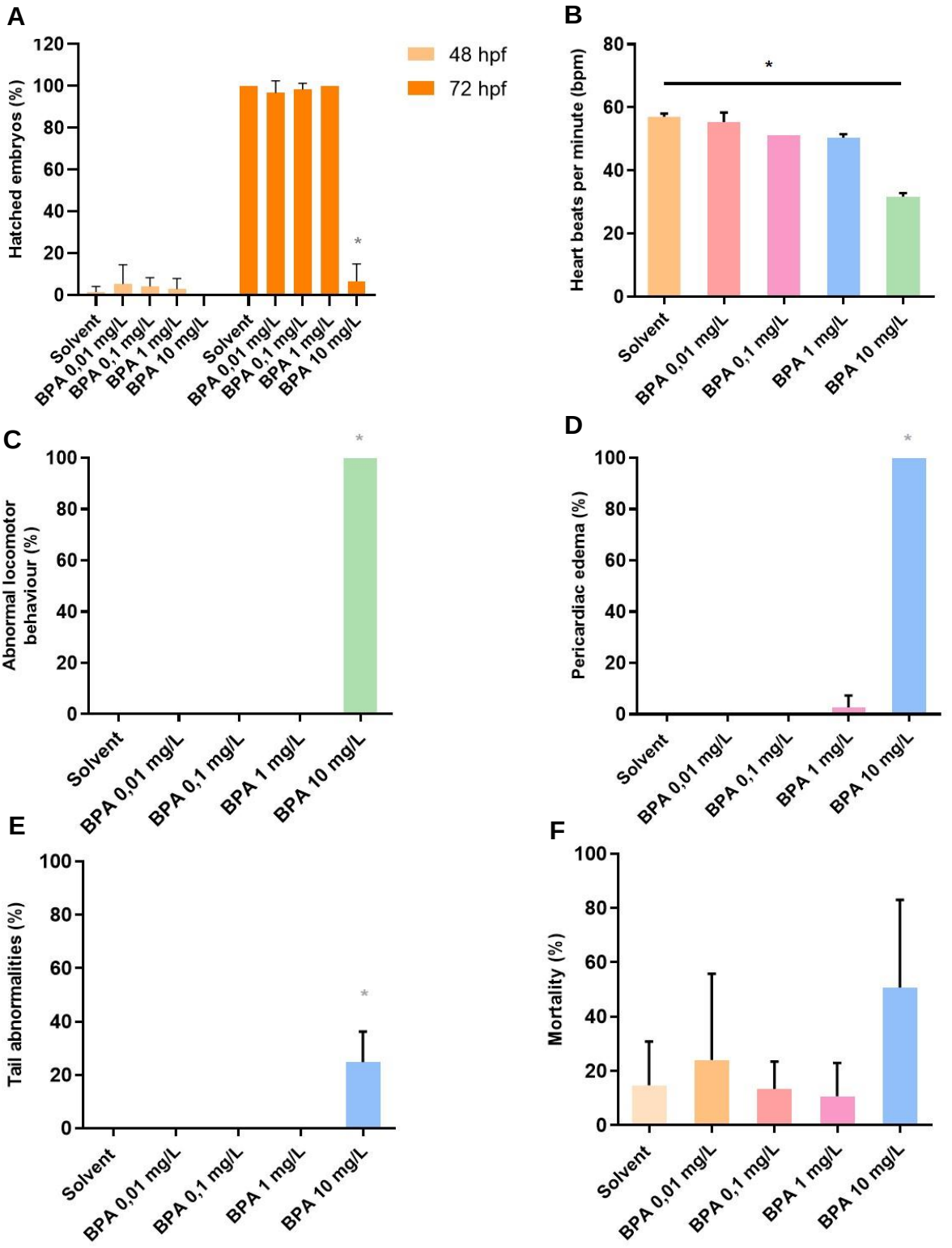

Figure 15 - Preliminary concentration assay of WT zebrafish exposed to BPA for a 7-day period. (A) Percentage of hatched embryos ($p<0.05$); (B) Heart rate at 72hpf ($p<0.05$); (C) Percentage of abnormal behaviour ($p<0.05$); (D) Percentage of pericardial edema ($p<0.05$); (E) Percentage of tail abnormalities ($p<0.05$); (F) Mortality rate ($p>0.05$).

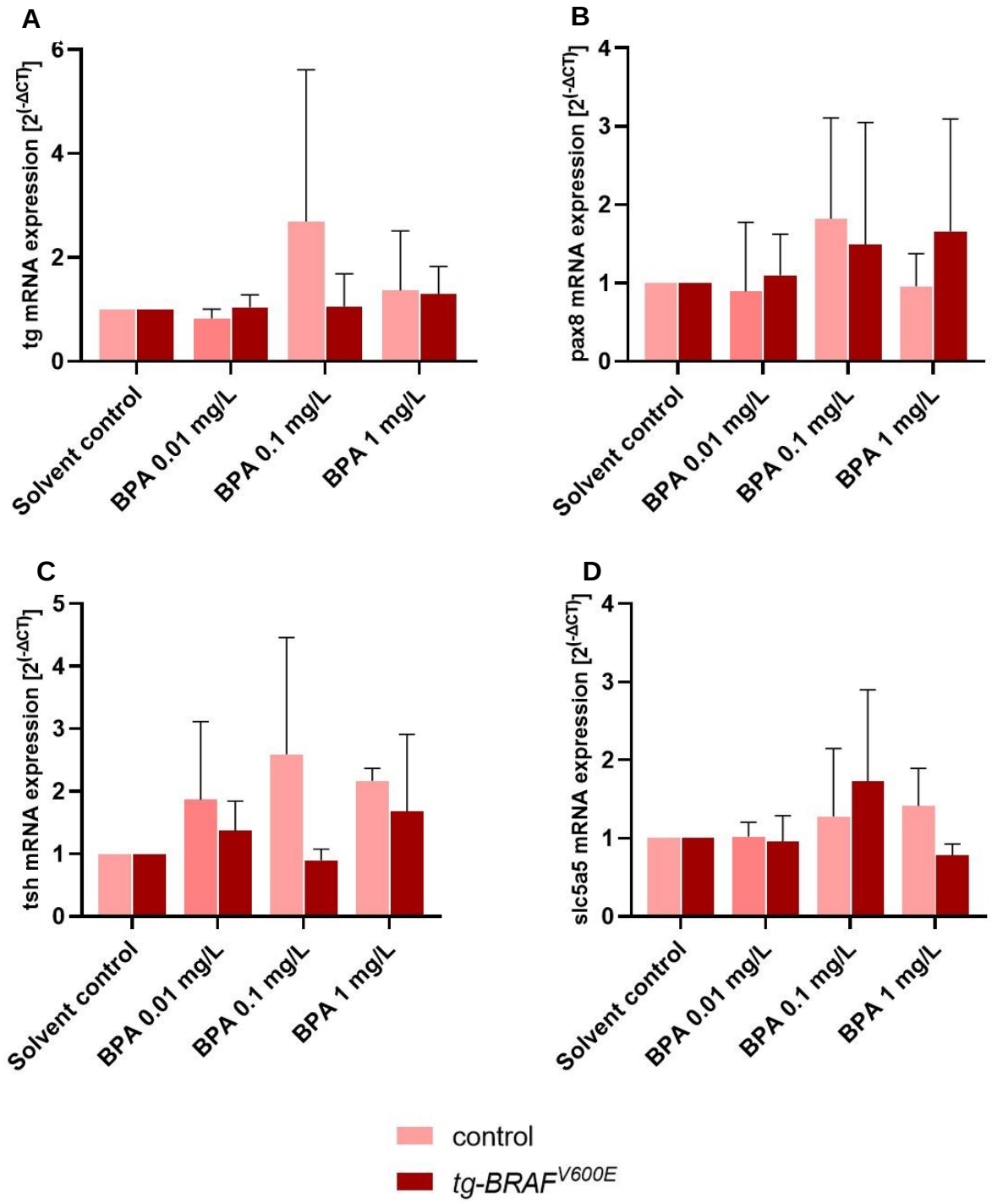

Figure 16 - BPA 21-day exposure to transgenic zebrafish larvae (*tg-BRAF^{V600E})* in comparison with control larvae (tg-mCherry). mRNA quantification of (A) tg, (B) pax8, (C) tsh, and (D) slc5a5. No statistically significant differences were detected ($p>0.05$).

Conclusion and future perspectives

Our preliminary investigation into the thyroid-disrupting effects of Bisphenol A (BPA) on $BRAF^{V600E}$-transgenic zebrafish embryos did not detected statistically significant alterations in thyroid differentiation markers, but a tendency to an opposite response was observed in tsh mRNA expression of *tg-*$BRAF^{V600E}$ when compared to the control fish. Also, alterations of pax8 and nis expression were noted, after BPA treatment. Overall, the effects were observed in both control and BRAF transgenic fish, specially at the intermediate BPA concentration of 0.1 mg/L. We aim to explore this model, performing more bioassays with different EDCs, to extend the assay to a longer time of exposure, until 3 mpf. This would allow us to understand if this endocrine disruptor is able to accelerate thyroid hyperplasia, already found in this model, or even to anticipate/promote tumourigenesis.

We hope that our study contributes to the understanding of the putative role of EDCs in thyroid neoplasia in the future.

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CHAPTER V | GENERAL DISCUSSION

In the recent years, the thyroid gland has emerged as a focal point in the complex landscape of public health challenges. This fact is of great concern since maintaining thyroid homeostasis is crucial not only for the first steps in human development but also for sustaining physiological and psychological well-being along life. Through this exploration of the thyroid's response to these multifaceted challenges, the work will hopefully contribute to the prevention, detection, and understanding of health threats posed by agents such as EDCs and events like the SARS-CoV-2 pandemic.

The emergence of the SARS-CoV-2 virus has marked a unique chapter in the current global health. Beyond the well-documented respiratory manifestations, the virus has revealed the ability to infiltrate various tissues and organs, including those crucial to endocrine function, such as the thyroid gland. Emerging evidence suggested a link between COVID-19 and thyroid disease (Brancatella et al., 2020; Lania et al., 2020; Rotondi et al., 2021). To understand the implications of the SARS-CoV-2 infection on thyroid function remains of great importance for the development of public health strategies, in order to improve patients' care, and was one of the main aims of this work. We have provided direct evidence of the viral presence in thyroid tissues from COVID-19 deceased patients (Article I), which can explain the acute thyroid dysfunction observed in some patients infected by the virus. Understanding the impact of the virus on the thyroid is important to comprehend the implications of the virus on endocrine system and in patients' health.

The patients' acute response to the viral infection was well described, however, the prolonged effects of the *post-COVID-19 condition*, were still not known (Shuwa et al., 2021). It is still not clear the role of the thyroid gland in the post-COVID-19 condition, and thyroid dysfunction seems to be mainly a secondary event of the disease and in most patients, transient (Article II). On top of the pandemic itself, the post-COVID-19 condition revealed to be a challenge for the health system. It is crucial to plan strategies for a more effective management of the latter symptoms of this disease in both healthcare and public health contexts.

Still in the scope of SARS-CoV-2 infection in thyroid, we were confronted with a lack of information about the expression of the molecules involved in the viral infection on the thyroid tissue. The literature already pointed out possible relationship between the virus and cancer (Almeida et al., 2019), and so, we delved into the unexplored territory of these molecules' potential involvement in thyroid tumourigenesis. Our findings, particularly the

correlation between increased Furin expression and worse prognostic features in thyroid cancer, such as lymph node metastization, not only contribute to our understanding of the virus-thyroid interaction but also shed light on a novel road for future research on Furin as a putative biomarker for thyroid malignancy (Article III).

The increased incidence of thyroid cancer worldwide has implications for public health and is an economic burden for the health care systems. While advancements in the diagnostic technologies and awareness have certainly contributed to the observed increase (Kitahara & Schneider, 2022), genuine rises in incidence, specifically due to novel risk factors, such as exposure to environmental pollutants seem to be open for discussion (Fiore et al., 2019; Pierozan et al., 2020; Soto & Sonnenschein, 2010). Additionally, shifts in lifestyle and dietary habits, including changes in iodine intake, may contribute to the rising prevalence (Kitahara & Schneider, 2022). Along with this, the growing incidence of endocrine-related tumours has driven the need to explore the potential role of Endocrine Disrupting Chemicals (EDCs) in this increase. Despite the discontinuation of certain EDCs, studies indicate their lasting impact on the rising occurrence of neoplasia (Soto & Sonnenschein, 2010). Our study revealed the thyroid as the endocrine organ presenting the highest cancer risk after EDC exposure which aligns with the observed increase in thyroid cancer. Despite this, the scarcity of detailed and controlled studies may account for conflicting results. Our study highlighted that multinational and multicentric human studies, strengthened by biochemical analysis, are imperative to establish a more robust and consistent evidence in this critical area of research (Article IV).

In the continuum of our research about EDCs impact in thyroid health, a collaborative work with a Brazilian research group brought the opportunity for an exploratory search of the impact of tributyltin (TBT) exposure on the thyroid gland. TBT emerged as a potent biocide with high potential of bioaccumulation throughout the food chain and thyroid disruptive properties (Marques et al., 2018; Santos-Silva et al., 2018). Although the use of TBT has been prohibited in numerous countries, including in Europe, despite the restrictions, high concentrations of TBT are still being found in several regions such as Brazil and other Latin American coastal areas (Bertuloso et al., 2015; Castro et al., 2018; Quintas et al., 2021). We observed thyroid histomorphological changes in our TBT exposure assay, using the mammal rat as model, at low doses and by short period of time, which underscores the potential public health threats posed by substances like TBT. Expectantly, this research contribution has implications for public health policies aimed at minimizing these risks and promoting thyroid health (Article V).

Study the EDCs impact in health is sometimes complex, since these chemicals act on the endocrine axis, while potentially disrupt other physiological systems, which cannot be recapitulated using cell-based models (Patisaul et al., 2018). It becomes evident that finding suitable models to study the impact of EDCs on health, particularly in mimicking human exposure, poses a significant challenge. The zebrafish model has emerged as a promising option for EDCs research (Patisaul et al., 2018), particularly in chemical screening due to its distinct advantages as similar genetic and physiologic traits, cost-effectiveness, rapid breeding and ease of manipulation. Taking these considerations into account and given the central role of the thyroid in this work, we developed a zebrafish transgenic model to investigate thyroid disease and tumourigenesis and, eventually, assess the impact of EDCs on thyroid function. In this study, our investigation of the role of the prevalent $BRAF^{V600E}$ mutation in PTCs in a transgenic zebrafish model revealed an abnormal thyroid morphogenesis and hyperplasia in young fish accompanied by growth impairment and later by goiter. Surprisingly, when examining the influence of p53 on cancer progression, a normal thyroid development until adulthood was present in transgenic p53 mutant fish harbouring the $BRAF^{V600E}$ mutation, suggesting a complex interplay between BRA^{FV600E} and p53 in thyroid cancer pathogenesis. This model offers valuable insights into the molecular events associated with promotion or suppression of neoplasia and allow us to have a model to study thyroid disease.

Given this data we were faced with a unique opportunity to understand the potential impact of EDCs in a context of a predisposition for thyroid disease such as the BRA^{V600E} mutation and understand the putative capacity of EDCs to induce or promote tumourigenesis in this setting. So far, our preliminary data on BPA 21-day exposure of these transgenic fish, allowed us to observe alterations in thyroid differentiation markers, in a non-monotonic dose manner, which is typically observed for EDCs. This implies that lower doses of BPA may have more pronounced effects than higher ones. Such observations are alarming for public health systems, emphasizing the need for further studies in this direction.

CHAPTER VI | CONCLUSION

In conclusion, this thesis, titled "Thyroid at the Crossroads of Public Health Challenges: from SARS-CoV-2 pandemic to Endocrine Disrupting Chemicals," has brought to light significant findings at the intersection of thyroid health and public well-being.

Our investigation revealed the susceptibility of thyroid follicular cells to SARS-CoV-2 infection, with Furin, one of the key players in SARS-CoV-2 infection, revealed the potential to be a poor prognostic factor for thyroid tumours.

Additionally, a comprehensive review spanning 40 years of research, established the thyroid as a major target for EDCs, with the higher number of studies revealing positive association with cancer, among 6 endocrine organs. To address the challenge of studying EDCs, we developed and characterized a transgenic fish model expressing $BRAF^{V600E}$ in thyroid cells, providing insights into abnormal thyroid morphogenesis and hyperplasia. This model serves as a valuable platform for exploring EDCs' role in the early onset of thyroid disease and cancer promotion within a genetically predisposed context.

Moving forward, this research highlights the need for focused interventions in providing awareness into the challenges associated with the thyroid gland within the broader context of public health. Future efforts should build upon strategies for mitigating the impact of infectious agents such as SARS-CoV-2 on thyroid health and addressing the interplay relationship between EDCs and thyroid neoplasia.

With this thesis we aimed to gain valuable insights into the complex dynamics of thyroid health and contribute to the broader understanding of how environmental factors, including diseases and EDCs, can impact the thyroid gland.

Overall, our studies contribute to support the crucial role of the One Health concept, highlighting the connection of human, animal, environmental health and stating the urgency for a comprehensive understanding and anticipation of the implications of different exposures across diverse populations. These policies are crucial for protecting human health and reduce health care burden.

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