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Unlocking the Clinicopathological and Molecular Profile of Thyroid Cancer: A Genomic Study of Sporadic and Familial Cases

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Unlocking the Clinicopathological and Molecular Profile of Thyroid Cancer: A Genomic Study of Sporadic and Familial Cases

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PUBLICATIONS

This thesis incorporates the collective work that was published between 2020 and 2024 in the form of original and review articles, as well as additional side projects.

All articles will be fully integrated in the body of the thesis, with exception of the BOOK CHAPTER and ARTICLE VI, which will be depicted in the Appendix Chapter.

ARTICLE I

Póvoa AA, **Teixeira E**, Bella-Cueto MR, Melo M, Oliveira MJ, Sobrinho-Simões M, Maciel J, Soares P. *Clinicopathological Features as Prognostic Predictors of Poor Outcome in Papillary Thyroid Carcinoma*. *Cancers* (Basel). Oct 29;12(11):3186 (2020). <https://doi.org/10.3390/cancers12113186>

ARTICLE II

Póvoa AA*, **Teixeira E***, Bella-Cueto MR, Batista R, Pestana A, Melo M, Alves T, Pinto M, Sobrinho-Simões M, Maciel J, Soares P. *Genetic Determinants for Prediction of Outcome of Patients with Papillary Thyroid Carcinoma*. *Cancers* (Basel). Apr 23;13(9):2048. (2021) ***these authors contributed equally to this work.** <https://doi.org/10.3390/cancers13092048>

ARTICLE III

Ramalho D, **Teixeira E**, Cueto R, Correia S, Rocha G, Oliveira MJ, Soares P, Póvoa AA. *Subcentimetric Papillary Thyroid Carcinoma: does diagnosis' kind impact prognosis?* *Cureus* 15(11): e49563. (2023) <https://doi:10.7759/cureus.49563>

ARTICLE IV

Amado A, Varanda J, Castro B, Graça S, Tavares A, Póvoa AA, Soares C, Canberk S, **Teixeira E**, Soares P, Oliveira M. *Subcentimetric papillary thyroid carcinoma with extensive lymph node and brain metastasis: case report & review of literature*. *Endocrinology, Diabetes & Metabolism Case Reports* 2023.4 (2023). <https://doi.org/10.1530/EDM-23-0025>

ARTICLE V

Teixeira E*, Fernandes C*, Paula AC, Lima RT, Batista R, Vinagre J, Bungardean M, Sobrinho-Simões M, Máximo V, Soares P. *Investigating USP42 mutation as underlying cause of Familial Non-Medullary Thyroid Carcinoma*. *International Journal of Molecular Sciences*. 25(3):1522. (2024) ***these authors contributed equally to this work.** <https://doi.org/10.3390/ijms25031522>

APPENDIX

The subsequent ARTICLE VI and BOOK CHAPTER while not integral part of this thesis, were used in INTRODUCTION, and GENERAL DISCUSSION of results.

ARTICLE VI

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*. Environmental Research, 114869. (2022) ***these authors contributed equally to this work.** <https://doi.org/10.1016/j.envres.2022.114869>

BOOK CHAPTER

Soares P*, Cameselle-Teijeiro JM*, **Teixeira E***, Póvoa AA*, Magalhães J, Amendoeira I, Sobrinho-Simões M. *Pathobiology, morphology and clinical significance of minimally invasive thyroid neoplasms* (Accepted for publication, 2023). ***These authors contributed equally to this work.**

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O meu mais sincero obrigada.

- Elisabete

“I became insane, with long intervals of horrible sanity.”

- Edgar Allan Poe

Index

Figure Index.....	XIX
Table Index.....	XXI
Abbreviations	XXIII
Abstract	XXVII
Resumo.....	XXIX
CHAPTER I – INTRODUCTION	1
The thyroid gland	1
1.1. Anatomy and histology.....	1
1.2. Embryogenesis	2
1.3. Thyroid hormones: function, regulation, and synthesis	2
1.3.1. General overview of thyroid hormone function	2
1.3.2. Thyroid hormone regulation – The Hypothalamus-Pituitary-Thyroid axis.....	3
1.3.3. Thyroid hormone synthesis in follicular cells: the major role of iodide (I ⁻) and the sodium-iodide symporter (NIS)	4
Thyroid disorders	7
1.4. Hashimoto’s Thyroiditis.....	7
1.5. Grave’s disease	8
1.6. Sporadic thyroid tumors: from benign to malignant nodules.....	8
1.6.1. Epidemiology	8
1.6.2. Etiology.....	9
1.6.2.1. Exposure to ionizing radiation	9
1.6.2.2. Incidental findings - Overdiagnosis	9
1.6.2.3. Iodide intake	10
1.6.2.4. Environmental pollutants.....	11
1.6.2.5. Other risk factors	11
1.6.3. Tumor Histotypes	11
1.6.3.1. Benign lesions	12
1.6.3.2. Low-risk neoplasms.....	13
1.6.3.3. Malignant Thyroid Neoplasms	13
1.6.3.3.1. Papillary Thyroid Carcinoma (PTC)	14
1.6.3.3.1.1. Infiltrative Follicular Variant PTC (IFV-PTC)	14
1.6.3.3.1.2. Tall cell PTC.....	14
1.6.3.3.1.3. Columnar cell PTC.....	14
1.6.3.3.1.4. Hobnail PTC	15
1.6.3.3.1.5. Solid PTC	15
1.6.3.3.1.6. Diffuse sclerosing PTC	15

1.6.3.3.1.7.	Warthin-like PTC.....	15
1.6.3.3.1.8.	Oncocytic PTC.....	15
1.6.3.3.2.	Follicular Thyroid Carcinoma (FTC).....	16
1.6.3.3.3.	Invasive Encapsulated Follicular Variant of Papillary Thyroid Carcinoma (IEFV-PCT)	16
1.6.3.3.4.	Oncocytic Thyroid Carcinoma (OCA)	17
1.6.3.3.5.	Differentiated High-Grade Thyroid Carcinoma (DHGTC).....	17
1.6.3.3.6.	Poorly-Differentiated Thyroid Carcinomas (PDTC).....	17
1.6.3.3.7.	Anaplastic Thyroid Carcinomas (ACA)	18
1.6.4.	Thyroid Cancer Staging.....	18
1.6.5.	Thyroid Cancer Treatment	18
1.6.6.	Thyroid cancer prognostic factors.....	19
1.6.6.1.	Patient characteristics.....	19
1.6.6.2.	Tumor characteristics.....	20
1.6.6.3.	Treatment response.....	20
1.6.6.4.	Molecular markers	20
1.6.7.	Familial Thyroid Cancer	25
CHAPTER II – AIMS		31
CHAPTER III – MATERIALS AND METHODS.....		35
CHAPTER IV – RESULTS.....		39
4.1.	ARTICLE I.....	39
4.2.	ARTICLE II.....	59
4.3.	ARTICLE III.....	81
4.4.	ARTICLE IV.....	97
4.5.	ARTICLE V.....	107
4.6.	<i>USP42</i> GENE KNOCKOUT SYNTHESIS USING CRISPR-RNP EDITING TECHNIQUE (in development).....	135
4.6.1.	MATERIALS AND METHODS.....	135
4.6.1.1.	Knockout of <i>USP42</i> gene using CRISPR-RNP	135
4.6.1.2.	Clonal selection of <i>USP42</i> and <i>CDK4</i> edited cells.....	136
4.6.1.3.	<i>USP42</i> edited cells DNA extraction	136
4.6.1.4.	PCR and Sanger sequencing validation of <i>USP42</i>	136
4.6.1.5.	Selection of <i>USP42</i> Knockout/Knockdown clones	137
4.6.1.6.	<i>USP42</i> CRISPR clones characterization (a preliminary analysis)	137
4.6.2.	RESULTS.....	137
4.6.2.1.	<i>USP42</i> CRISPR-RNP transfection evaluation	137
4.6.2.2.	<i>USP42</i> CRISPR-RNP clone cells morphology.....	138
4.6.2.3.	<i>USP42</i> CRISPR-RNP candidate clones' selection	140

4.6.2.4.	SeqScreener Gene Edit Confirmation	140
4.6.2.5.	Mutation Surveyor® DNA Variant Analysis	143
4.6.2.6.	USP42 mRNA expression on <i>USP42</i> clones	145
4.6.2.7.	USP42 protein expression evaluation	146
4.6.2.8.	β-actin protein expression evaluation	146
4.6.2.9.	Cell cycle-related protein expression evaluation	147
4.6.3.	DISCUSSION	149
4.7.	EVALUATION OF THE PATHOGENIC ROLE OF <i>KCNB2</i> AS A PUTATIVE GENE IN NSFNMTC (in development)	153
4.7.1.	INTRODUCTION	153
4.7.2.	MATERIALS AND METHODS	155
4.7.2.1.	<i>Family presentation</i>	155
4.7.2.2.	<i>In silico</i> studies	156
4.7.2.3.	<i>In vitro</i> studies	156
4.7.2.3.1.	FRTL-5 cell line	156
4.7.2.3.2.	FRTL-5 transfected cells clonal selection	157
4.7.2.3.3.	<i>KCNB2</i> edited cells DNA extraction	157
4.7.2.3.4.	PCR and Sanger Sequencing validation of <i>KCNB2</i> transfected cells	157
4.7.2.3.5.	FRTL-5 proliferation assessment	158
4.7.2.3.6.	RNA extraction and cDNA conversion	158
4.7.2.3.7.	Real-Time quantitative Polymerase Chain Reaction	158
4.7.2.3.8.	Mycoplasma spp. contamination elimination	159
4.7.3.	RESULTS	159
4.7.3.1.	<i>In silico</i> results	159
4.7.3.2.	<i>In vitro</i> results	162
4.7.3.2.1.	<i>KCNB2</i> transfected cell clonal selection results	162
4.7.3.2.2.	Cell proliferation analysis	163
4.7.3.2.3.	<i>KCNB2</i> mRNA expression evaluation	164
4.7.3.2.4.	Thyroid cell markers mRNA expression evaluation	165
4.7.4.	DISCUSSION	165
CHAPTER V – GENERAL DISCUSSION		171
CHAPTER VI – GENERAL CONCLUSIONS		181
REFERENCES		185
Appendix		201
ARTICLE VI		201
BOOK CHAPTER		249

Figure Index

Figure 1 - A) Anatomy and B) histology of the thyroid gland.....	1
Figure 2 - The Hypothalamus-Pituitary-Thyroid (HPT) axis - regulation of T3 and T4 thyroid hormone synthesis.....	4
Figure 3 - Thyroid hormone synthesis by thyroid follicular cells and cellular homeostasis maintenance.....	6
Figure 4 - Histologic subtypes of thyroid tumors. A) Thyroid tumors major groups and origin cell type. B) Further subdivision of thyroid tumors originated in thyroid follicular cells.....	12
Figure 5 - Simple representation of the Mitogen-Activating Protein Kinase (MAPK), and the Phosphatidylinositol-3-kinase (PI3K)/AKT-pathways.....	25
Figure 6 - Images of the pool of Nthy-ori 3-1 cells grew up to T75 flasks, post-transfection and before clonal selection.....	138
Figure 7 - Nthy-ori 3-1 parental cells morphology in comparison to CRISPR-RNP <i>USP42</i> edited cells.....	138
Figure 8 - Nthy-ori 3-1 parental cells morphology in comparison to CRISPR-RNP <i>USP42</i> edited cells four weeks after cell stabilization	139
Figure 9 - Electrophoresis gels of the DNA of the <i>USP42</i> viable clones	140
Figure 10 - CRISPR- RNP edited <i>USP42</i> clones eletropherograms - A) Clone P1C12; B) Clone P2F5; C) Clone P2F12; D) Clone P4C5; and E) Clone P5D2	144
Figure 11 - <i>USP42</i> mRNA expression of CRISPR-RNP <i>USP42</i> edited clones in comparison to the parental cell-line.....	145
Figure 12 - A) <i>USP42</i> protein expression of CRISPR-RNP <i>USP42</i> edited clones in comparison to the parental cell-line	146
Figure 13 - A) β -actin protein expression of CRISPR-RNP <i>USP42</i> edited clones in comparison to the parental cell-line	147
Figure 14 - Cell cycle-related protein expression of CRISPR-RNP <i>USP42</i> edited clones in comparison to the parental cell-line. A) p53 protein expression; B) Cyclin D1 protein expression; C) p21 protein expression; D) p27 protein expression.	148
Figure 15 - kv2.2 channel and predicted mutation location	154
Figure 16 - vgKCNs location in the human genome	154
Figure 17 - Normal ionic conductivity in thyroid follicular cells.....	155
Figure 18 - Heredogram of family C	156
Figure 19 - vgKCN genetic alterations found in TCGA database from Cell, 2014, and in an in-house database composed of oncocyctic tumors (OA and OCAs).....	160
Figure 20 - Analysis of clinicopathological factors in vgKCN-altered (orange) and in vgKCN-WT cases (grey) - A) Patient sex, B) Clinical stage, C) Distant metastases, D) Extrathyroidal extension, E) Tumor location, F) RAI treatment, G) Disease recurrence, H) Disease-free survival, and I) Overall survival.....	161
Figure 21 - Electropherograms from the analysis of transfection in the pool of cells. A) Electrophoresis results of <i>KCNB2</i> WT and Mutated sequences and of pCMV6 vector, B) <i>KCNB2</i> WT hotspot region, C) <i>KCNB2</i> Mutated hotspot region, D) pCMV6 vector representative region	162
Figure 22 - Electrophoresis results of antibiotic and single cell clonal selection in 1% agarose gel. A) Empty vector clone G5 amplification, B) <i>KCNB2</i> WT and Mutated clones' amplification of <i>KCNB2</i> hotspot region, C) Amplification of the middle and end section of the ORF of <i>KCNB2</i> WT and Mutated pool of cells and of tested <i>KCNB2</i> WT G5 and <i>KCNB2</i> Mutated D8 clones.....	163
Figure 23 - Cell proliferation assay using Empty vector transfected cells. Fold change [(Final cell count/Initial cell count)-1] was calculated at 24h, 48h and 72h after plating.....	164

Figure 24 – Representative images of the six tested cell concentrations of Empty vector transfected cells at 48h timepoint. A) 2×10^4 , B) 3×10^4 , C) 4×10^4 , D) 5×10^4 , E) 7.5×10^4 , and F) 1×10^5 cells/well 164

Figure 25 – KCNB2 mRNA expression evaluation 165

Figure 26 - Thyroid markers mRNA expression. A) Rat slc5a5 expression on FRTL-5 transfected pool of cells, B) Rat tsh receptor expression on FRTL-5 transfected pool of cells 165

Table Index

Table 1 - Molecular and immunohistochemical markers associated with thyroid cancer subtype.	21
Table 2 - Syndromic and Non-syndromic forms of FNMTTC, respective altered genes and associated thyroid lesions.	27
Table 3 – Sequences of guides and primers used for the CRISPR-RNP assay to obtain a <i>USP42</i> knockout cell line	135
Table 4 - SeqScreener Gene Edit Confirmation software results of the eletropherograms of the pool of cells collected after CRIPR-RNP transfection and before clonal selection.	141
Table 5 - SeqScreener Gene Edit Confirmation software general results of the eletropherograms of the CRIPR-RNP <i>USP42</i> edited cells after clonal selection.	142
Table 6 - Mutation Surveyor DNA Variant Analysis software results of the eletropherograms of the CRIPR-RNP <i>USP42</i> clones most likely to be <i>USP42</i> knockouts.....	145
Table 7 – Characteristics of <i>KCNB2</i> and <i>pCMV6</i> primers used for clonal selection – primer sequence, amplicon size and annealing temperature.	158

Abbreviations

Acronym

¹²³ I ⁻	Iodine-123 radioactive isotope
¹³¹ I ⁻	Iodine-131 radioactive isotope
^{99m} TcO ₄ ⁻	Pertechnetate
ACA	Anaplastic Thyroid Carcinoma
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ALK	Anaplastic lymphoma kinase
APC	APC regulator of WNT Signaling Pathway
ARID2	AT-Rich Interaction Domain 2
ATA	American Thyroid Association
ATK1	AKT Serine/Threonine Kinase 1
ATM	ATM Serine/Threonine Kinase
BMI	Body Mass Index
bp	base pair
BPA	Bisphenol-A
BPAF	Bisphenol-AF
BPE	Bisphenol-E
BRAF	B-Raf Proto-Oncogene
cAMP	Cyclic Adenosine Monophosphate
CCDC6	Coiled-Coil Domain Containing 6
CD31	Platelet endothelial cell adhesion molecule (PECAM-1)
CDC123	Cell Division Cycle 123
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B
CHEK2	Checkpoint kinase 2
ClO ₃ ⁻	Chlorate
ClO ₄ ⁻	Perchlorate
COSMIC	Catalogue Of Somatic Mutations In Cancer
CRISPR-RNP	Clustered Regularly Interspaced Short Palindromic Repeats - Ribonucleoprotein
CS	Carney Syndrome
CT scan	Computed Tomography scan
CTNNB1	Catenin Beta 1
D2-40	Podoplanin
DEHAL1	Iodotyrosine dehalogenase 1
DEHP	Di(2-ethylhexyl)phthalate
DHGTC	Differentiated High-Grade Thyroid Carcinoma
DICER 1	Dicer 1, Ribonuclease III
DIT	Diiodotyrosine
DM	Distant Metastases
DRP1	Dynamin related protein 1
DUB	Deubiquitinating enzyme
DUOX	Dual Oxidase
EGFR	Epidermal growth factor receptor
EIF1AX	Eukaryotic Translation Initiation Factor 1A X-Linked
EDC	Endocrine-Disrupting Chemical
EDTA	Ethylenediaminetetraacetic Acid
ETE	Extrathyroidal Extension

Ets-1	ETS Proto-Oncogene 1, Transcription Factor
ETV6	ETS Variant Transcription Factor 6
EV	Empty vector
EZH1	Enhancer Of Zeste 1 Polycomb Repressive Complex 2 Subunit
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFPE	Formalin-Fixed Paraffin-Embedded
FMTC	Familial Medullary Thyroid Carcinoma
FNAB	Fine-Needle Aspiration Biopsy
FND	Follicular Nodular Disease
FNMTCT	Familial Non-Medullary Thyroid Carcinoma
FOXE1	Forkhead Box E1
FTA	Follicular Thyroid Adenoma
FTC	Follicular Thyroid Cancer
FT-UMPs	Follicular Thyroid Tumors of Uncertain Malignant Potential
GD	Grave's disease
GLIS1	GLIS Family Zinc Finger 1
GLIS3	GLIS Family Zinc Finger 3
GNAS	GNAS Complex Locus
gnomAD	Genome Aggregation Database
GPCR	G Protein-Coupled Receptor
H&E	Hematoxylin and eosin
HDR	Homology-Directed Repair
HHEX	Hematopoietically-expressed homeobox protein
HPT	Hypothalamus-Pituitary-Thyroid
HT	Hashimoto's Thyroiditis
HTT	Hyalinizing Trabecular Thyroid Tumors
I-	Iodide
IEFV-PCT	Invasive Encapsulated Follicular Variant of Papillary Thyroid Carcinoma
IFV-PTC	Infiltrative Follicular Variant PTC
IntOGen	Integrative OncoGenomics
KCNA2	Potassium Voltage-Gated Channel Subfamily A Member 2
KCNA10	Potassium Voltage-Gated Channel Subfamily A Member 10
KCNB2	Potassium Voltage-Gated Channel Subfamily B Member 2
KCNC4	Potassium Voltage-Gated Channel Subfamily C Member 4
KCND3	Potassium Voltage-Gated Channel Subfamily D Member 3
KCNE3	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 3
KCNH1	Potassium Voltage-Gated Channel Subfamily H Member 1
KCNH2	Potassium Voltage-Gated Channel Subfamily H Member 2 (HERG)
KCNIP2	Potassium Voltage-Gated Channel Interacting Protein 2
KCNIP4	Potassium Voltage-Gated Channel Interacting Protein 4
KI	Knockin
KO	Knockout
LNM	Lymph Node Metastases
LOH	Loss of Heterozygosity
LT4	Levothyroxine
MAP2K5	Mitogen-Activated Protein Kinase Kinase 5
MAPK pathway	Mitogen-Activating Protein Kinase pathway
MCT8	Monocarboxylate transporter 8 (SLC16A2)
MEHP	Mono(2-ethylhexyl) phthalate
MEK	Mitogen-activated protein kinase kinase

MI-FTC	Minimally-invasive Follicular Thyroid Carcinoma
MIT	Monoiodotyrosine
MMP9	Matrix metalloproteinase-9
MNG1	Multinodular Goiter-1
mPTC	Papillary microcarcinoma
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic Acid
MTC	Medullary Thyroid Carcinoma
mTOR	Mammalian target of rapamycin
Mut	Mutated
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCOA4	Nuclear Receptor Coactivator 4
NDUFA13	NADH:Ubiquinone Oxidoreductase Subunit A13
NF1	Neurofibromin 1
NF2	NF2, Moesin-Ezrin-Radixin Like (MERLIN) Tumor Suppressor
NHEJ	Non-Homologous End Joining
NIFTP	Non-Invasive Follicular Thyroid neoplasm with Papillary-like nuclear features
NIS	Sodium-Iodide Symporter
NKX2.1	NK2 Homeobox 1
NMTC1	Non-Medullary Thyroid Cancer 1
NOP53	NOP53 Ribosome Biogenesis Factor
NOTCH1	Neurogenic locus notch homolog protein 1
NSFNMTC	Non-syndromic Familial Non-Medullary Thyroid Carcinoma
NTN4	Netrin 4
NTRK	Neurotrophic Tyrosine Receptor Kinase
NTRK3	Neurotrophic Receptor Tyrosine Kinase 3
OA	Oncocytic Adenoma
OCA	Oncocytic Thyroid Carcinoma
ORF	Open Reading Frame
OS	Overall survival
TP53	Tumor protein P53
PAX8	Paired Box 8
PCBs	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
PDTC	Poorly-Differentiated Thyroid Carcinomas
PI3K/AKT	Phosphatidylinositol-3-kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein Kinase A
PPARG	Peroxisome Proliferator Activated Receptor Gamma
PRKAR1A	Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha
PRN	Cytosolic Iron-Sulfur Assembly Component 3
PTC	Papillary Thyroid Cancer
PTCSC3	Papillary Thyroid Carcinoma Susceptibility Candidate 3
PTEN	Phosphatase and Tensin homologue
RAI	Raidioiodine therapy (131I)
RAS	RAS Proto-Oncogene (included NRAS, HRAS and KRAS)
RB1	RB Transcriptional Corepressor 1
ReO4⁻	Perrhenate
RET	RET Proto-Oncogene
RFS	Recurrence-Free Survival
RON	Registo Oncológico Nacional

RT-PCR	Real-time Polymerase Chain Reaction
SCL5A5	Solute Carrier Family 5 Member 5
SCN⁻	Thiocyanate
SFNMTC	Syndromic Familial Non-Medullary Thyroid Carcinoma
SLC16A2	Solute Carrier Family 16 Member 2
SLC26A4	Solute Carrier Family 26 Member 4 (Pendrin)
SNP	Single-Nucleotide Polymorphism
SRGAP1	SLIT-ROBO Rho GTPase Activating Protein 1)
T3	Triiodothyronine
T4	Thyroxine
TC	Thyroid Cancer
TCGA	The Cancer Genome Atlas
TCO	Thyroid Carcinoma, Nonmedullary, With Cell Oxyphilia
TERTp	Telomerase reverse transcriptase promoter
Tg	Thyroglobulin
THADA	THADA Armadillo Repeat Containing
TIMM44	Translocase Of Inner Mitochondrial Membrane 44
TKI	Tyrosine Kinase Inhibitor
TNM	Tumour, Node, Metastasis
TPO	Thyroid Peroxidase
TRH	Thyrotropin-Releasing Hormone
TSH	Thyroid Stimulating Hormone
TSHr	Thyroid Stimulating Hormone receptor
TITF-1	Thyroid Transcription Factor-1
UMPs	Uncertain Malignant Potential
UNICEF	United Nations Children's Fund
USP	Ubiquitin Specific Peptidase
USP6	Ubiquitin Specific Peptidase 6
USP13	Ubiquitin Specific Peptidase 13
USP15	Ubiquitin Specific Peptidase 15
USP18	Ubiquitin Specific Peptidase 18
USP26	Ubiquitin Specific Peptidase 26
USP30	Ubiquitin Specific Peptidase 30
USP39	Ubiquitin Specific Peptidase 39
USP42	Ubiquitin Specific Peptidase 42
USP9X	Ubiquitin Specific Peptidase 9 X-Linked
vgKCN	Voltage-gated Potassium Channel
WES	Whole-Exome Sequencing
WHO	World Health Organization
WI-FTC	Widely-invasive Follicular Thyroid Carcinoma
WRN	WRN RecQ Like Helicase
WS	Werner Syndrome
WT	Wild-type
WTD-UMPs	Well-Differentiated Tumors of Uncertain Malignant Potential

Abstract

Thyroid cancer (TC) remains understudied when compared to other cancers, resulting in persistent gaps in patient risk assessment, prognostic, and management decisions. Identifying low-risk tumors and predicting the risk of recurrence/persistent disease, structural disease and disease-specific mortality remains challenging for both sporadic and familial forms of TC.

Papillary Thyroid Carcinoma (PTC) usually presents with good prognosis, however a number of cases present with high aggressiveness and distant metastases. Also, the genetics behind Non-Syndromic Familial Non-medullary Thyroid Carcinoma (NSFNMTTC) is rather heterogeneous and poorly understood, leaving many affected families without identified genetic risk *loci*. The establishment of clinicopathological and molecular markers is essential for adjusting patient management, genetic counseling and for contributing to the development of new therapies. Disease prevention and active surveillance are key factors in reducing aggressive treatments, comorbidities, invasive surgeries, and the burden to the healthcare system.

This work aims to advance early diagnosis, risk stratification, and personalized medicine for thyroid cancer patients through a comprehensive study of both sporadic and familial cases.

In a first approach, our team showed that clinicopathological factors like sex, presence of psammoma bodies, extrathyroidal extension, lateral compartment lymph node metastasis, lymph vessel invasion, venous invasion, necrosis, and incomplete surgical resection are crucial to predict TC outcome. We further showed that tumor molecular status also has implications in disease progression. We demonstrate *BRAF* mutation to be associated with locoregional tumor aggressiveness, while *TERT promoter* mutations are linked to structural disease and PTC-related mortality. Analysis of concomitant *BRAF* and *TERT promoter* mutations were shown to be associated with disease progression and poorer prognosis. No association between *RAS* mutations and patient worse prognosis was found.

In our study, we also compare the outcomes of subcentimetric PTC tumors (PTC tumor size ≤ 1 cm) that were clinically diagnosed *versus* incidental tumors. We suggest that, for subcentimetric tumors, less invasive surgery and post-operative surveillance can be applied for low-risk malignancies. However, this work further showed that subcentimetric PTC tumors, although frequently being associated with favorable disease outcome, sometimes may result in distant metastases and PTC-related death. We explored and reported in this thesis a case report of a patient with a lethal subcentimetric PTC tumor that presented with aggressive clinicopathological and molecular markers. Hence, emphasizing that PTC prognosis should consider patient characteristics, histological components, molecular landscape, and the risk of infiltration or invasion into surrounding tissues and distant sites.

Familial Non-Medullary Thyroid Carcinoma (FNMTc) is still a largely unexplored disease and our team employed Whole-Exome Sequencing (WES) in two families (Family R and Family C) in order to progress in the understanding of the clinical situation. Although both families comprised the criteria for TC heredity (>3 first-degree relatives affected by the disease and diagnosis at early age), no syndromic forms of the disease were detected. Therefore, our team, aiming to explore the pathogenesis and genetics of NSFNMTC, by WES analysis, identified in these families two novel germline, possibly pathogenic, mutations in the *USP42* gene in family R and in the *KCNB2* gene in family C. Our *in silico* and *in vitro* assays demonstrate the putative pathogenic role of these germline mutations in NSFNMTC. To our knowledge, we describe for the first time mutations in *USP42* and *KCNB2* genes in the context of NSFNMTC

With our in-depth exploration of sporadic and familial thyroid carcinomas we contribute with new data that can lead to better patient risk stratification and prognostication, as well as improve genetic risk assessment, providing patients with a clearer understanding of their specific situation and facilitate a more informed and confident decision-making process by the physicians.

Resumo

O cancro da tiroide (CT) permanece pouco estudado em comparação com outros cancros, resultando em lacunas persistentes na estratificação de risco, prognóstico e tratamento clínico adequado a cada paciente. Identificar tumores de baixo risco e prever o risco de recorrência/persistência de doença, doença estrutural e mortalidade, permanece um desafio tanto para formas esporádicas assim como para formas familiares do CT.

O Carcinoma Papilar da Tiroide (CPT) geralmente apresenta um bom prognóstico, no entanto, alguns casos manifestam alta agressividade e metástases à distância. Além disso, a genética por detrás do Carcinoma Não Medular Familiar da Tiroide Não Sindrómico (CNMFTNS) é bastante heterogénea e pouco compreendida, deixando muitas famílias afetadas sem *loci* genéticos de risco identificados. O estabelecimento de marcadores clinicopatológicos e moleculares é essencial para ajustar o manejo clínico do paciente, auxiliar o aconselhamento genético e contribuir para o desenvolvimento de novas terapias. A prevenção da doença e a vigilância ativa são fatores chave na redução de tratamentos agressivos, comorbidades, cirurgias invasivas e no encargo económico para o sistema de saúde.

Este trabalho tem como objetivo auxiliar no diagnóstico precoce, na estratificação de risco e na medicina personalizada para pacientes com CT, através de um estudo abrangente de casos esporádicos e familiares.

Numa primeira abordagem, a nossa equipa demonstrou que fatores clinicopatológicos como sexo, presença de corpos psamomatosos, extensão extratiroideia, metástases em gânglios linfáticos no compartimento lateral, invasão de vasos linfáticos, invasão venosa, necrose e ressecção cirúrgica incompleta são cruciais para prever o desfecho do CT. Demonstramos ainda que o estado molecular do tumor também tem implicações na progressão da doença. Demonstramos que a mutação *BRAF* está associada à agressividade tumoral locorregional, enquanto que, as mutações no promotor da *TERT* estão ligadas à doença estrutural e à mortalidade relacionada com o CPT. A análise de mutações concomitantes *BRAF* e promotor da *TERT* mostrou estarem associadas à progressão da doença e a um pior prognóstico. Não foi encontrada nenhuma associação entre mutações *RAS* e um pior prognóstico do paciente.

No nosso estudo, nós também comparamos o desfecho de tumores CPT subcentimétricos (tamanho ≤ 1 cm) diagnosticados clinicamente em comparação a tumores incidentais. Sugerimos que, para tumores subcentimétricos, podem ser aplicadas cirurgias menos invasivas e vigilância pós-operatória para malignidades de baixo risco. No entanto, este trabalho mostrou ainda que os tumores CPT subcentimétricos, embora frequentemente associados a um desfecho favorável da doença, por vezes podem resultar em metástases à

distância e morte relacionada com CPT. Exploramos e reportamos nesta tese um caso clínico com um tumor CPT subcentimétrico letal que apresentava marcadores clinicopatológicos e moleculares agressivos. Assim, enfatizando que o prognóstico do CPT deve considerar características do paciente, componentes histológicos, estadió molecular e o risco de infiltração ou invasão em tecidos circundantes e à distância.

O Carcinoma Não Medular Familiar da Tireoide (CNMFT) é ainda uma doença pouco explorada e a nossa equipa aplicou *Whole-Exome Sequencing* (WES) em duas famílias (Família R e Família C) de forma a contribuir para o conhecimento da situação clínica. Embora ambas as famílias cumprissem os critérios de hereditariedade (>3 familiares de primeiro grau afetados pela doença e diagnóstico em idade precoce), não foram detetadas formas sindrómicas da doença. Assim, a nossa equipa, com o objetivo de explorar a patogénese e genética do CNMFTNS, através da análise WES, identificou nessas famílias duas novas mutações germinativas, possivelmente patogénicas, no gene *USP42* na família R e no gene *KCNB2* na família C. As nossas análises *in silico* e *in vitro* demonstram o potencial papel patogénico destas mutações germinativas no CNMFTNS. Dentro do nosso conhecimento, descrevemos pela primeira vez mutações nos genes *USP42* e *KCNB2* no contexto do CNMFTNS.

Com a nossa abordagem aprofundada dos carcinomas da tireoide esporádicos e familiares, contribuímos com novos dados que podem melhorar a estratificação do risco e prognóstico dos pacientes, bem como melhorar a avaliação do risco genético, proporcionando aos pacientes uma compreensão mais clara da sua situação específica e facilitando um processo de tomada de decisão mais informado e confiante pelos clínicos.

CHAPTER I

INTRODUCTION

CHAPTER I – INTRODUCTION

The thyroid gland

1.1. Anatomy and histology

The thyroid is a butterfly shaped gland located in the neck, below the hyoid bone and anterior to the trachea (**Figure 1, A**) [1]. This vital endocrine organ produces hormones that play major roles in the human organism, being implicated in organ development, carbohydrate, lipid and protein synthesis, as well as body homeostasis regulation [2, 3].

The thyroid is divided macroscopically into 3 parts: right lobe, left lobe and the connective part, the isthmus (**Figure 1, A**) [1]. The lobes present a conical shape, with a base, an apex, three surfaces (lateral, medial, and posterolateral) and two borders (anterior and posterior). The isthmus presents two surfaces (anterior and posterior) and two borders (inferior and superior) [1].

Microscopically, the lobes are composed of two endocrine cell types: follicular cells and parafollicular cells (C cells) (**Figure 1, B**). Follicular cells are cuboid cells organized in spherical structures, that constitute the functional units of the thyroid gland (**Figure 1, B**). These functional units, the thyroid follicles, contain a gelatinous substance in their interior, denominated colloid, where the thyroid hormones T3 (triiodothyronine) and T4 (thyroxine) are produced and stored. The colloid is mostly constituted by thyroglobulin (Tg), an acidophilic secretory glycoprotein (**Figure 1, B**) [1]. Scattered between the thyroid follicles, are present parafollicular cells which are derived from neural crest cells [1]. These cells secrete calcitonin, a vital hormone in calcium regulation (**Figure 1, B**). These structures are held together by a delicate network of reticular fibers with an extensive capillary bed [4].

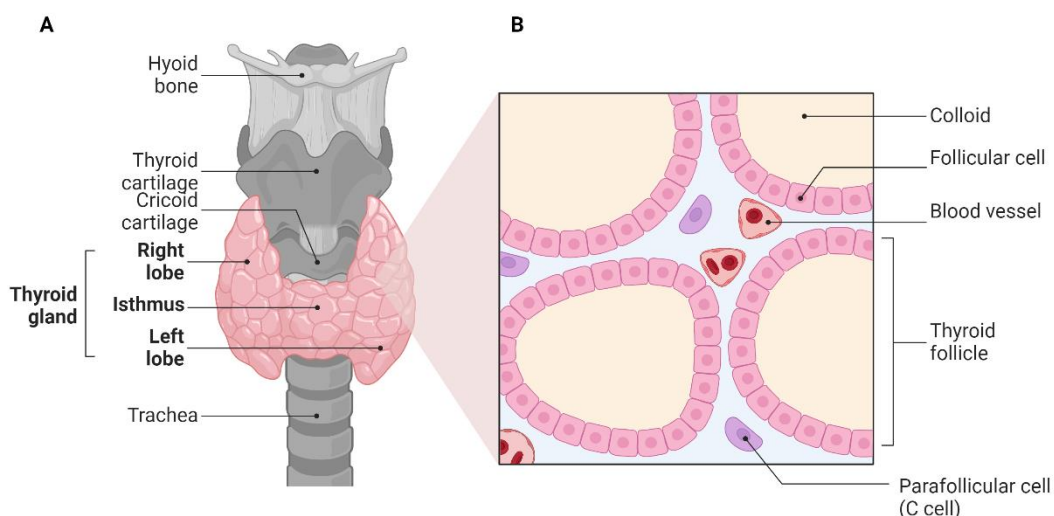


Figure 1 - A) Anatomy and B) histology of the thyroid gland. Created with Biorender.com

1.2. Embryogenesis

The thyroid is the first endocrine organ to appear during embryogenesis, at the 3rd week of gestation [2, 5, 6], with transcription factors such as NKX2.1, PAX8, FOXE1, and HHEX, being expressed at the 4th week [7]. It has two embryological origins: the primitive pharynx and the neural crest.

Proliferation of endodermal epithelial cells that form a diverticulum in the pharyngeal floor start thyroid embryogenesis [5]. First, the embryonic thyroid develops caudal to the tuberculum impar (the median tongue bud) and after several stages of transformation (e.g. enlargement, bifurcation, lobulation), finally sets in the neck as a bilobed encapsulated structure by the 7th week of gestation [2, 5, 6]. The thyroid stimulating hormone receptor (TSHr) begins to be expressed at the 8th week of gestation [7]. The diverticulum finally descends to its final adult position by the 11th week of gestation and almost in its final form is already capable of producing colloid and uptake radioiodine (¹³¹I) [2, 5]. Genes such as SCL5A5 (which codes for the sodium-iodide symporter – NIS), Thyroglobulin (Tg), Thyroid peroxidase (TPO) and dual oxidase (DUOX) genes are expressed at this stage of development [7].

The histological differentiation of the thyroid gland can be divided into three stages: pre-colloid - which occurs between the 7th and the 13th week of gestation; colloid - which occurs between the 13th and the 14th week and finally the follicular stage, which occurs after the 14th week of gestation [2]. Interestingly, despite organ formation is completed after 11 weeks, fetuses continue to receive maternal thyroid hormones, which are critical for their development, until they are capable of producing hormones for themselves, which occurs by the 16th week of pregnancy [5, 8]. The fetal thyroid stimulating hormone (TSH) begins its production at the 10th week of gestation, although in low circulating levels, reaching its peak at week 30 [7]. However, already in week 20, the TSHr is already sensitive to fetal TSH. Levels of T4 are described to be detected at the 18th week of gestation, reaching its peak between the 34th and 36th weeks [7].

It is important to note that full maturation of the thyroid gland is only achieved 30 days after birth, when TSH and T4 levels in the blood are at similar concentration as in adults [7].

1.3. Thyroid hormones: function, regulation, and synthesis

1.3.1. General overview of thyroid hormone function:

The thyroid hormones T3 and T4, perform fundamental roles in orchestrating a multitude of physiological functions.

The receiving and further production of thyroid hormones brings great implications in the development of the brain of fetuses and newborns, continuing to have major implications during

growth and throughout lifetime [9]. T3 and T4 hormones have been shown to impact neuronal and glial cell differentiation and migration, synaptogenesis, and myelination [9]. The lack of hormone was shown to result in neurocognitive impairment and retardation [9]. Even a slight reduction in maternal T4 production during gestation is sufficient to impair cognitive development in the newborn and result in decreased IQ in an irreversible manner [10-12]. In adults, the lack or excess of hormone production also brings great consequences that will be further explored in this thesis [5, 13]. Hormone fluctuations can influence mood, cognition, and mental well-being, being associated with mental disorders such as depression and anxiety [14, 15]. T3 and T4 also stimulate the breakdown of carbohydrates, proteins, and fats, fostering energy production for various bodily activities [16]. Regulation of body temperature and thermogenesis is also regulated by thyroid hormones [17]. By affecting heart rate, contractility, and blood pressure, these hormones have also been shown to be important for the cardiovascular system [5]. Dysregulation of thyroid hormones can also contribute to a spectrum of conditions from menstrual irregularities and infertility, both in men and women [18, 19]. Beyond these, proper levels of T3 and T4 play a role in skin texture, hair growth, bone density, and the overall balance of endocrine functions in the body [20-22].

1.3.2. Thyroid hormone regulation – The Hypothalamus-Pituitary-Thyroid axis

The Hypothalamus-Pituitary-Thyroid (HPT) axis is a complex hormonal system that regulates the function of the thyroid gland and the synthesis of thyroid hormones (**Figure 2**). The process begins in the hypothalamus, which produces the Thyrotropin-Releasing Hormone (TRH), under a feedback mechanism, when receiving signals that thyroid hormones are in low levels in the blood (**Figure 2, A**). TRH stimulates the pituitary gland to produce TSH (**Figure 2, B**), which then enters the blood stream and reaches the thyroid gland (**Figure 2, C-D**). TSH recognizes TSHr, a G-protein-coupled seven-transmembrane receptor, that locates at the basolateral membrane of follicular cells [23]. In response to TSHr activation, a series of molecular mechanisms are activated inside the follicular cells, leading to the production and storage of T3 (20%) and T4 (80%) hormones in the colloid (**Figure 2, E**), that are later released into the blood stream (**Figure 2, F**) in order to reach cells (**Figure 2, G**) and resulting in the transcription and activation of target genes (**Figure 2, H**). When the circulating levels of T3 and T4 are high, the negative feedback mechanism is activated, resulting in the inactivation of TSH and TRH hormone synthesis (**Figure 2, I-J**). The tight regulation of this system allows to keep homeostasis [5, 24, 25].

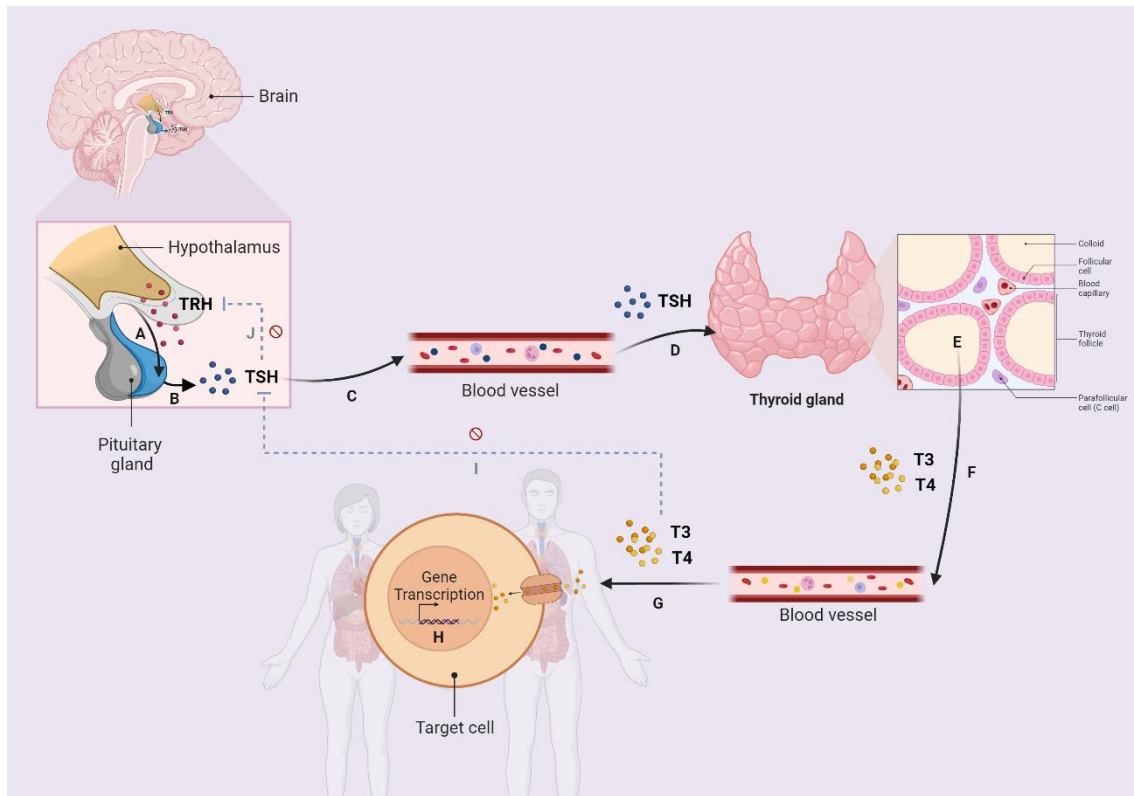


Figure 2 - The Hypothalamus-Pituitary-Thyroid (HPT) axis - regulation of T3 and T4 thyroid hormone synthesis. Created with Biorender.com

1.3.3. Thyroid hormone synthesis in follicular cells: the major role of iodide (I^-) and the sodium-iodide symporter (NIS)

The synthesis of thyroid hormones is dependent on the capacity of the follicular cells to incorporate iodide (I^-), the limiting component for the hormone synthesis [26]. In fact, the ability of the thyroid to accumulate I^- has long provided the diagnostic and therapeutic tools for the management of thyroid diseases, in particular thyroid cancer (TC) [27]. In 1896, Baumann described for the first time that the thyroid gland concentrates I^- by a factor of 20-40 times with respect to plasma under physiological conditions [28]. In 1996, the Sodium-Iodide Symporter (NIS), which mediates I^- uptake in follicular cells was first cloned in rats (*X. laevis* oocytes) using cDNA libraries derived from FRTL-5 cells and then in humans by Smanik and colleagues [29, 30].

I^- deficiency can often bring great consequences in human health since it affects the production and concentration of thyroid hormones, as previously described (Chapter 1.3.1) [12].

Ion channels and transporters rarely function in isolation, instead, they often regulate each other (**Figure 3**) [12]. The major determinants of I^- retention in follicular cells are NIS mediated I^- uptake, organification and efflux [31].

When reaching the thyroid parenchyma through the blood stream, TSH binds to TSHr, activating the cAMP Signaling Pathway, through G protein-coupled receptor (GPCR) signaling

(**Figure 3**) [31]. Then, cAMP activates protein kinase A (PKA) that activates NIS transcription [10, 26, 32]. Upon activation, and in normal conditions, NIS imports I^- together with 2 Na^+ ions (**Figure 3**). This transport is permitted by the gradient created by the Na^+/K^+ ATPase that exports 3 Na^+ ions from the cell, followed by the import of 2 K^+ ions (**Figure 3**) [31]. The excess of K^+ ions creates a hyperpolarization of the cell membrane, activating potassium channels to export K^+ ions to the exterior of the cell. Disruption of this chemical balance could be sufficient to impair NIS function, even though no mutations in NIS are present [33]. Even though some NIS mutations have been reported in the literature, these seem little relevant for the study of iodine deficiencies [34, 35]. Interestingly, NIS presents a very low affinity for I^- , which increases 10 times when the 2 Na^+ molecules bind to the transporter. Almost 80% of NIS proteins are occupied with 2 Na^+ ions, enabling an efficient I^- transport [36].

Also important for maintaining the membrane potential, at the apical membrane, pendrin (SLC26A4), an 1:1 electroneutral iodide/chloride, iodide/bicarbonate, and chloride/bicarbonate exchanger), and another yet unidentified transporter, mediate I^- efflux to the colloid (**Figure 3**) [37]. Thyroid peroxidase (TPO), using hydrogen peroxide (H_2O_2) generated by the DUOX2/DUOX2 system (NADPH oxidase complex of dual oxidase 2 and dual oxidase maturation factor 2, respectively) mediates the oxidation, organification and coupling reaction between tyrosine residues, Tg and I^- that result in the synthesis of T3 and T4 (**Figure 3**). Of note, TPO has no biological activity in the absence of H_2O_2 which is likely to be the limiting factor for Tg iodination when I^- supply is normal [10, 27].

Iodinated Tg is taken into the cell by micro- and macropinocytosis and digested in lysosomes. Monoiodotyrosine (MIT) and diiodotyrosine (DIT) are dehalogenated by DEHAL1 (iodotyrosine dehalogenase 1) and together with Tg and I^- are recycled for new organification processes (**Figure 3**). T3 and T4 are excreted via the monocarboxylate channel MCT8 (SLC16A2) and other transporters [31, 34, 38, 39].

Importantly, NIS can be regulated by several pathways. TSH is the primary regulator of NIS in the thyroid, modulating its expression and function through transcriptional and post-transcriptional events [40, 41]. TSH increases NIS transcription, modulates its half-life (5 days in the presence of TSH and 3 days in its absence) and also regulates its targeting and/or retention in the plasma membrane and its phosphorylation status at the carboxy terminus [42-45]. Other main regulator of NIS expression is I^- itself [31]. High concentration of I^- blocks T3 and T4 synthesis via inhibition of organification (Wolff-Chaikoff effect) [46]. Escaping this effect will lead indirectly to decreased NIS gene transcription, increased NIS protein degradation and decreased activity [39].

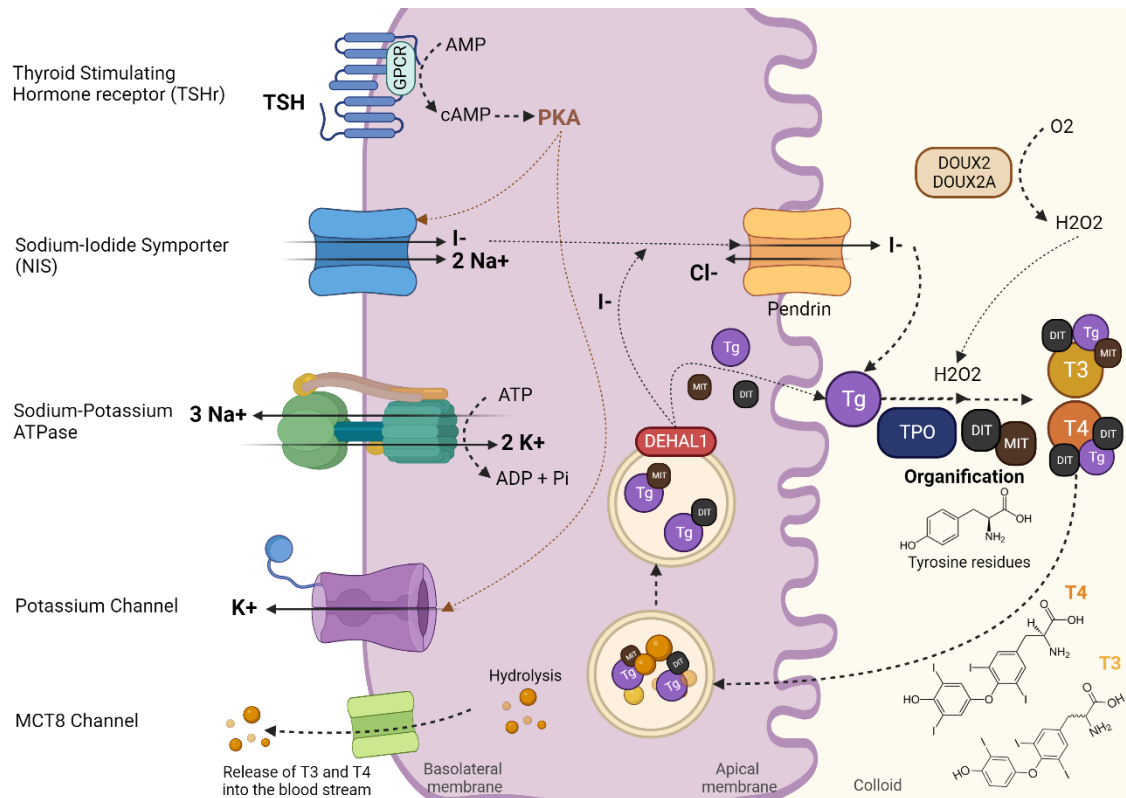


Figure 3 - Thyroid hormone synthesis by thyroid follicular cells and cellular homeostasis maintenance. Created with Biorender.com

Tg can also regulate NIS, being proposed to explain the morphologic heterogeneity of the thyroid follicles and its differential expression of thyroid genes, such as decrease in NIS, TPO and DUOX [47, 48]. The Transforming Growth Factor- β (TGF- β) has also been shown to downregulate I^- transport, inhibiting mRNA expression of TSHr, TPO, NIS, Na^+/K^+ -ATPase and Tg [49, 50]. Insulin-like growth factor 1 (IGF-1) affects thyroid hormone synthesis by downregulating the expression of NIS and cytokines, such as TNF- α and IL-1 that were shown to inhibit NIS mRNA [51, 52]. Another downregulator of NIS is estradiol, which could explain why women present a higher incidence of hypothyroidism and cancer [39, 53, 54]. This hypothesis is still controversial. A recent study by Yuk *et al.* (2022) hypothesized that I^- deficiency leads to increased estrogen activity in women, resulting in increased risk for uterine fibroids and thyroid dysfunction [55]. Yet another recent study by Xu *et al.* (2023) found no difference in thyroid function of post-menopausal women receiving menopause hormone therapy [56]. Estradiol has also been shown to upregulate Tg synthesis [54].

NIS glycosylation was also demonstrated to modulate both NIS membrane translocation and function, through cAMP-mediated glycosylation [57].

NIS is predicted to be composed of 13 transmembrane segments, an extracellular N-terminal region and an intracellular carboxy terminus, passive of being phosphorylated. In the

thyroid cell, the carboxy terminus faces the lumen [27, 31]. A total of 275 organisms have been described to present NIS orthologs, such as dogs and zebrafish [58]. This transporter is expressed in stomach and intestine for I⁻ absorption, which is then cleared by the kidney and thyroid gland, which stores most I⁻. NIS is also present in the lacrimal sac, choroid plexus and salivary glands. Additionally, in the placenta, NIS is expressed in the apical membrane of cytotrophoblasts, being later expressed in lactating breast tissue to provide I⁻ to the infant [10, 12].

Thyroid disorders

Thyroid disorders comprise a group of distinct illnesses, from autoimmune disorders to tumors [5].

The most prevalent are hypothyroidism, which is characterized by a reduced concentration of T3 and T4 and hyperthyroidism, which results in the excessive production of the same hormones. Both these conditions can result from autoimmune disorders, namely Hashimoto's thyroiditis, and Grave's disease, respectively [59].

1.4. Hashimoto's Thyroiditis

Hashimoto's Thyroiditis (HT) is a chronic inflammatory condition in which the immune system produces antibodies against follicular cells [60, 61]. HT is one of the most common autoimmune diseases in humans, being the main cause of hypothyroidism and goiter worldwide [60, 61]. The gradual destruction of the thyroid tissue will further lead to insufficient thyroid hormone production (hypothyroidism). Patients with HT often presented higher levels of circulating TSH [15, 62]. This illness is clinically evident in only 0.1-2.0% of the population, being subclinical in 10-15% [63]. HT is eight times more frequent in women than in men, reaching a peak of incidence between the 45 and 55 years of age [63, 64]. Common symptoms of hypothyroidism include fatigue, weight gain, difficulty in paying attention or executing tasks, cognitive impairment, altered sexual drive, overall reduction in the quality of life, anxiety, and depression disorders [15, 65-67]. Treatment often includes hormone therapy (LT4 - levothyroxine) [15].

Other inflammation conditions in thyroid are, for instance, painless postpartum thyroiditis and painless sporadic thyroiditis however, contrary to HT, these conditions are subacute rather than chronic [61, 68].

1.5. Grave's disease

Grave's disease (GD) is a chronic illness characterized by antibody-mediated overstimulation of the thyroid gland, leading to excessive production of thyroid hormones (hyperthyroidism). Antibodies that mimic TSH are produced by the immune system causing a continuous stimulation of the hormone synthesis cascade (**Figure 3**). Symptoms include lack of concentration, irritability, sweating, weight loss, rapid heart rate, and anxiety. GD often presents with follicular nodular disease (FND) and may be associated with eye comorbidities, known as Graves' ophthalmopathy [5, 59, 69]. Treatment includes antithyroid drugs, radioiodine therapy and thyroidectomy [69, 70].

1.6. Sporadic thyroid tumors: from benign to malignant nodules

1.6.1. Epidemiology

TC is the most common endocrine malignancy worldwide and its incidence has been increasing over the past decades [71]. TC ranks as the 9th most incident cancer worldwide and as 24th in mortality [72]. Incidence varies per geographical region, with higher incidence in more developed countries for both genders but generally showing a 3-fold difference in incidence between females and males (3:1 ratio) [72, 73].

High-income Asian countries present the highest numbers of diagnosed TC cases, which may be due to overdiagnosis resulting from high surveillance [74]. In Portugal, a study by the National Cancer Registry (Registo Oncológico Nacional - RON) demonstrated that between the 1989 and 2009, TC incidence increased over 8% in both men and women in the northern region *per year* [75]. Hence the need to better comprehend this illness at the clinicopathological and molecular level.

Regarding age at diagnosis, most studies describe a “bell-like” shaped curve, beginning at the end of adolescence and peaking at the ages of 55 in women and 65 in men, declining with older age [76, 77]. Other studies suggest a bimodal distribution with two peaks, one before the 3rd decade of life and other after the 7th decade [78, 79].

Despite TC incidence continuous increase over the years (and is yet predicted to continue to increase), TC related mortality has remained stable over the years (<https://seer.cancer.gov/statfacts/html/thyro.html>, accessed in December 2023).

1.6.2. Etiology

TC risk factors can be subdivided into two main groups: non-modifiable and modifiable risk factors [73]. Non-modifiable risk factors are essentially related to gender and inherited genetic traits and family clusters that might predispose for thyroid disease. These will be further elaborated in subsequent sections of this thesis. Modifiable (or potentially modifiable) risk factors are those in which the individual may intervene or modify its behavior [73], which will now be addressed:

1.6.2.1. Exposure to ionizing radiation

The most well studied risk factor for TC is exposure to ionizing radiation during childhood [72]. The first association was reported in 1950, and has been consolidated over time, especially with data from World War II bombing of Hiroshima and Nagasaki and from the Chernobyl incident in 1986 [80, 81]. More recent data from the Fukushima earthquake and tsunami that hit the nuclear power plant, further infer that TC and its outcome is dose-dependent [82].

1.6.2.2. Incidental findings - Overdiagnosis

Overdiagnosis is defined as the “diagnosis of a condition that would not have caused harm to the individual over their lifetime if left undetected” [73]. In virtue of the evolution of imaging techniques in the medical field (e.g. ultrasound, MRI, CT scan), many subclinical thyroid nodules were detected. Between 1998 and 2012 there was a 3-4-fold increase in thyroidectomy surgeries [83]. Most detected nodules were indolent – non-palpable and asymptomatic. Furthermore, several studies show that occult TCs are prevalent upon autopsy on death by other causes [76, 84, 85]. This might point out that in fact a great number of cancer cases are being overdiagnosed and overtreated [83].

However, the hypothesis of incidental findings explaining the large increase of TC incidence has some limitations. Although the incidence of other cancers has also been increasing, we fail to observe such abrupt increase as we see for TC, despite high surveillance, extensive use of imaging techniques and biomarker identification [72, 86]. This leads to the belief that we might be searching for a thyroid specific cause. Additionally, for overdiagnosis to be the only explanation of incidence increase, it would be expected a more prominent decrease in the incidence of advanced stage and large thyroid carcinomas, which was not observed. Plus, a higher incidence of TC, would lead to an increase in TC-specific mortality. However, TC-specific mortality remained stable over the years [86, 87].

1.6.2.3. Iodide intake

Iodine deficiency was recognized as a major cause of morbidity in human and animal population around the globe, particularly in Europe and Southeast Asia. Already in the 19th century it was recognized that I⁻ deficiency was associated with FND, development delay and irreversible mental retardation in children in several worldwide countries [88, 89]. Both I⁻ deficit and excess have been described to be associated with FND, Follicular Thyroid Adenomas (FTA), HT, GD, and TC [68, 90].

The diet is the only source of I⁻ and the current recommended daily dosage of I⁻ is of 150 µg for healthy adults, 220 µg during pregnancy and 290 µg during lactation [88, 91]. Between 70-80% of dietary I⁻ is stored in the thyroid gland [12, 39].

Political actions have been taken to decrease I⁻ deficiency through salt iodization programs. In 1994 WHO and UNICEF officially recommended salt iodization as the main strategy to eliminate I⁻ deficiency [88, 92]. Other sources of I⁻ are sea water, fish, milk, and infant formula [93]. It has been reported however, that excess I⁻ can also be harmful in humans and other animals [94]. It seems that I⁻ concentration correlation with thyroid illness can be represented in a U-shaped curve [90].

In mice studies, association between I⁻ excess and autoimmune disease has been demonstrated. The same study stated that I⁻ enhances disease in a dose-dependent manner and further explored the possible mechanisms by which I⁻ incorporation in Tg may increase its antigenicity and promote inflammation [95].

The prevalence of FND, as well as thyroid nodules has been reported to be higher in I⁻ deficient populations [96]. Of note, while in I⁻ rich regions, Papillary Thyroid Carcinoma (PTC) is the most prevalent histotype, in I⁻ deficient regions, Follicular Thyroid Carcinoma (FTC) seems to be more prevalent [97]. Iodine prophylaxis in a FND endemic area in Poland was shown to cause a shift from FTC to PTC tumors [98]. Furthermore, animal studies, such as the one conducted by Yamashita *et al.* (1990), exclusively detected PTC in animals that were provided with an iodine-enriched diet [99]. Hence, I⁻ concentration might not only influence the incidence of TC but also impact the tumor's histology [97]. Importantly, mice studies indicate that when in the condition of I⁻ deficiency or excess, only mice exposed to carcinogens or radiation developed tumors [100]. This seems to indicate that I⁻ concentration may be a carcinoma promoter but not an initiator [96].

Interestingly, high I⁻ intake was previously associated with the highly prevalent *BRAF V600E* mutation in PTCs [101]. However, a study by Vuong *et al.* (2016) comparing TC in an iodine-rich region in Japan and an iodine-deficient region in Vietnam, found no differences in the tumor at

a molecular level, stating that I⁻ concentration does not impact on the occurrence of *BRAF* and *RAS* point mutations, as well as *RET/PTC* translocations [102].

1.6.2.4. Environmental pollutants

Environmental pollutants have been in the spotlight when it comes to study the higher incidence of all cancers that we have witnessed over the years [72, 103]. A systematic review conducted by our group, evaluating the effect of endocrine disrupting chemicals on endocrine cancer tumorigenesis, showed that the thyroid gland was the endocrine organ with a higher number of studies showing a positive correlation for cancer risk (67%) [104] (**APPENDIX – ARTICLE VI**). Even though most studies focus on the effects of pesticides, flame retardants and Polychlorinated Biphenyls (PCBs), exposure to phthalates showed higher risk for TC development [104], with exposure to DEHP and MEHP metabolites showing 14 and 16 times increased risk, respectively [105, 106]. Also, the anion ClO₄⁻, an environmental pollutant, previously known as a competitive inhibitor of NIS and that is actively transported by NIS as a substrate, may compromise thyroid normal function [107].

1.6.2.5. Other risk factors

Female gender presents increased risk of thyroid disease. Alcohol consumption and smoking are habits also associated with thyroid carcinogenesis [108]. High body mass index (BMI) and obesity have been strongly correlated with TC [73, 109]. In fact, higher weight and adiposity were shown to be associated with more aggressive forms of the disease and even TC-related mortality [110]. Autoimmune conditions such as HT and GD, due to their inflammatory condition, were also indicated to promote carcinogenesis [108]. Estrogen also seems to play a role in TC. This may explain why females are more predisposed for the disease. However, the mechanisms behind this correlation are still unclear [111].

1.6.3. Tumor Histotypes

Tumor histotypes will be presented according to the 2022 WHO classification of thyroid tumors (5th edition) [112-114].

Thyroid tumors may originate in two cell types that compose the thyroid gland (**Figure 4, A**). Medullary thyroid carcinoma is the only tumor that originates solely from parafollicular cells, however, tumors rising from follicular cells may be divided into three major groups: benign lesions, low-risk neoplasms, and malignant thyroid neoplasms (**Figure 4, A**) [112-114]. (Of note:

Mixed medullary and follicular cell–derived carcinomas rise from both thyroid cell types, although being a rare entity, with less than 60 cases reported in the literature [115]).

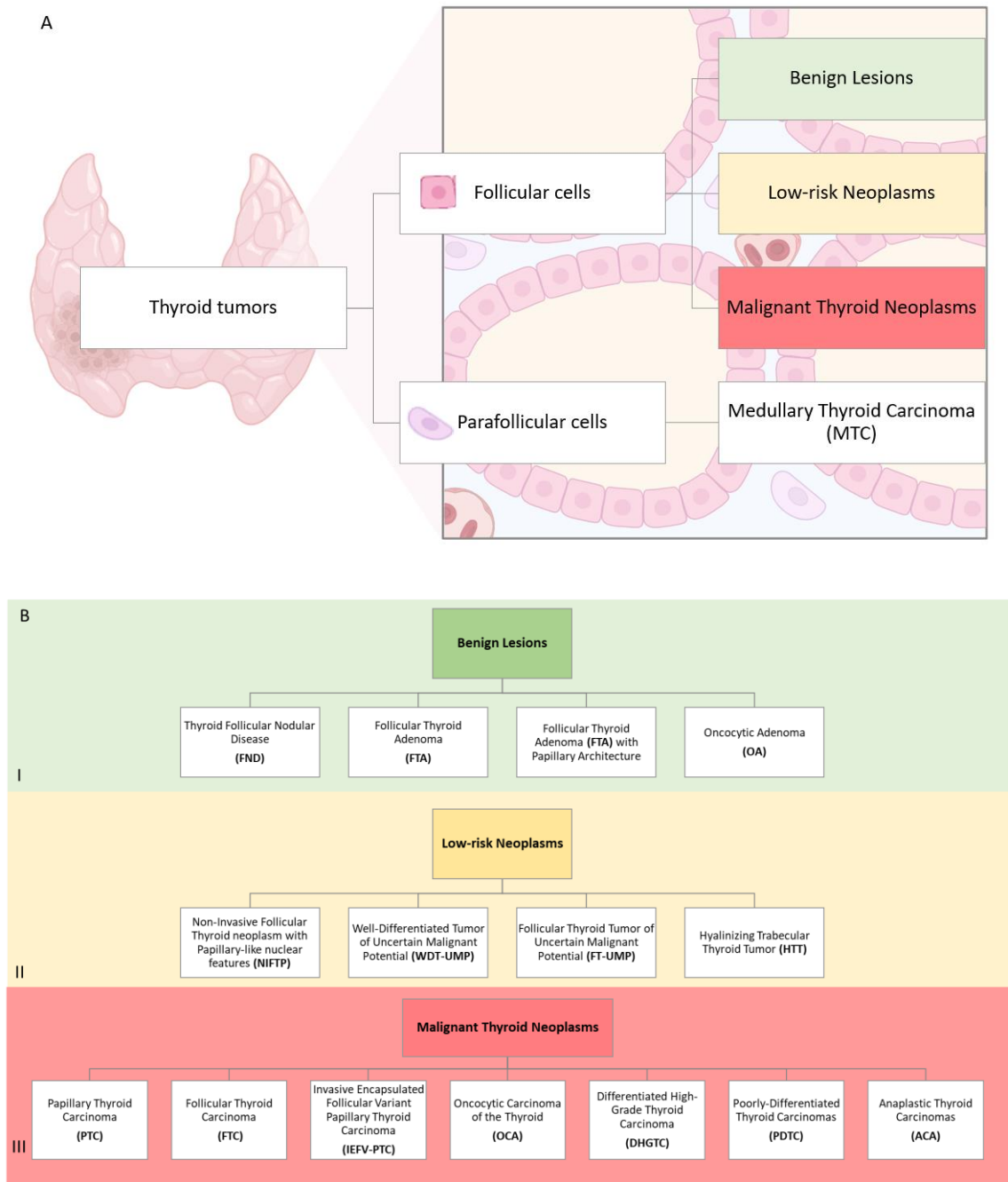


Figure 4 - Histologic subtypes of thyroid tumors. A) Thyroid tumors major groups and origin cell type. B) Further subdivision of thyroid tumors originated in thyroid follicular cells. Created with Biorender.com

1.6.3.1. Benign lesions

Benign lesions of the thyroid gland comprise Follicular Nodular Disease (FND), previously designated as multinodular adenomatous goiter, Follicular Thyroid Adenoma (FTA), FTA with Papillary Architecture, previously known as papillary adenomatous/hyperplastic nodule and

Oncocytic Adenoma (OA) (**Figure 4, B, I**). Usually, FTA with Papillary Architecture tumors are associated with thyroid hyperfunction, appearing as warm nodules in thyroid scans [115].

1.6.3.2. Low-risk neoplasms

Low-risk neoplasms of the thyroid comprise Non-Invasive Follicular Thyroid neoplasm with Papillary-like nuclear features (NIFTP), Well-Differentiated Tumors of Uncertain Malignant Potential (WDT-UMPs), Follicular Thyroid Tumors of Uncertain Malignant Potential (FT-UMPs) and Hyalinizing Trabecular Thyroid Tumors (HTT) (**Figure 4, B, II**). These tumors are classified regarding their pathological features and molecular profile, which reflects their clinical behavior, which is benign-like, despite having a risk to progress to invasive (malignant) tumors. NIFTP is a non-invasive follicular neoplasm that presents PTC atypia, and that is rarely associated with lymph node metastases (LNM), being classified as a low-risk neoplasm (**Figure 4, B, II**). NIFTP presents very low risk of recurrence and an excellent prognosis [116]. Tumors of Uncertain Malignant Potential (UMPs), are those in which despite thorough analysis of the tumor, the existence of capsular and/or vascular invasion is equivocal, therefore these tumors require vigilance and new assessment [113]. Given that the risk of progression is low, less aggressive treatment is recommended for low-risk neoplasms [113, 117].

1.6.3.3. Malignant Thyroid Neoplasms

Given that this thesis centers around malignant thyroid neoplasms, these will be more extensively explored in this subchapter:

Malignant thyroid neoplasms present a broad spectrum of histologic subtypes, from differentiated lesions: Papillary Thyroid Carcinoma (PTC), Follicular Thyroid Carcinoma (FTC), Invasive Encapsulated Follicular Variant of Papillary Thyroid Carcinoma (IEFV-PTC), Oncocytic Thyroid Carcinoma (OCA), and Differentiated High-Grade Thyroid Carcinoma (DHGTC); to less differentiated or undifferentiated lesions: Poorly-Differentiated Thyroid Carcinomas (PDTC) and Anaplastic Thyroid Carcinomas (ACA) (**Figure 4, B, III**).

FTC, IEFV-PTC and OCA are usually encapsulated lesions, and according to the extension of invasion can be further classified into minimally invasive (capsular invasion only), encapsulated angioinvasive and widely invasive [112, 113]. Minimally invasive tumors are categorized as low-grade, and surgical removal alone may suffice for treatment approach, while angioinvasive and widely invasive tumors may require complete thyroidectomy and adjuvant therapy to prevent loco-regional recurrence and/or distant metastasis (DM) [112]. [This subchapter is extensively

explored in the **APPENDIX - BOOK CHAPTER: Pathobiology, morphology and clinical significance of minimally invasive thyroid neoplasms**].

Papillary Thyroid Carcinoma (PTC), Differentiated High-Grade Thyroid Carcinoma (DHGTC), Poorly-Differentiated Thyroid Carcinomas (PDTC) and Anaplastic Thyroid Carcinomas (ACA) are cancers with a clear infiltrative/invasive behavior [112, 113].

1.6.3.3.1. Papillary Thyroid Carcinoma (PTC)

Classic PTC is composed of well-formed papillae in which tumor cells show specific nuclear features (PTC atypia): enlargement of the nucleus with clear nucleoplasm, peripheral concentration of the chromatin and irregular nuclear shape, resulting in cytoplasmic pseudoinclusions [112]. In PTC, the presence of lymphatic invasion is common, often resulting in lymph node invasion, while vascular invasion is less common. Due to multifocality and the presence of local invasion, PTC recurrence is high, reaching 35% of all cases [118].

PTC accounts for up to 85% of all TC diagnoses, being the most common TC [118]. It seems to be the most prevalent histotype in I⁻ rich regions [97].

PTC may be subclassified into different subtypes, with differential characteristics, growth pattern, prognosis, and molecular profile. Over 15 histopathologic PTC subtypes have been described [119] but in this subchapter we will approach only the most common:

1.6.3.3.1.1. Infiltrative Follicular Variant PTC (IFV-PTC)

IFV-PTC presents with follicular growth, lacking the common PTC papillae presentation. However, the nuclei atypia of PTC is present. This subtype is also characterized by the presence of psammoma bodies and fibrosis [112]. Sometimes, small papillae may be found [112]. IFV-PTC is classified as a *BRAF-like* tumor, similar in behavior to classic PTC [116, 120].

1.6.3.3.1.2. Tall cell PTC

Tall cell PTC is diagnosed when more than 30% of tumor cells are columnar, present a height 2-3 times its width, and the nuclei atypia of PTC is present [112, 121]. This subtype is more common in older patients and is associated with higher aggressiveness and higher mortality risk, when comparing to classic PTC, being itself a prognostic factor in TC [112, 121].

1.6.3.3.1.3. Columnar cell PTC

Columnar cell PTC is a rare and aggressive subtype of PTC characterized by columnar cells that present PTC nuclear atypia and a mixed morphology of papillae and follicles [112, 122]. An

eosinophilic cytoplasm and subnuclear vacuoles are characteristic of this subtype [112]. Tendency for invading the surrounding tissues and poor prognosis has been described [122].

1.6.3.3.1.4. Hobnail PTC

The Hobnail PTC is characterized by tumors with a papillary or micropapillary growth pattern that present $\geq 30\%$ hobnail cells, meaning tumors cells of abundant cytoplasm and enlarged nuclei that is pressured into the apical surface of the cell (high nuclei/cytoplasm ratio) [112, 123]. This complex subtype is more frequent in women and associated with higher mortality and worse prognosis [123].

1.6.3.3.1.5. Solid PTC

Solid PTC (or Trabecular PTC) is characterized histologically by nests of tumor cells that can be solid, trabecular and insular [112, 124]. Solid PTC is rather similar to PDTC, however, maintaining the nuclear atypia of PTC and lacking the presence of high grade features [112, 124]. This subtype is rare and indicated as associated with higher risk of local metastases and DM, presenting poorer prognosis when comparing to classic PTC [125].

1.6.3.3.1.6. Diffuse sclerosing PTC

Diffuse sclerosing PTC is another rare subtype of PTC that is characterized by affecting a large area of the thyroid, sometimes being present in both lobes (bilateral disease) [112, 119]. It is more common in women and at young age (< 30 years) [119]. Dense sclerosis, abundant psammoma bodies, fibrosis and lymphocytic infiltration are common findings on histologic evaluation [112, 119]. Association with HT has been reported in up to 75% of the cases [119].

1.6.3.3.1.7. Warthin-like PTC

Warthin-like PTC presents papillary growth, with the papillae lined with oncocytic cells [112, 126]. This subtype is more common in middle-aged women and is characterized by marked infiltration of lymphocytes and plasma cells [112, 126]. It is often associated with thyroiditis [126]. The outcome of Warthin-like PTC is similar to that of classical PTC, not being associated with tumor aggressiveness or worse outcome [126].

1.6.3.3.1.8. Oncocytic PTC

Oncocytic PTCs, previously designated as “Hürthle cell” carcinomas, are characterized by papillary growth and over 75% of oncocytic tumor cells [112]. Over the years, the different

classifications attributed to oncocytic tumors, lead to misconceptions regarding this entity, since all tumors with over 75% of oncocytic tumor cells were grouped as “Hürthle cell” carcinomas [127]. This included OCAs and even PDTs, which can explain why patient prognosis and therapy response analysis differed so markedly across studies [127]. The most recent WHO classification has allowed to group these tumors according to their clinicopathological risk, allowing a better clinical management [112, 113].

Of note, PTC ≤ 1 cm were frequently incidental cases, and presenting good prognosis. Previously to the 2022 WHO update, PTC ≤ 1 cm were grouped as an independent subtype: papillary microcarcinoma (mPTC). However, some cases of mPTC were highly metastatic and aggressive, leading to DM and PTC-related mortality. Therefore, in the 5th edition of the WHO classification of thyroid neoplasms, these tumors are no longer grouped by size in a subtype of PTC, but rather evaluated by their histopathologic characteristics, invasiveness and aggressiveness [112]. In this thesis, we will present a study evaluating subcentimetric PTC prognosis between cases that were clinically diagnosed and those that were incidental findings (**ARTICLE III**). We will also present an example of an aggressive case of lethal papillary “microcarcinoma”, now designated subcentimetric PTC (**ARTICLE IV**).

1.6.3.3.2. Follicular Thyroid Carcinoma (FTC)

FTC is a differentiated malignant epithelial tumor that accounts for approximately 10% of all TCs [128, 129]. FTC presents a follicular growth pattern, ranging from small-to-medium-sized follicles, while lacking the nuclei features of PTC [129, 130]. Solid or trabecular growth patterns can also be present [129]. This histotype seems to be more frequent in I⁻ deficient areas [95]. Despite presenting a good prognosis, 20-30% of FTC cases show vascular invasion, local recurrence and DM, being associated with poorer prognosis [130, 131]. Histologically, FTC is similar to FTA, what distinguishes both entities is the presence of invasion (capsular and/or angioinvasive) in FTC, which also determines disease prognosis [130].

1.6.3.3.3. Invasive Encapsulated Follicular Variant of Papillary Thyroid Carcinoma (IEFV-PCT)

The new WHO classification has brought to light some TC subtypes that despite histologically similar, showed rather different prognosis, and allowed to better group these entities regarding their risk, invasion and clinical behavior [112, 113]. The distinction between FTC and IEFV-PCT is based on nuclear morphology, since IEFV-PCT presents PTC nuclear atypia [112]. IEFV-PCT is an

invasive tumor that can be further classified as minimally invasive, encapsulated angioinvasive or widely invasive, falling in the category of malignant tumor [112, 120, 132]. This entity was shown to present higher recurrence and metastatic potential [120]. However, IEFV-PTC presents better prognosis than IFV-PTC, that behaves more similarly to classic PTC [120, 132]. Another distinction between IEFV-PTC and IFV-PTC is their molecular profile, since IEFV-PTC is a *RAS-like* tumor and IFV-PTC is a *BRAF-like* tumor [116].

1.6.3.3.4. Oncocytic Thyroid Carcinoma (OCA)

OCA is an invasive tumor that is composed of at least 75% of oncocytic cells [112]. Oncocytic cells are composed of abundant granular eosinophilic cytoplasm, that results from the accumulation of altered mitochondria. OCA presents capsular and/or vascular invasion and is absent of high-grade features [tumor necrosis and/or high mitotic grade (≥ 5 mitosis/2mm²)] [109, 110]. OCA can be further classified into minimally invasive (capsular invasion only), encapsulated angioinvasive and widely invasive [109, 110]. This subtype is the malignant counterpart of OA and is more frequent in women, representing 3-5% of all TC cases, and tends to develop DM [133]. At diagnosis, up to 27% of the cases show DM at diagnosis, which relates to an association with extensive vascular invasion [109, 110].

1.6.3.3.5. Differentiated High-Grade Thyroid Carcinoma (DHGTC)

DHGTC can be comprised of PTC, FTC or OCA tumors that despite also presenting a differentiated histology, are classified as high-grade given the presence of tumor necrosis and/or high mitotic grade (≥ 5 mitosis/2mm²), which has been associated with poorer prognosis [112, 113]. DHGTC tumors present either PTC-related nuclear atypia or a follicular growth pattern, not falling in the PDTC category (**Figure 4, B, III**) [113].

1.6.3.3.6. Poorly-Differentiated Thyroid Carcinomas (PDTC)

PDTC diagnosis is made according to Turin criteria: (1) presence of a solid/trabecular/insular growth pattern, while in the (2) absence of the conventional nuclear features of PTC and (3) at least one characteristic of poor prognosis - convoluted nuclei; > 3 mitoses per 2 mm², and tumor necrosis [112, 113, 134]. PDTC comprises a small subset of thyroid tumors, representing 3-5% of all TC [135]. Given its loss of tumor differentiation and aggressiveness, it contributes significantly to TC-related death (**Figure 4, B, III**) [134].

1.6.3.3.7. Anaplastic Thyroid Carcinomas (ACA)

ACA is the most aggressive subtype of TC, comprising less than 2% of all cases [136] (**Figure 4, B, III**). Despite rare, this disease presents with rapid growth and high metastatic rate [137]. DM are usually present at diagnosis [136]. ACA is undifferentiated but some characteristics such as: squamous morphology, giant cells, and pleiomorphic morphology can be found [137]. Due to its rapid and highly lethal behavior, ACA usually requires multimodal therapy: excisional surgery (whenever possible), radiation therapy, chemotherapy and/or targeted therapy, namely Tyrosine Kinase Inhibitors (TKI) [137].

1.6.4. Thyroid Cancer Staging

TC treatment is dependent on TC staging and prognosis. TC staging can be performed according to several guidelines. The TNM staging evaluates tumor size as well as the presence and number of LNM and DM [138]. TNM classification and age are considered for patient staging and tumor classification by the American Joint Committee on Cancer (AJCC) guidelines, which allows a good communication between clinicians worldwide [139].

In 2015, the American Thyroid Association (ATA) published the guidelines for management of differentiated thyroid neoplasms [140]. ATA guidelines present recommendations for the management of differentiated TC depending on tumor and patient characteristics, collecting the overall knowledge obtained over the years [140]. The guidelines cover clinical and ultrasound criteria for fine-needle aspiration biopsy (FNAB), molecular markers, staging, surgery, radioiodine therapy, thyrotropin suppression therapy, surveillance, and management of recurrent and metastatic TC [140].

1.6.5. Thyroid Cancer Treatment

According to the ATA guidelines, standard treatment for TC usually comprises surgery, that may be the complete removal of the thyroid gland (total thyroidectomy) or partial removal (hemithyroidectomy, when only one lobe is excised), in some cases followed by radioiodine (RAI) ablation of remnant thyroid tissue using ^{131}I [140, 141]. For low-risk patients, the ATA guidelines establish that active surveillance is now an option within standard treatment [142].

RAI is possible given NIS's ability to uptake ^{131}I [143]. Inside the follicular cell, ^{131}I decays, emitting β -radiation with a mean tissue penetration of 1 mm and γ -radiation which presents a deeper penetration capacity, being visualized in scintigraphy. Preserved NIS expression and functionality allows RAI to be specifically delivered in the targeted tissue [144]. The effectiveness

of RAI therapy depends both on the radiation dose and the I⁻ concentrating ability of the cells through the organification of RAI and its retention [145, 146].

Of note, NIS presents the ability to transport other substrates, such as thiocyanate (SCN⁻), chlorate (ClO₃⁻), perrhenate (ReO₄⁻), and perchlorate (ClO₄⁻) [147]. This has provided the basis for diagnostic scintigraphy imaging of the thyroid using inexpensive radioisotopes with affinity for NIS (¹³¹I⁻, ¹²³I⁻, ^{99m}TcO₄⁻), however ¹³¹I contrary to other isotopes, is the only radioisotope that allows not only imaging but also a therapeutic effect by inducing cell death [148].

For tumors that are not RAI avid, or that develop RAI resistance, molecular-targeted therapies with several tyrosine kinase inhibitors (TKIs) can be applied [140, 141, 149]. RAI refractory patients are described as: those whose tumors show no ¹³¹I uptake in the post therapy scan; patients with multiple lesions, with at least one not showing ¹³¹I uptake; patients whose tumors progressed even if showing ¹³¹I uptake (12-16 months after treatment); and patients submitted to accumulated doses of ¹³¹I ≥ 600 mCi and with no remission [150].

In undifferentiated tumors, such as ACA, high spectrum TKIs (e.g. Lenvatinib and Sorafenib), BRAF or MEK inhibitors (Dabrafenib, Trametinib) are usually required, since these have shown effectiveness in RAI refractory tumors, some of which are irresectable [151].

Since the thyroid gland is a vital organ, its surgical removal/ablation implicates treatment with hormonal substitution, namely LT4, a synthetic analogous of the T4 hormone, which is also used to treat hypothyroidism [152].

1.6.6. Thyroid cancer prognostic factors

Prognostic factors in TC may be related to patient characteristics, tumor characteristics, treatment response and/or molecular markers. Here, a brief overview of prognostic factors will be given, since clinicopathological and molecular factors will be further explored in **ARTICLE I** and **ARTICLE II**, respectively.

1.6.6.1. Patient characteristics

As previously discussed, patient sex impacts prognosis. Although TC incidence is higher in women, males tend to present poorer prognosis [153, 154]. Patient's age, as shown by Kim *et al.* (2018) when applying the AJCC/TNM age cut-off of ≥ 55 years is also an important prognostic factor. Patients with 55 years or older presented lower overall survival (OS) and recurrence-free survival (RFS) when comparing to younger patients, furthermore, the genetic background of patients <55 years of age was shown to be different from patients ≥55 years of age [139, 155].

1.6.6.2. Tumor characteristics

As shown in chapter 2.3.3. tumor histology has a great impact on prognosis. While differentiated tumors tend to present a better prognosis, undifferentiated tumors tend to be rapidly lethal [112, 113]. However, even differentiated tumors may present dismal prognosis. The separation of minimally invasive encapsulated angioinvasive and widely invasive tumors has come to help with the evaluation of the tumor's capacity to form LNM and/or DM, which are major prognostic factors (see **APPENDIX – BOOK CHAPTER**) [112, 113]. The presence of extrathyroidal extension (ETE) and tumor size are also appointed as important clinicopathological factors [156].

1.6.6.3. Treatment response

A study by Durante *et al.* (2006) showed that patients with RAI avid tumors presented a 10-year survival rate of 92%, while patients without any RAI uptake only had 10% [157]. Recent studies have attempted to re-differentiate RAI refractory tumors in order to make them RAI avid using TKI inhibitors [158, 159]. In fact, between 7-23% of TC patients will develop DM and from those, around 60% will become I⁻ refractory [160, 161]. Patients who remain with structural disease 6-24 months after initial therapy, present double-to-triple risk of disease recurrence, which compromises their recovery and quality of life [150, 162]. NIS function and expression alterations that could be associated with worse prognosis have been studied over time [163, 164]. But as demonstrated by Mian *et al.* (2008) tumors with no RAI uptake show reduced Tg, TPO and pendrin gene expression, while showing an increase in GLUT-1 gene expression. This may show that alterations in molecules that are involved in follicular cell intracellular metabolism may also contribute to tumor recurrence [164].

1.6.6.4. Molecular markers

Knowledge of TC genetic and epigenetic alterations has been shown as fundamental for the management and treatment of the disease, being not only acknowledged in the 2015 ATA guidelines but also strongly considered in the 5th edition of WHO Classification of thyroid tumors [112, 116, 140, 165]. Molecular markers have been shown to assist with TC histologic subtype determination, allowing to avoid overtreatment in low-risk tumors, particularly upon FNAB (**Table 1**) [116, 165, 166].

Benign lesions are associated with *TSHr*, *RAS* and *DICER 1* mutations [167-169]. Gene fusions including the *PPARG* and *THADA* genes have been reported in NIFTP lesions [170]. HTT, at the molecular level, presents *PAX8::GLIS1* and *PAX8::GLIS3* gene fusions [112].

Encapsulated follicular neoplasms, either benign lesions or low-risk neoplasms do not present *BRAF V600E* mutation, usually being called *RAS-like* lesions, that are similar to FTC (**Table 1**) [112]. The type of genetic alterations found in benign and low-risk neoplasms is similar, however, NIFTP presents the highest rate of *RAS* mutations (up to 60%) (**Table 1**) [112]. For this entity, the presence of a *BRAF V600E* mutation would exclude a NIFTP diagnosis, since this alteration is essentially associated to malignancy (**Table 1**) [112]. The malignant neoplasm IEFV-PTC, is actually more genetically related to FTC than PTC, presenting almost the same genetic landscape as NIFTP and FTC (**Table 1**) [112]. The *BRAF K601* mutations, however, seem to be common in encapsulated follicular neoplasms (**Table 1**) [112, 170].

Rather than diagnostic determination, molecular markers also allow to stratify patients according to aggressiveness, prognosis and risk of recurrence and mortality [166]. *RAS* mutations can be found either in benign, low-risk and malignant lesions, however, *RAS-like* mutations appear to be more frequent in benign and low-risk neoplasia, being usually present in encapsulated lesions with follicular pattern. Hence these are normally associated with better patient outcome, although some studies are controversial (**Table 1**) [112, 171]. Nevertheless, it is recommended that *RAS* mutations are not evaluated alone, but with the presence of other genetic alterations that might be present in the tumor [172]. The presence of other molecular alterations, such as *TERT promoter (TERTp)* mutations and *p53* alterations has long been associated with DM and worse patient prognosis, usually indicating a subset of tumors that will progress over time (**Table 1**) [112].

The presence of RET fusions, like the ones found in classic PTC, Diffuse sclerosing PTC and Solid PTC are frequently radiation induced (**Table 1**) [112, 173]. These gene fusions or mutations can also result from RAI treatment applied to TC, or be spontaneously acquired [173].

Further detail on molecular and immunohistochemical markers associated with TC subtype is depicted in **Table 1**.

Table 1 - Molecular and immunohistochemical markers associated with thyroid cancer subtype.

Type of thyroid cancer	Thyroid cancer Subtype	Molecular markers and IHC markers	References
Benign lesions	FTA	RAS mutations BRAF K601E, EIF1AX, EZH1, DICER1, PTEN, rTSH mutations PAX8::PPARG and THADA fusions Absence of BRAF V600E	[112, 165]
Low-risk neoplasms	FT-UMP	RAS mutations BRAF K601E, EIF1AX, EZH1, DICER1, PTEN, rTSH mutations PAX8::PPARG Absence of BRAF V600E	[112]

Type of thyroid cancer	Thyroid cancer Subtype	Molecular markers and IHC markers	References
Malignant thyroid neoplasms	WDT-UMP	RAS mutations BRAF K601E, EIF1AX, EZH1, DICER1, PTEN, rTSH mutations PAX8::PPARG, THADA fusions (rare) Absence of BRAF V600E	[112]
	NIFTP	RAS mutations BRAF K601E, EIF1AX, EZH1, DICER1, PTEN, rTSH mutations PAX8::PPARG, THADA fusions Absence of BRAF V600E	[112, 170]
	HTT	PAX8::GLIS3 (the most frequent type) and PAX8::GLIS1 fusions Tg, TTF1, and PAX8 expression Absence of BRAF V600E	[112]
	IFV-PTC	BRAF V600E and K601E mutation NRAS and CTNNB1 mutations (less common) RET translocation NTRK and ALK fusions	[112]
	Tall cell PTC	BRAF V600E mutations TERT promoter mutations TP53 mutations	[112]
	Columnar cell PTC	BRAF V600E mutations BRAF fusions and deletions CDKN2A loss Chromosome 1q gain RAS mutations (rare) TERT promoter (rare) TP53 mutations (rare)	[112, 122]
	Hobnail PTC	BRAF V600E mutations TERT promoter mutations TP53 mutations PIK3CA mutations RET rearrangements (rare) EGFR, ATK1, ATM, ARID2, and NOTCH1 (rare)	[112]
	Solid PTC	CCD6::RET and NCOA4::RET rearrangements (radiation induced) ETV6::NTRK3 fusions BRAF V600E mutations (rare)	[112, 125]
	Difuse sclerosing PTC	NCOA4::RET rearrangements (radiation induced) RET/PTC rearrangements BRAF V600E mutations (up to 20%) ALK rearrangements (up to 10%) LOH of 3p24, 9p21, 17q21, 21q22, and 22q13 (frequent)	[112, 174]
	Warthin-like PTC	BRAF V600E mutations	[112]

Type of thyroid cancer	Thyroid cancer Subtype	Molecular markers and IHC markers	References
	Oncocytic PTC	BRAF V600E mutations NDUFA13 germline mutations (rare) RET rearrangements (rare)	[112]
	FTC	RAS PAX8::PPARG PTEN, PIK3CA, EIF1AX, DICER1, rTSH mutations TERT promotor mutations TP53 mutations	[112, 166]
	IEFV-PTC	RAS mutations BRAF K601E, EIF1AX, EZH1, DICER1, PTEN, rTSH mutations PAX8::PPARG THADA fusions BRAF, RET, NTRK, or ALK fusions (rare) BRAF V600E (uncommon)	[112, 116]
	OCA	TERT promotor mutations Mitochondrial DNA mutations	[166, 175, 176]
	DHGTC	BRAF (BRAF V600E) mutation RAS mutations RET or NTRK3 fusions TERT promoter mutations PIK3CA mutations TP53 mutations	[112]
	PDTC	<i>BRAF (BRAF V600E)</i> mutation RAS mutations <i>RET, ALK, NTK1</i> and <i>NTRK3</i> gene fusions <i>TERT promoter</i> mutations <i>PIK3CA</i> mutations <i>TP53</i> mutations	[112, 165]
	ACA	<i>BRAF V600E</i> mutations (frequent) RAS mutations <i>TERT promoter</i> mutations (frequent) TP53 (frequent) <i>PTEN</i> and <i>PIK3CA</i> mutations <i>NF1, NF2, ATM, CDKN2A, CDKN2B</i> and <i>RB1</i> mutations PAX8 expression (frequent) TTF1 expression	[112, 165]

Based on Overview of the 2022 WHO Classification of Thyroid Neoplasms by Baloch et al. (2022) [112]; **FTA** - Follicular Thyroid Adenoma, **FT-UMP** - Follicular Thyroid Tumor of Uncertain Malignant Potential, **WDT-UMP** - Well-differentiated Tumor of Uncertain Malignant Potential, **NIFTP** - Non-Invasive Follicular Thyroid neoplasm with Papillary-like nuclear features, **HTT** - Hyalinizing Trabecular Thyroid Tumor, **IEFV-PTC** - Invasive Encapsulated Follicular Variant of Papillary Thyroid Carcinoma, **FTC** – Follicular Thyroid Carcinoma, **OCA** – Oncocytic Thyroid Carcinoma, **IFV-PTC** – Infiltrative Follicular Variant Papillary Thyroid Carcinoma, **PTC**- Papillary Thyroid Carcinoma, **DHGTC** - Differentiated High-Grade Thyroid Carcinoma, **PDTC** - Poorly-Differentiated Thyroid Carcinomas, **ACA** - Anaplastic Thyroid Carcinomas.

Molecular markers have been fundamental for predicting the efficacy of treatment, encouraging the production of novel therapeutic targets [165, 166]. Identification of tumor driver mutations has allowed to select specific TKIs to treat TC when standard RAI treatment fails or is not applicable [166]. In some cases, even allowed to obtain tumor re-differentiation so that RAI therapy could be applied [165].

Most mutations identified in TC affect essentially two signaling pathways: the mitogen-activating protein kinase (MAPK), and the Phosphatidylinositol-3-kinase (PI3K/AKT) pathways [166]. These pathways are activated when a ligand (e.g. growth factor) binds to the Receptor Tyrosine Kinase (RTK) [177]. In the MAPK pathway, activation of RAS after RTK binding sets off a protein chain reaction designated the MAPK cascade (**Figure 5**) [177]. This cascade involves a series of protein phosphorylation events, namely the activation of BRAF, followed by MEK and ERK proteins (**Figure 5**). Activated ERK migrates to the cell nucleus, regulating gene expression through transcription factors that result in cell growth, proliferation, motility and migration (**Figure 5**) [177]. For the PI3K/AKT-pathway, the activated RTK signals PI3K to phosphorylate cell membrane lipids, forming PIP3 (phosphatidylinositol (3,4,5)-trisphosphate), which then activates AKT (protein kinase B) that by itself can regulate several cellular processes, but can also activate mTOR (mammalian target of rapamycin), which further regulates cell growth and protein synthesis (**Figure 5**) [178]. Mutations in *BRAF* and *RAS* genes have been shown to lead to the constitutive activation of these pathways, resulting in excessive cellular proliferation and migration [112, 166]. Of note, RAS also activates the PI3K/AKT pathway (**Figure 5**) [178]. Mutations in the other proteins belonging to MAPK and PI3K/AKT pathways can also be found [112, 166].

MAPK and PI3K/AKT pathways have been intensely studied regarding the development of targeted therapies to surpass RAI resistance [177, 178]. For instance, Dabrafenib, Sotorasib and Selumetinib have been FDA approved as BRAF, RAS and MEK inhibitors, respectively [177].

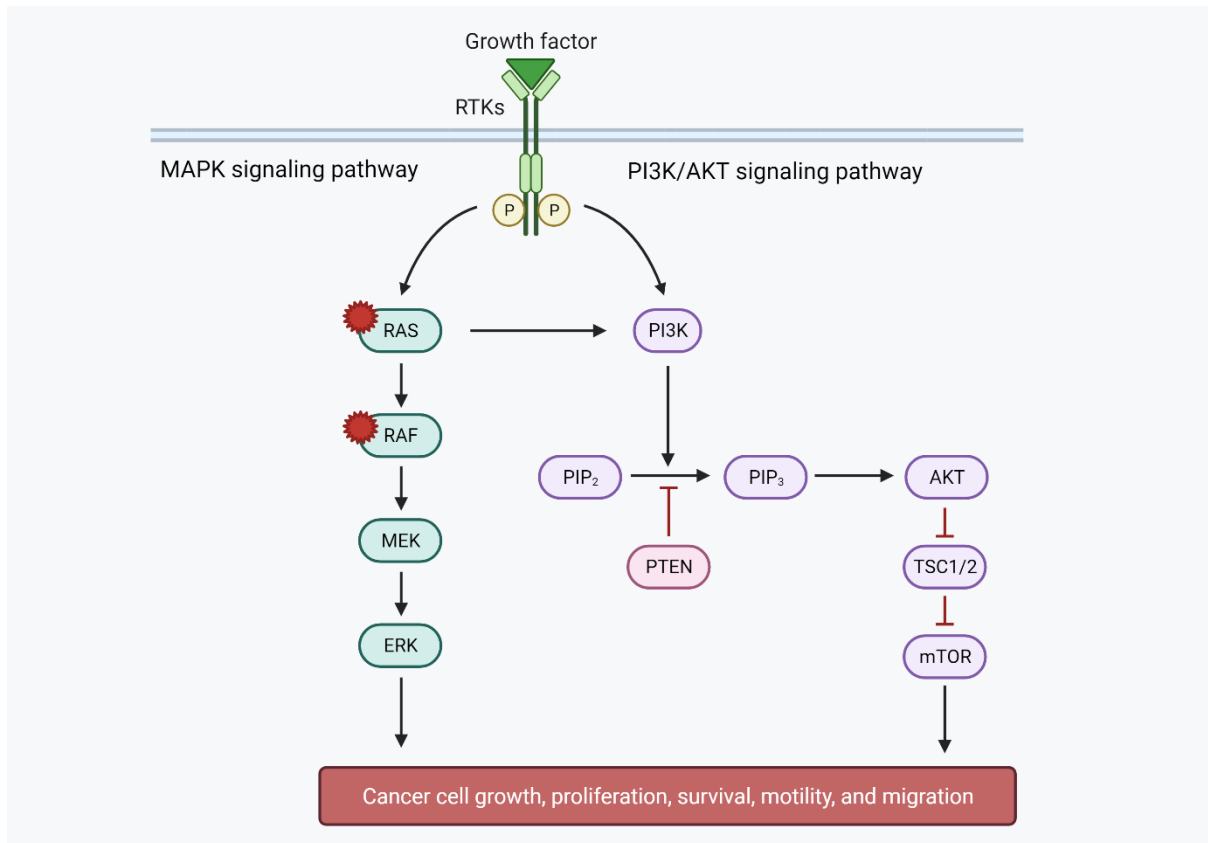


Figure 5 – Simple representation of the Mitogen-Activating Protein Kinase (MAPK), and the Phosphatidylinositol-3-kinase (PI3K)/AKT-pathways. Mutated proteins marked with a red asterisk shape. Created with Biorender.com

1.6.7. Familial Thyroid Cancer

Familial forms of TC account for up to 10% of all TC [179]. In familial forms, cancer diagnosis occurs at an early age, usually presenting as a multifocal disease, and some authors advance that the course of the disease can be aggressive, with local invasion, lymph nodes metastasis, increased risk of recurrence and decrease survival rates when comparing to sporadic TC [180]. However, some authors argue that the clinical outcome of familial cases does not differ from sporadic cases [181].

Familial forms can commonly arise from parafollicular cells and are frequently syndromic forms related to RET mutations (25% of the cases), being designated Familial Medullary Thyroid Carcinoma (FMTc) [181]. Since tumors, sporadic or familial, rising from parafollicular cells are not the focus of this thesis, these will not be further explored.

Follicular cells also give rise to familial forms of TC, being designated Familial Non-Medullary Thyroid Carcinoma (FNMTc) [182, 183]. Through clinicopathological findings, familial forms of TC are divided into two categories: syndromic and non-syndromic [179, 184, 185].

Syndromic FNMTc (SFNMTc) comprehends several syndromes where TC is not the main phenotypic expression, but rather a less common manifestation. Some of these syndromes are:

Familial Adenomatous Polyposis (FAP), PTEN-hamartoma tumor syndrome, Werner Syndrome (WS), Carney Syndrome (CS), DICER1 syndrome, and others [183, 184] (**Table 2**).

Non-syndromic FNMTC (NSFNMTTC) presents TC has the main phenotypic manifestation [182, 185]. FNMTC is challenging to differentiate from sporadic NMTC due to three main reasons: 1 - FNMTC is known for being a rare condition; 2 - histologically speaking, FNMTC tumors do not differ considerably from sporadic cancers; and 3 - the genetics behind FNMTC is rather heterogeneous and poorly understood [182, 185].

NSFNMTTC has been hypothesized to be a polygenic disorder usually coupled with various low- to moderate-susceptibility genes and incomplete penetrance [182, 183, 186-189]. Although the genetic component is poorly understood, there has been a few reports that advance several genes that may play a role in NSFNMTTC, such as germline mutations in Forkhead Box E1 (*FOXE1*) gene [187, 190], in Thyroid Transcription Factor-1 (*TTF-1*) [188, 190], in Mitogen-Activated Protein Kinase Kinase 5 (*MAP2K5*) gene [190, 191] and in the *USP13* gene (**Table 2**) [192].

According to the WHO classification, in the absence of previous irradiation and known syndromic cancer, NSFNMTTC is clinically suspected when one of three conditions are found in a family: 1 - presence of follicular cell-derived thyroid carcinoma in at least three first-degree relatives, 2 - presence of PTC in two or more first-degree family members, or 3 – presence of FND in three or more first- or second-degree relatives [181, 182, 184, 185]. NSFNMTTC is frequently concomitant with benign lesions. FND, FTA, inflammatory diseases, e.g. HT, and oncocytic change have been reported (**Table 2**) [181]. Also, synchronous PTC with other subtypes, namely FTC and OCA can be found in NSFNMTTC (**Table 2**) [181, 187]. Comparing to SNMTC, NSFNMTTC has been associated with increased risk of lateral compartment LNM, ETE and disease recurrence [181].

The emergence of Next Generation Sequencing (NGS) techniques, namely Whole-Exome Sequencing (WES), has allowed genetic analysis with extraordinary coverage and has permitted to overcome some genetic barriers [193]. **Table 2** explores syndromic and non-syndromic forms of FNMTC, their respective altered genes and thyroid lesions that were reported in familial cases.

Table 2 - Syndromic and Non-syndromic forms of FNMTC, respective altered genes and associated thyroid lesions.

	FNMTC	Gene (gene location)	Thyroid lesions	Reference
Syndromic	Familial adenomatous polyposis (autosomal dominant)	<i>APC</i> (5q22.2)	Cribiform-morular PTC (16%)	[179, 181, 183]
	PTEN-hamartoma tumor syndrome (autosomal dominant)	<i>PTEN</i> (10q23.31)	FND (43–75%), FTA (25%), OA, classic PTC and IFV-PTC (60%), FTC (14–45%), OCA (≈ 1%), ACA (< 1%), C-cell hyperplasia, Lymphocytic thyroiditis (55%)	[179, 181, 183]
	DICER1 syndrome (autosomal dominant)	<i>DICER1</i> (14q32.13)	FND, FTA PTC, FTC and PDTC (rare)	[179, 181, 183]
	Carney complex (autosomal dominant)	<i>PRKAR1A</i> (17q24.2)	FND, FTA PTC and FTC (15%)	[179, 181, 183]
	Werner syndrome (autosomal recessive)	<i>WRN</i> (8p12)	FTA FTC, PTC and ATC (18%)	[179, 181, 183]
	McCune–Albright syndrome	<i>GNAS</i> (20q13.2–q13.3)	FTA with Papillary Architecture PTC, FTC	[179]
Non-syndromic	Familial papillary thyroid carcinoma (autosomal dominant)	<i>TCO</i> (19p13)	FTA PTC (classic and oncocytic), OCA	[179, 181, 183, 194]
	Familial papillary thyroid carcinoma with papillary renal cell neoplasia (unknown)	<i>PRN</i> (1q21)	PTC	[179, 183]
	Familial non-medullary thyroid carcinoma type 1 (unknown)	<i>NMTC1</i> (2q21)	PTC (follicular variant)	[179, 181, 183]
	Familial papillary thyroid carcinoma and multinodular goitre (autosomal dominant)	<i>MNG1</i> <i>PTCSC3</i> (14q32)	Nodular hyperplasia PTC	[179, 183]
	-	<i>FOXE1</i> (9q22)	FND classic PTC, Tall cell PTC IFV-PTC, FTC	[181, 183, 187, 190]
	-	<i>TITF-1</i> (14q13)	FND PTC	[183, 188, 190]

FNMTc	Gene (gene location)	Thyroid lesions	Reference
-	<i>MAP2K5</i> (15q23)	PTC	[183, 190, 191]
-	<i>USP13</i> (3q26.33)	FND, HT, Thyroiditis PTC	[192]
-	<i>SRGAP1</i> (12q14.2)	PTC	[183, 190, 195]
-	<i>CHEK2</i> (22q12.1)	PTC	[181, 196]
-	<i>TIMM44</i> (19p13.2)	FTA, OA OCA, classic PTC, Oncocytic PTC	[181, 194, 197]
-	<i>NTN4</i> (12q22)	OCA, Classic PTC, IFV-PTC	[181, 198]
-	<i>NOP53</i> (19q13.33)	OCA, PTC	[181, 199]

FND – Follicular Thyroid Disease, **PTC**- Papillary Thyroid Carcinoma, **FTA** - Follicular Thyroid Adenoma, **FTC** – Follicular Thyroid Carcinoma, **OA** – Oncocytic Adenoma, **OCA** – Oncocytic Thyroid Carcinoma, **IFV-PTC** – Infiltrative Follicular Variant Papillary Thyroid Carcinoma, **PDTC** - Poorly-Differentiated Thyroid Carcinomas, **ACA** - Anaplastic Thyroid Carcinomas, **HT** - Hashimoto thyroiditis

CHAPTER II

AIMS

CHAPTER II – AIMS

Comparing to other cancers (e.g. breast, lung, colorectal), TC remains understudied. Over the last years, several improvements in TC research were made, however, gaps persist in prognostication and patient management decisions. Establishing clinicopathological and genetic markers would improve genetic counseling and contribute to the development of new therapies. Disease prevention and active surveillance are crucial to evade aggressive treatments, reduce comorbidities, avoid invasive surgeries, and alleviate the burden to the healthcare system.

Distinguishing TC low-risk tumors from those at risk of recurrence and TC-related mortality remains challenging. This is true for both sporadic and familial forms of the disease, with the latter posing additional challenges. In familial TC, a substantial void exists on the knowledge of the genetic factors associated with disease. This limited understanding is partially due to the absence of identified genes associated with the disease, as well as the small size of some studied families and the variable disease phenotype within affected individuals. It appears that in some families', mutations attributable to familial disease segregation are unique of that family, hampering our understanding of the genetic background of the disease.

MAIN AIM:

The main aim of this work is to contribute to offer to thyroid cancer patients an early and better diagnosis, a more accurate risk-stratification, and a personalized medicine approach. For that we perform an in-depth exploration of sporadic and familial thyroid cancer, analyzing clinicopathological aspects as well as the molecular landscape of these tumors.

SPECIFIC AIMS:

1. In-depth exploration of sporadic thyroid cancer, with emphasis in Papillary Thyroid Carcinoma (PTC):
 - 1.1. First, we aim to identify clinicopathological factors that can contribute to better predict the outcome of PTC patients, considering recurrent/persistent disease, structural disease and disease-specific mortality outputs. We aim to contribute to the knowledge of PTC biological behavior and to optimize patient management during follow-up (**ARTICLE I** and **ARTICLE III**).
 - 1.2. Second, we aim to identify molecular markers that can contribute to predict the outcome of PTC patients. Here, we aim to establish the role of *BRAF*, *RAS* (*NRAS*,

HRAS and *KRAS*) and *TERT* promoter genes in TC progression and how mutational status may contribute to predict disease recurrence/persistence, structural disease, development of DM and PTC-related mortality (**ARTICLE II** and **ARTICLE IV**).

2. In-depth exploration of familial thyroid cancer: Investigation of two families

Our team identified two families that presented familial TC (Family R and Family C). Both families comprised the criteria for TC heredity (>3 first-degree relatives affected by the disease and diagnosis at early age), but no syndromic forms of the disease were detected.

Resorting to Whole-Exome Sequencing, we identified in these families two novel germline, possibly pathogenic, mutations in the *USP42* gene (a ubiquitin-specific protease) in Family R and in the *KCNB2* gene (a voltage-gated potassium channel) in Family C.

Therefore, our specific goals were to find evidence for *USP42* and *KCNB2* gene alterations as underlying causes of NSFNTMC. Therefore, we aim:

- 2.1. To evaluate the pathogenic role of *USP42* as a putative gene in NSFNTMC. For that we will study in *in silico* databases, public and in-house, the relevance of ubiquitin-specific peptidases (USPs) in TC, and we will also carry out *in vitro* studies to evaluate the impact of *USP42* detected mutation's impact in thyroid cell biology (**ARTICLE V**).
- 2.2. To evaluate the pathogenic role of *KCNB2* as a putative gene in NSFNTMC. For that we will study in *in silico* databases, public and in-house, the relevance of voltage-gated potassium channels (vgKCNs) in TC, and we will also carry out *in vitro* studies to evaluate the impact of the *KCNB2* detected mutation's impact in thyroid cell biology. We further aim to establish a correlation between vgKCN mutations, I- uptake and patient response to treatment (**Section 4.7; Manuscript in development**).

CHAPTER III

MATERIALS AND METHODS

CHAPTER III – MATERIALS AND METHODS

3.1. Sporadic Thyroid Cancer (AIM 1)

Materials and methods regarding the study of AIM 1, are depicted in **ARTICLES I-IV**.

3.2. Familial Thyroid Cancer (AIM 2)

Materials and methods regarding AIM 2, the study of familial TC, are depicted in **ARTICLE**

V. Subsequent materials and methods related to ongoing work are described in segments 4.6 and 4.7 of the RESULTS chapter.

CHAPTER IV

RESULTS

CHAPTER IV – RESULTS

4.1. ARTICLE I

Even though PTC presents a good prognosis, some cases are metastatic and highly aggressive, several times resulting in patient loss. There is a need to understand which patients to treat with a more conservative approach and which patients require a more aggressive treatment. This way, our team performed a study in which investigated clinicopathological factors that may influence patient outcomes, namely recurrent/persistent disease, structural disease, and disease-specific mortality. The main goal was to identify pre-surgical factors linked to adverse outcomes in a consecutive series of PTC patients.

Article

Clinicopathological Features as Prognostic Predictors of Poor Outcome in Papillary Thyroid Carcinoma

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Simple Summary: Thyroid cancer incidence is increasing, with overdiagnosis being the major driver of the thyroid cancer “epidemic”. Papillary thyroid carcinoma, usually with excellent prognosis, sometimes has an aggressive metastatic pattern. This heterogeneity in progression makes it difficult to tailor treatment strategies for an individual patient. We aimed to identify clinicopathological factors associated with papillary thyroid carcinoma recurrence, persistence, and specific mortality. Our study supports that both pre-surgical factors, such as male gender, presence of psammoma bodies, gross extra-thyroidal extension, and lateral compartment lymph node metastases, as well as lymph vessel invasion, venous invasion, presence of necrosis, and incomplete surgical resection, should be taken into consideration regarding treatment and follow-up of PTC patients. The same is true when analysis is restricted to stage I patients. The importance of this report is to emphasize clinical and imaging pre-surgical thyroid cancer patients’ evaluation for an appropriate surgical treatment and patient prognosis.

Abstract: Papillary thyroid cancer (PTC) has an indolent nature and usually excellent prognosis. Some PTC clinicopathological features may contribute to the development of aggressive metastatic disease. In this work, we want to evaluate PTC clinicopathological features that are presurgical prognostic predictors of patients’ outcomes and find which indicators are more adequate for tailoring surgical procedures and follow-up. We studied a series of 241 PTC patients submitted to surgery. All patients’ files and histological tumor samples were reviewed. The 8th edition AJCC/UICC (American Joint Committee on Cancer/Union for International Cancer) Controlstaging system and the 2015 American Thyroid Association risk stratification system were used. Total thyroidectomy was performed in 228 patients, lymphadenectomy in 28 patients. Gross extrathyroidal extension (ETE) was present in 10 patients and 31 tumor resection margins were incomplete. Cervical lymph node metastases (LNMs) were present in 34 patients and distant metastases at diagnosis in four patients.

In multivariate analysis, male gender (OR = 15.4, $p = 0.015$), venous invasion (OR = 16.7, $p = 0.022$), and lateral compartment LNM (OR = 26.7, $p = 0.004$) were predictors of mortality; psammoma bodies (PBs) (OR = 4.5, $p = 0.008$), lymph vessel invasion (OR = 6.9, $p < 0.001$), and gross ETE (OR = 16.1, $p = 0.001$) were predictors of structural disease status; male gender (OR = 2.9, $p = 0.011$), lymph vessel invasion (OR = 2.8, $p = 0.006$), and incomplete resection margins (OR = 4.6, $p < 0.001$) were predictors of recurrent/persistent disease. Our study supports that the factors helping to tailor patient's surgery are male gender, presence of PBs, gross ETE, and lateral compartment LNM. Together with pathological factors, lymph vessel invasion, venous invasion, necrosis, and incomplete surgical resection, should be taken into consideration regarding treatment and follow-up of patients.

Keywords: thyroid cancer; prognosis; clinicopathological; outcomes

1. Introduction

Thyroid cancer (TC) is the most common endocrine malignancy [1,2]. According to National Cancer Institute surveillance, epidemiology, and end results (SEER), the yearly TC incidence has nearly tripled since 1975. Almost the entire change has been attributed to an increase in the incidence of papillary thyroid microcarcinoma (mPTC) [2,3]. In 2020, TC is the fifth most common cancer in women [4] and overdiagnosis is a major driver of the TC “epidemic” [5].

PTC is the most common malignancy originating from the thyroid (80–90% of all TC types [1]). Treatment includes surgical resection of the thyroid gland with radioiodine (RAI) ablation in selected cases. PTC generally has an indolent nature and good, or even excellent, prognosis after surgery, with survival rates for adults of 92–98% at 10-year follow-up [6]. Yet, some PTC patients (5–10%) develop highly aggressive metastatic disease [7], require further treatment, have poor quality of life, and a higher mortality rate. In order to differentiate patients with a worse prognosis from patients with excellent outcomes, if possible, at a pre-surgical stage, it is essential to evaluate appropriately the clinicopathological features of PTC, in an attempt to better predict patient prognosis and offer a more adequate treatment. Many risk factors and indicators of poor prognosis, including male gender, older age, increased tumor size (≥ 4 cm), gross extrathyroidal extension (ETE) [8], presence of lymph node metastases (LNMs) ≥ 3 cm and distant metastases (DMs) [9], that independently affect disease-free survival (DFS) and disease-specific survival (DSS) [10].

In the present study, we aimed to evaluate the specific association of clinicopathological features as prognostic predictors of recurrent/persistent disease, structural disease status and disease specific mortality, in a consecutive series of PTC patients. We also aimed to find which surgical procedures are more adequate to treat and follow patients. Patients' quality of life is of paramount importance, so it is important to aggressively treat an aggressive cancer, as is taking off the psychological burden of an indolent one. Determining which clinicopathological features are prognostic predictors of each of the established outcomes is important since they have different impacts on patients' quality of life.

2. Results

A detailed presentation of data can be accessed in Table S2.

2.1. Demography and Histopathology

Tumors were classified according to the WHO classification [7], and subdivided into four categories: classical PTC ($n = 157$), follicular variant PTC ($n = 55$), oncocytic (classical oncocytic, follicular oncocytic, and Warthin-like) variant PTC ($n = 18$), and aggressive (tall cell, hobnail, solid/trabecular, and diffuse sclerosing) variant PTC ($n = 9$). The primary tumor was not available for revision in three cases.

Our cohort constituted 202 (83.8%) females and 39 (16.2%) males, the mean age of the patients was 51.7 ± 15.2 years. Gender distribution did not reveal differences between histological subtypes. Patients presenting with aggressive variant PTC were older (mean 57.6 ± 14.8 years).

The most frequent type of surgery was total thyroidectomy ($n = 228$, 94.6%). Lymphadenectomy was performed in 28 (11.6%) patients. Patients presenting with aggressive variant PTC and classical PTC were more frequently submitted to associated lymphadenectomy ($n = 3$, 33.3% and $n = 20$, 12.7%, respectively) than the remaining patient population ($p = 0.035$).

Median tumor size was 12.0 ± 10.0 mm, and 97 (40.2%) were mPTC. The presence of solid components was a rare event ($n = 19$, 8.0%), more often present in aggressive variant PTC ($n = 3$, 33.3%, $p < 0.001$). Inflammatory infiltrate was a frequent finding ($n = 212$, 89.5%) that was present in all aggressive variant PTC ($n = 9$, $p = 0.005$). PBs appeared in 82 (34.6%) tumors, they predominated in classical PTC and aggressive variant PTC ($n = 72$, 46.2% and $n = 3$, 33.3%, respectively), with statistically significant differences ($p < 0.001$). Dystrophic calcification (DC) was found in 57 (23.9%) and necrosis in five (2.1%) tumors.

The majority of tumors were unilateral ($n = 199$, 83.3%), unifocal ($n = 151$, 62.7%), and with an expansive growth pattern ($n = 133$, 55.6%). Lymph vessel invasion was present in 47 tumors (19.8%). Classical PTC ($n = 36$, 23.1%) and aggressive variant PTC ($n = 4$, 44.4%) evidenced a higher occurrence of lymph vessel invasion ($p = 0.023$). Venous invasion was a rare event ($n = 16$, 6.7%) that was more often in follicular variant PTC and oncocytic variant PTC ($n = 8$, 4.5% and $n = 3$, 16.7%, respectively; $p = 0.003$).

Most tumors ($n = 168$, 70.6%) were located at the gland periphery or contacted the gland capsule, but only 96 (41.7%) tumors extended beyond the capsule. Histological extension to the perithyroidal soft tissue was present in 96 (41.7%) tumors. Gross ETE was present in 10 (4.2%) patients, three (33.3%) had aggressive variant PTC ($p = 0.009$). Resection margins were incomplete in 31 (12.9%) patients.

The histological examination of the thyroid gland revealed associated lesions in 158 (65.8%) patients. Multinodular goiter and lymphocytic thyroiditis were the most common lesions found ($n = 63$, 39.9%, and $n = 79$, 50.0%, respectively).

2.2. Post-Operative Staging and Radioiodine (RAI)

There were 193 (80.0%) tumors smaller than 20mm. Cervical LNMs were present in 34 (14.1%) patients. Despite not reaching statistical significance, classical PTC and aggressive variant PTC had higher proportions of tumors with LNMs ($n = 25$, 15.9% and $n = 2$, 22.2%, respectively). Central LNMs were present in 18 (7.5%) patients, and lateral LNMs were present in 16 (6.6%) patients. Extranodal disease extension was present in eight (44.4%) of the central LNMs and five (31.2%) of the lateral LNMs.

DMs were present at diagnosis in four (1.7%) patients, two cases of classical PTC and two cases of follicular variant PTC. The majority of our population were stage I ($n = 225$, 93.4%), there were 12 (5.0%) stage II patients and four (1.7%) stage IVB patients.

RAI treatment was performed in 182 (75.5%) patients, 26 (14.3%) patients were submitted to more than one treatment. The median RAI dose administered was 100.0 ± 28.0 mCi (0–740 mCi).

We subdivided our cohort into two groups, depending on whether they were submitted to RAI. In the RAI group ($n = 182$), half of patients ($n = 96$) had intermediate risk of recurrence. The majority ($n = 44$, 74.6%) of non-RAI-treated patients ($n = 59$) had low risk of recurrence.

2.3. Follow-Up

The median follow-up was 71.8 ± 40.4 months (2.1–197.4 months).

We maintained the two group subdivision during follow-up evaluation, since Tg (thyroglobulin) levels are different concerning the type of surgery and RAI administration.

The first dynamic risk stratification (FDRS) analysis was made 24 months after treatment, as proposed by Tuttle et al. [11]. At that time, 118 (68.2%) RAI treated patients revealed an excellent

response to treatment. Nineteen (11.0%) patients had a structural incomplete response. At FDRS, 41 (71.9%) of the non-RAI-treated patients had an excellent response to treatment.

At the end of follow-up, 14 (7.7%) RAI-treated patients had a structural incomplete response. Most non-RAI-treated patients had excellent or indeterminate response to treatment ($n = 56$, 96.5%), none had structural incomplete response or needed to be submitted to further treatment.

2.4. Association Between Clinicopathological Features and Recurrent/Persistent Disease

In our series, recurrence was observed in 12 (5.0%) patients. Persistence was noticed in 45 (18.7%) patients. A detailed presentation of data can be accessed in Table S3.

We found a significant association between increased risk of recurrent/persistent disease and male gender, lymph vessel invasion, presence of necrosis, gross ETE, incomplete surgical resection, lateral compartment LNM, and extranodal extension of lateral LNM in the whole group (Table 1). All the clinicopathological features mentioned above significantly lowered DFS at 10-year follow-up (Figure 1A–G).

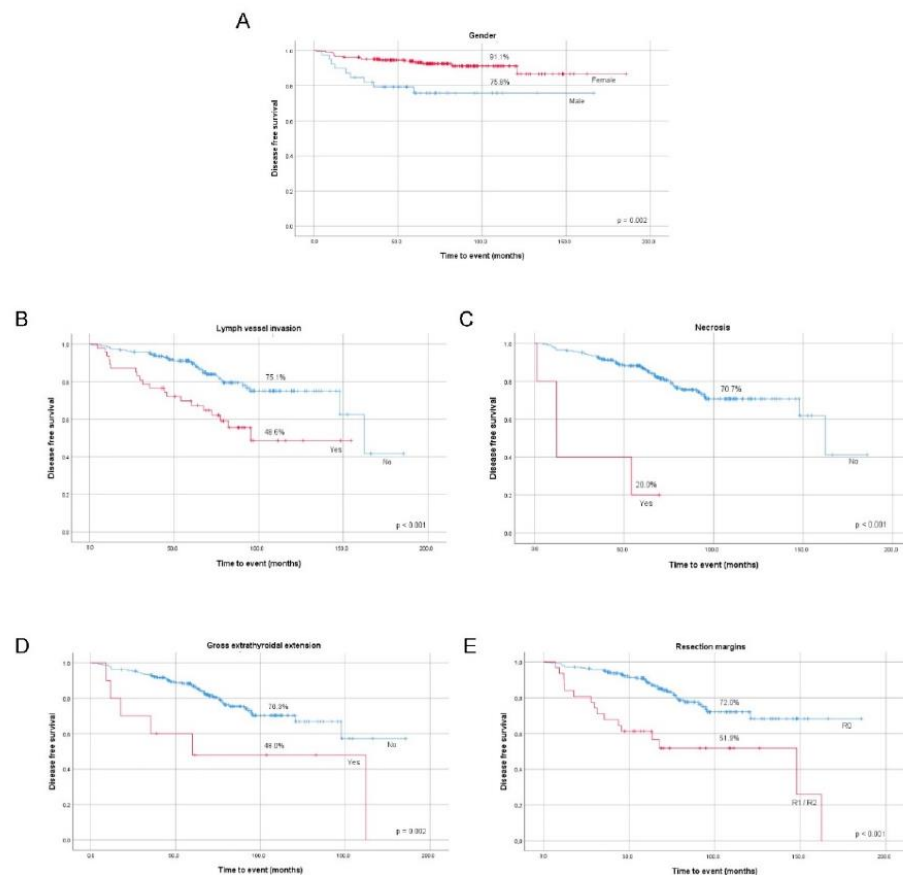


Figure 1. Cont.

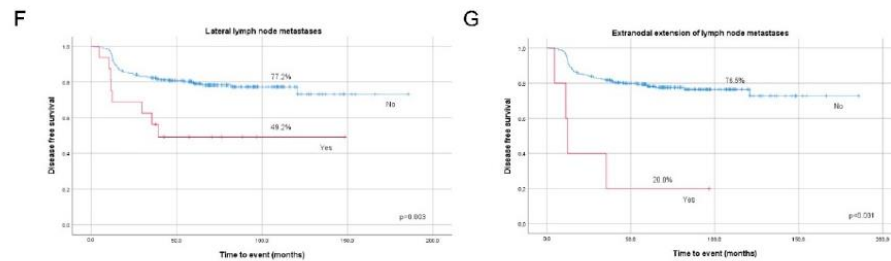


Figure 1. Kaplan–Meier curves of papillary thyroid cancer (PTC) recurrent/persistent disease status according to gender (A), lymph vessel invasion (B), necrosis (C), gross extrathyroidal extension (D) and resection margins (E), metastasized lateral lymph nodes (F), and extranodal extension of metastasized lateral lymph nodes (G).

Table 1. Univariate analysis regarding recurrent/persistent disease.

Clinicopathological Features	Univariate Analysis	
	OR (95% CI)	p-Value
Gender	2.732 (1.324–5.637)	0.007
Necrosis	14.560 (1.592–133.196)	0.018
Lymph vessel invasion	3.399 (1.710–6.756)	<0.001
Gross extrathyroidal extension	5.400 (1.467–19.881)	0.011
Resection margins	4.507 (2.057–9.872)	<0.001
Lateral lymph node metastases	3.592 (1.283–10.059)	0.015
Extranodal extension of lateral lymph node metastases	13.811 (1.511–126.219)	0.020

In classical PTC, a significant association was found between increased risk of recurrent/persistent disease and larger tumor size, lymph vessel invasion, presence of necrosis, incomplete surgical resection, and extranodal extension of lateral LNM.

Patients with recurrent/persistent disease more often needed further RAI treatment ($p < 0.001$).

The multivariate logistic regression analysis after adjustment for age at diagnosis demonstrated that male gender (OR = 2.9 (95% CI 1.27–5.83)), lymph vessel invasion (OR = 2.8 (95% CI 1.34–5.83)), and incomplete surgical resection (OR = 4.6 (95% CI 1.98–10.67)) were independent risk factors for recurrent/persistent disease (Table 2).

Table 2. Multivariate logistic regression analysis regarding recurrent/persistent disease.

Clinicopathological Features	Multivariate Analysis	
	OR (95% CI)	p-Value
Gender	2.870 (1.273–5.833)	0.011
Lymph vessel invasion	2.795 (1.340–5.833)	0.006
Resection margins	4.596 (1.980–10.671)	<0.001

2.5. Association Between Clinicopathological Features and Recurrent/Persistent Disease, in Stage I Patients

Considering that 225 (93.5%) patients in our series were at stage I, according to the AJCC/UICC (American Joint Committee on Cancer/Union for International Cancer Control), and that this is the stage that fits the majority of PTC patients, we performed the above analysis in this subgroup of patients.

We found a significant association between increased risk of recurrent/persistent disease and nodule size, lymph vessel invasion, gross ETE, and incomplete surgical resection.

The multivariate logistic regression analysis after adjustment for age and gender at diagnosis demonstrated that gender (OR = 2.6 (95% CI 1.05–6.22); $p = 0.039$), lymph vessel invasion (OR = 2.9

(95% CI 1.33–6.31); $p = 0.007$), and incomplete surgical resection (OR = 4.4 (95% CI 1.83–10.70); $p = 0.001$) remained independent risk factors for recurrent/persistent disease in stage I patients.

2.6. Association Between Clinicopathological Features and Structural Disease Status

A detailed presentation of data can be accessed in Table S4.

We found a significant association between increased risk of structural disease status and male gender, tumors bigger than 10mm, presence of PBs, DC, necrosis, lymph vessel invasion, gross ETE, incomplete surgical resection, lateral compartment LNM, and extranodal extension of lateral LNM in the whole group (Table 3). All the clinicopathological features mentioned above significantly lowered DFS at 10-year follow-up (Figure 2A–I).

Table 3. Univariate analysis regarding structural disease status.

Clinicopathological Features	Univariate Analysis	
	OR (95% CI)	p-Value
Gender	3.487 (1.413–8.605)	0.007
Nodule size (cut-off 10 mm)	2.968 (1.074–8.201)	0.036
Psammoma bodies	3.783 (1.515–9.445)	0.004
Dystrophic calcification	2.750 (1.134–6.666)	0.025
Necrosis	47.556 (5.045–448.261)	0.001
Lymph vessel invasion	7.690 (3.048–19.402)	<0.001
Gross extrathyroidal extension	11.105 (2.950–41.801)	<0.001
Resection margins	5.291 (2.074–13.500)	<0.001
Lateral lymph node metastases	8.944 (2.981–26.841)	<0.001
Extranodal extension of lateral lymph node metastases	14.591 (2.312–92.076)	0.004

In classical PTC, a significant association was found between increased risk of structural disease status and presence of PBs and DC, lymph vessel invasion, incomplete surgical resection, and lateral compartment LNM.

The multivariate logistic regression analysis after adjustment for age at diagnosis and gender demonstrated that PBs (OR = 4.5 (95% CI 1.49–13.45)), lymph vessel invasion (OR = 6.9 (95% CI 2.47–19.22)), and gross ETE (OR = 16.1 (95% CI 3.30–78.68)) are independent risk factors for structural disease status (Table 4).

Table 4. Multivariate logistic regression analysis regarding structural disease status.

Clinicopathological Features	Multivariate Analysis	
	OR (95% CI)	p-Value
Psammoma bodies	4.478 (1.491–13.451)	0.008
Lymph vessel invasion	6.887 (2.467–19.224)	<0.001
Gross extrathyroidal extension	16.118 (3.302–78.678)	0.001

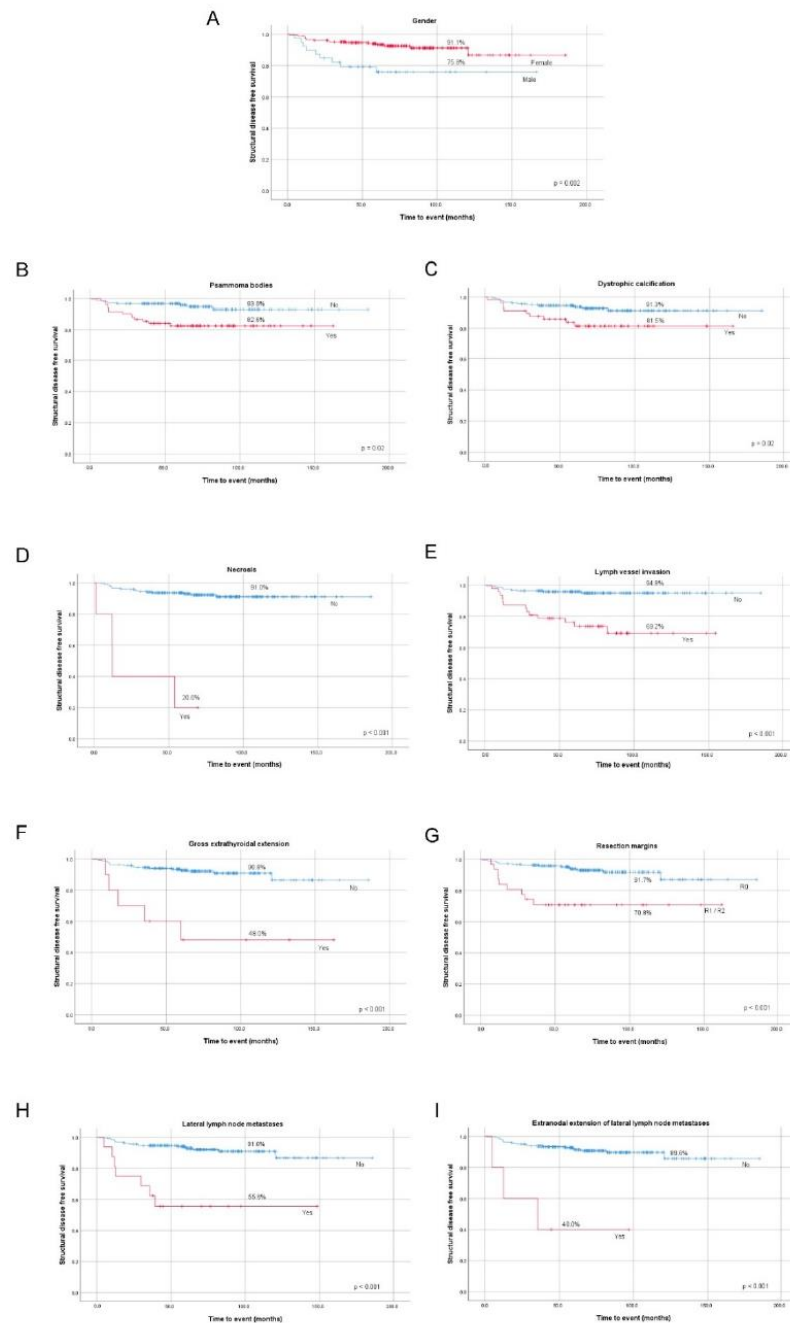


Figure 2. Kaplan–Meier curves of PTC structural disease status by gender (A), presence of psammoma bodies (B), presence of dystrophic calcification (C), presence of necrosis (D), lymph vessel invasion (E), gross extrathyroidal extension (F), resection margins (G), presence of metastasized lateral lymph nodes (H), and extranodal extension of metastasized lateral lymph nodes (I).

2.7. Association Between Clinicopathological Features and Structural Disease Status, in Stage I Patients

In stage I patients, we found a significant association between increased risk of structural disease status and nodule size, presence of PBs, DC, lymph vessel invasion, gross ETE, incomplete surgical resection, and lateral compartment LNM in the whole group.

The Cox regression analysis after adjustment for age at diagnosis and gender demonstrated that PBs (OR = 4.86 (95% CI 1.37–17.30); $p = 0.015$), lymph vessel invasion (OR = 7.76 (95% CI 2.35–25.65); $p = 0.001$), and gross ETE (OR = 24.13 (95% CI 3.55–163.91); $p = 0.001$) are independent risk factors for structural disease status in stage I patients.

2.8. Association Between Clinicopathological Features and Disease-Specific Mortality

A detailed presentation of data can be accessed in Table S5.

A significant association was found between increased risk of DSM (disease-specific mortality) and older age at diagnosis, male gender, larger tumors, presence of necrosis, lymph vessel invasion, venous invasion, gross ETE, lateral compartment LNM, and extranodal extension of lateral LNM in the whole group (Table 5). All the clinicopathological features mentioned above significantly lowered DSS at 10-year follow-up (Figure 3A–G).

Table 5. Univariate analysis regarding PTC disease-specific mortality.

Clinicopathological Features	Univariate Analysis	
	HR (95% CI)	p-Value
Age	1.067 (1.012–1.124)	0.016
Gender	7.279 (1.861–24.481)	0.004
Nodule size	1.079 (1.035–1.124)	<0.001
Necrosis	68.100 (9.248–501.489)	<0.001
Gross extrathyroidal extension	16.000 (3.306–77.440)	0.001
Resection margins	6.044 (1.529–23.899)	0.010
Lymph vessel invasion	7.421 (1.706–32.273)	0.008
Venous invasion	8.346 (1.872–37.205)	0.005
Lateral lymph node metastases	14.667 (3.484–61.745)	<0.001
Extranodal extension of metastasized lateral lymph nodes	57.500 (8.065–409.938)	<0.001

In classical PTC, a significant association was found between increased risk of DSM and older age and larger tumors. In follicular variant PTC, a significant association was found between increased risk of DSM and venous invasion. DSM events were not observed in oncocytic variant PTC.

All patients that had DSM were treated with RAI. All of them had disease persistence and received higher RAI doses ($p < 0.001$). They either had DMs at presentation or (the majority) developed DMs ($p < 0.001$) during follow-up.

The multivariate logistic regression analysis after adjustment for age at diagnosis demonstrated that gender (HR = 15.4 (95% CI 1.69–140.84)), venous invasion (HR = 16.7 (95% CI 1.50–185.60)), and lateral compartment LNM (HR = 26.7 (95% CI 2.88–246.87)) are independent risk factors for DSM (Table 6).

Table 6. Multivariate logistic regression analysis regarding PTC-specific mortality.

Clinicopathological Features	Multivariate Analysis	
	HR (95% CI)	p-Value
Age	1.077 (1.010–1.148)	0.023
Gender	15.426 (1.690–140.844)	0.015
Venous invasion	16.667 (1.497–185.606)	0.022
Lateral lymph node metastases	26.686 (2.885–246.870)	0.004

The analysis was not done for stage I patients since only three patients had disease-specific mortality (DSM).

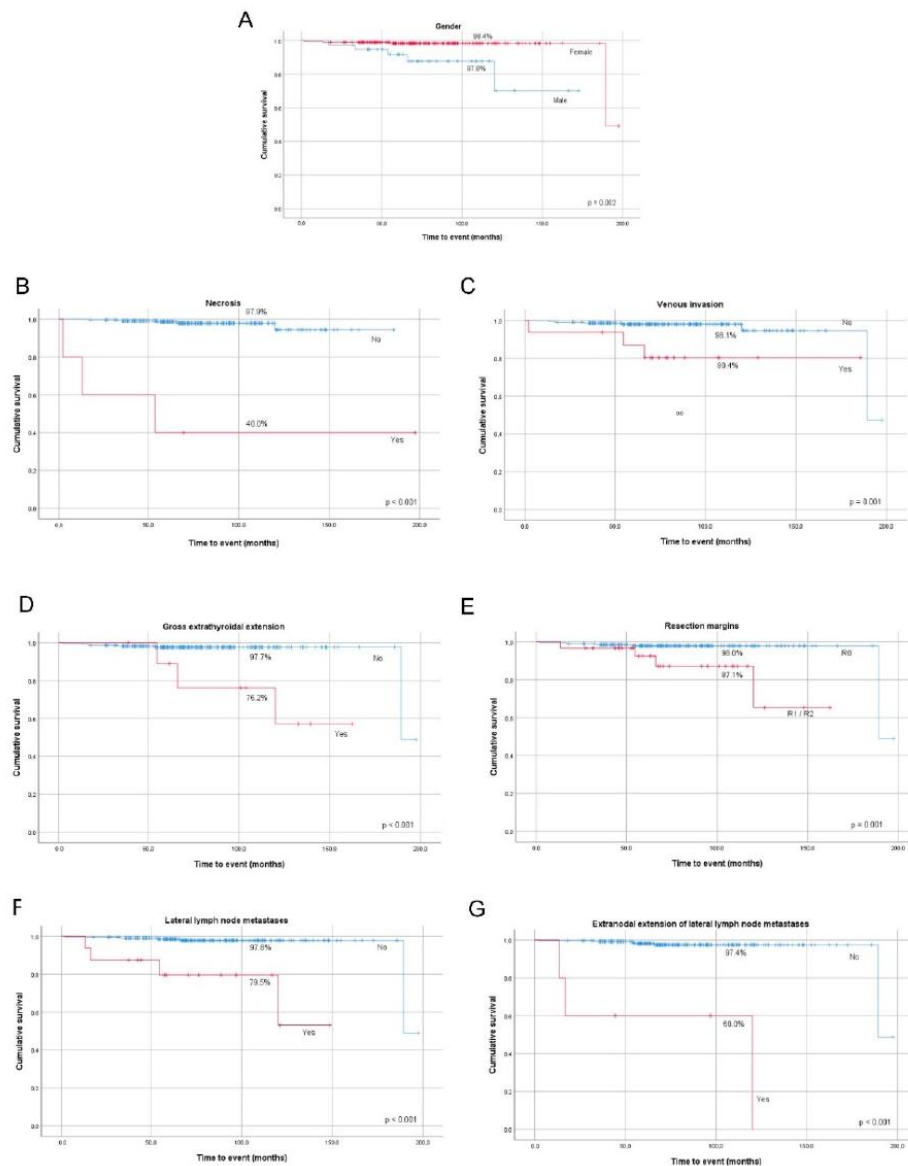


Figure 3. Kaplan–Meier survival curves of PTC disease-specific survival (DSS) by gender (A), presence of necrosis (B), venous invasion (C), gross extrathyroidal extension (D) resection margins status (E), metastases to lateral lymph nodes (F), and extranodal extension of lateral lymph node metastases (G).

3. Discussion

Thyroid cancer is a disease carrying a good or very good prognosis. However, recurrent/persistent disease can be present in a substantial proportion of these patients and there is lack of effective indicators that can be used in the daily clinical practice to stratify patients. In most cases, clinicians have difficulty separating a low risk disease from patients at risk of persistence/progression/death.

Our study was done in a central hospital, based upon a series of consecutive patients (more than 80% of the patients were middle-aged women) submitted to thyroid surgery between January 2002 and December 2015. All the patients had a diagnosis of PTC, without selection bias other than the inclusion/exclusion criteria.

At 10-year follow-up, our cohort of patients displayed an overall survival of 96.3%, with a recurrent/persistent disease rate of 23.7% (in accordance with the literature) [6,12]. Seventy-eight percent of the patients did not have evidence of disease at the end of follow-up. Most patients presented stage I or II disease. The above data indicate that our PTC series is composed of low-risk cases and is a reliable series to study the prognosis influence of the clinicopathological features, in an unbiased daily clinical setting.

Total thyroidectomy and RAI ablation were the most frequent treatments for PTC patients. In 2015, ATA (American Thyroid Association) guidelines [2] emphasized the role of lobectomy and isthmectomy and limited the RAI role in PTC treatment, but these guidelines were not used in our cohort. The patients who were treated without receiving RAI had excellent outcomes, supporting observations that in selected cases, RAI is not needed to assure definitive PTC treatment [13].

Previous studies attributed to male gender a significant role in worse PTC prognosis [14,15]. In our series, male patients displayed a higher risk of persistent/recurrent disease and of DSM. Our results confirm male gender as an independent prognostic factor, affecting recurrent/persistent DFS and DSM [10].

An association between matrix calcification and thyroid malignancy has been previously proposed [3]. Older patients will have increased tumor size and more frequent calcifications and this may explain why tumors with calcifications are associated with more aggressive features [12]. In our work, we evaluated the presence of DC and PBs. The presence of PBs may be seen either within PTC or in adjacent thyroid tissue [16], which is useful for diagnosing classical PTC, but whether or not the presence of PBs is associated to PTC prognosis [3,17–20] still remains controversial.

The aforementioned controversy stems from the fact that PBs are frequently found in mPTC [18] and associated with the indolent course of this type of cancer [19]. We have described a significant correlation between PTC with PBs and younger patients, ETE, and LNM [3,20] and other authors also reported a significant association with high stage grouping (stage IVa) and poorer disease-free survival [17]. In our series, the presence of PBs was an independent factor for structural disease status in the multivariate analysis. Our result indicates, for the first time, a relevant role of PBs as an indicator of patient outcome, providing one rule out the mPTC.

The presence of PBs can be revealed by a careful ultrasonography, because there is a strong association between PBs and microcalcifications [12,17,20]. In fine needle aspiration cytology, PBs are an excellent marker of PTC diagnosis [3]. Pre-operative knowledge of PBs' relation to microcalcifications and to structural disease status, observed in our series, can provide meaningful information in surgeons' decision-making processes.

A well-known factor associated with an adverse outcome regardless of architectural pattern or nuclear features is necrosis [21]. The presence of necrosis increased the risk for all the evaluated outcomes. Although present in five cases, the prognosis importance of such a finding (necrosis) was so high that we had to remove it from the multivariate analysis; otherwise, necrosis would be the only significantly predictive factor for any outcome.

The impact of lymph vessel invasion and venous invasion on tumor behaviour is recognized by the ATA risk stratification system [2]. Taking in consideration the above, we realize the importance of analyzing lymph vessel invasion and venous invasion in patient outcome. We performed this analysis in our series, despite recognizing the difficulty and limitation to distinguishing venous invasion from lymph vessel invasion by H&E (Hematoxylin and eosin) studies alone [21,22]. In the multivariate analysis, lymph vessel invasion remained an independent prognostic factor for recurrent/persistent disease and structural disease status. In multivariate analysis, venous invasion is associated with DSM.

This issue has recently been emphasized by the International Collaboration on Cancer Reporting (ICCR). The dataset developed by the ICCR for the pathology reporting of thyroid resection specimens for carcinoma declares that it is mandatory to specifically report on the status of venous invasion in PTC [23].

Tumor location in the thyroid gland is suggested by some authors to predispose to ETE, since superficial tumor location may facilitate tumor spread into lymphatic channels and regional lymph nodes [24]. We did not find a significant association between tumor location or minimal ETE and any of the outcomes. This observation fits with other authors that stated that minimal ETE is not an independent prognostic factor for DFS or DSS [21,25], and is associated with a low risk (3–9%) of recurrence [26]. Our result also supports the recent revision and downgrading of staging in cases with minimal ETE [2].

Gross ETE is described by some authors as an independent prognostic factor, affecting patients' DFS and DSS [27]. Other authors suggested that gross ETE particularly affects DFS of patients with tumors measuring >20 mm [28]. In our series, in which 80% of the tumors were <20 mm, gross ETE had a deleterious impact on patients' evaluated outcomes. In the multivariate analysis, gross ETE remained a significant independent factor for structural disease status. This observation fits with other authors that described gross ETE association with an increased recurrence risk (23–40%) [21,26] being a major predictor for poor outcome in PTC [25].

The assessment of ETE is crucial in pre-operative staging of TC [29] to decide the extent of the surgery; if ETE is present, RAI treatment will be performed. Ultrasonography is routinely done in every thyroid patient and has high sensitivity for assessing gross ETE [29]. Given the risk of gross ETE for structural disease observed in our series, pre-operative knowledge can be of major significance in surgeons' decision-making processes.

Having usually an indolent growth and excellent prognosis, the quality of surgery is pivotal in PTC treatment [30]. The surgeon is an important part of a personalized approach and a cornerstone involving the patient, the clinician, and the pathologist. A detailed surgical report is crucial for proper staging and further treatment and incomplete surgical resection is a high risk factor for recurrence, as well as DSM [11,25]. In our series, incomplete surgical resection was reported in 12.9% patients (in accordance with the literature [31]), and remained a significant independent prognostic feature for recurrent/persistent disease in the multivariate analysis.

Cervical LNM is a common finding in PTC, affecting 20% to 80% of patients [20,21]. Some authors state that clinically evident cervical LNM seems to be the fundamental indicator in terms of prognosis [32]. PTC frequently recurs in regional lymph nodes [28] and cervical LNM, at initial surgery, increases the incidence of recurrent disease during follow-up [30,32] and decreases DSS in some studies. Controversy exists regarding appropriate surgical management of cervical lymph nodes without evidence of metastases. Some authors prefer prophylactic central compartment lymphadenectomy because second surgery for recurrence in the central compartment has a greater risk of recurrent laryngeal nerve injury and permanent hypoparathyroidism. Ito et al. highlighted that a large number of central LNMs (>5), not just their presence, has an independent prognostic impact [33]. Other authors have emphasized that prophylactic central lymphadenectomy does not improve patients' outcomes [34]. ATA recommends prophylactic central lymphadenectomy in the presence of advanced tumors and lateral compartment LNM [2].

In our series, central LNM was not associated with patient outcome. The absence of an association between central LNM and prognosis can be related to the tumors' small size (only 4/18 tumors were ≥ 21 mm) or the small number of central LNMs (13/18 tumors had fewer than five LNMs). The absence of an association between central LNM and prognosis can also indicate that central LNM has no significant association with patient prognosis. In contrast, we observed a significant impact of lateral compartment cervical LNM and of extranodal extension of lateral LNM in all evaluated outcomes, as is described in the literature [30]. In the multivariate analysis, only the presence of lateral compartment LNM remained a significant independent prognostic feature, increasing the risk of DSM.

Ultrasonography evaluation of lateral LNM is mandatory in pre-operative staging of thyroid cancer to plan the surgery extension. In our series, we observed a significant risk of lateral LNM for DSM and confirmed its pre-operative importance in surgeons' decision-making processes.

The overall good prognosis of PTC is expressed in patients' long-term survival and in the fact that most PTC patients are stage I, according to the AJCC/UICC, at diagnosis. In our series, 225 (93.5%) patients were stage I at diagnosis. Our results are of even greater importance since the same clinicopathological factors remained as statistically significant outcome predictors when statistical analysis, for recurrence/persistent disease and structural disease, was performed when restricted to stage I patients.

4. Materials and Methods

4.1. Patient Cohort and Tissue Samples

Each file of 1652 consecutive patients, with thyroid tumors, submitted to thyroid surgery at the central hospital of Vila Nova de Gaia/Espinho (CHVNG/E), from January 2002 to December 2015, was reviewed by a single medical doctor (A.A.P.). TC was diagnosed in 461 (26%) patients. PTC was present in 423 (91.8%) of them. We excluded 158 patients: 29 patients had tumors smaller than 2 mm, 16 patients had not enough data and/or follow-up, and 113 patients had no material available to be rescued. In the present series ($n = 265$), we diagnosed 24 non-invasive follicular thyroid neoplasms with papillary-like nuclear features, which were excluded, following the recommendations of the World Health Organization (WHO) [7]. Inclusion criteria were the diagnosis of PTC in patients older than 18 years, followed for a minimum of two years (unless recurrence or disease-specific mortality occurred earlier), in which there were thyroid specimens for histological re-evaluation ($n = 241$).

Formalin-fixed paraffin-embedded (FFPE) material was collected, and all tumor samples were reviewed by a single pathologist, M.R.B., according to the WHO classification, 4th edition [7]. All doubtful tumor samples were reviewed by two senior pathologists (M.S.S., M.R.B.) for consensual diagnosis. Each patient was staged according to the 8th edition AJCC/UICC staging system [35]. Histological criteria adopted are summarized in Table S1.

All procedures described in this study were in accordance with national and institutional ethical standards. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of CHVNG/E (Project investigation 30/2016, 2016-01-28, Comissão de Ética do Centro Hospitalar de Vila Nova de Gaia/Espinho). According to Portuguese law, informed consent is not required for retrospective studies.

4.2. Patient Follow-Up

PTC patients underwent surgery with or without RAI treatment. Surgical options depended on tumor characteristics (size, ETE, clinically identified LNM, DM), and patient's age and gender. Lymphadenectomy was performed whenever LNMs were found before surgery or intra-operatively. RAI treatment was dependent on type of surgery performed (total vs. lobectomy/lobectomy and isthmectomy, surgery completeness), post-surgery pathological findings (size, ETE, lymph vessel and venous invasion, LNM), presence of DM, and patient's data.

RAI therapy was given 4 weeks after levothyroxine (LT4) withdrawal or using a recombinant thyroid stimulating hormone (rhTSH) preparation. This treatment was followed by post-therapy scintigraphy seven days later. Stimulated thyroglobulin (sTg) was evaluated at the time of RAI therapy and it was defined as the thyroglobulin (Tg) level measured when serum TSH was >30 mIU/L. The post-ablation Tg level was assessed approximately 12 months after surgery.

During the first follow-up year, patients were followed every three months and, in subsequent years, twice a year. Serum biochemical evaluation (TSH, free LT4, Tg, anti-Tg antibodies (TgAb)) and neck ultrasonography (US) were routinely performed. Computed tomography (CT) scans, 18-fluorodeoxyglucose positron emission tomography (18-FDG-PET) scans or scintigraphy were

performed only if previous exams were doubtful or suspicious. During follow-up, we used the 2015 ATA risk stratification system [2] to predict the risk of recurrent or persistent disease, in patients who were treated with RAI. In non-RAI-treated patients, we used the risk stratification system for patients treated without RAI published by Momesso et al. [36]. All patients were stratified as having four levels of possible response to treatment: excellent, indeterminate, biochemically incomplete, and structurally incomplete [2,36].

Structural disease status was detected by appropriate imaging modalities and, whenever possible, confirmed by cytology. Additional surgery was performed whenever feasible. If disease was unresectable, patients received RAI therapy. One patient with RAI-refractory disease was submitted to tyrosine kinase inhibitor treatment.

4.3. Clinical Endpoints

No clinical evidence of disease (NED) at final follow-up was established if patients had Tg levels that fit an excellent response [2], no detectable TgAb, and no structural evidence of disease. Patients were classified as having persistent disease whenever Tg values fit an indeterminate or incomplete response (elevated basal or sTg values alone, without structural correlation), or there was any evidence of disease on cross-sectional imaging (US, CT scan), functional imaging (RAI scan or 18-FDG-PET scan), or biopsy-proven disease (cytology or histology).

Patients were considered to have a positive structural disease status if any of the following conditions were met: (1) positive cytology/histology, (2) highly suspicious lymph nodes or thyroid bed nodules in the neck US (hypervascularity, cystic areas, heterogeneous content, rounded shape, or enlargement on the follow-up), (3) findings in scintigraphy, 18-FDG-PET scans, or other cross-sectional imaging highly suspicious for metastatic disease. Recurrence was defined if new biochemical, structural, and/or functional evidence of disease was detected following any period of NED. Recurrent/persistent disease ($n = 57$) was considered if patients had indeterminate or incomplete response to treatment, whether disease was only biochemical ($n = 33$) or biochemical and structural ($n = 24$). Disease-specific mortality (DSM) was also an endpoint (patients dying of unrelated conditions had the final status determined based on data available before their demise).

4.4. Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics version 25 (IBM, New York, NY, USA). The results were expressed in absolute frequency, percentage, mean $\pm$ standard deviation (Std), or median $\pm$ interquartile range (IR). Univariate analysis and correlation between recurrent/persistent disease, structural disease status, DSM, and clinicopathological features (age, gender, tumor size, ETE, lymph vessel invasion, venous invasion, for instance) were performed using a chi-squared test, Fisher's exact test, and logistic regression analysis. Unpaired *t*-tests or Mann–Whitney tests were applied when adequate. Multivariate analysis was performed using Cox regression. In the regression models, all the features significantly associated with each specified outcome in the univariate analysis were included in the multivariate analysis. The model was adjusted for gender and age at the time of surgery. Results were considered statistically significant at a *p* value < 0.05 .

5. Conclusions

Our study was performed in a consecutive series of patients submitted to surgery in a single institution, in a defined period, using clinical data collected by a single medical doctor and tumors reviewed by a single pathologist. The patients were followed for a median of 72 months. With this methodology, we achieved an unbiased population from a single institution, and it was rewarding to realize that we observed an excellent prognosis and low mortality rates as major conclusions points.

Having been referred to as predictors of poor outcome in previous studies, the evaluated features had not been previously addressed in a systematic way in relation to patient outcomes (recurrent/persistent disease, structural disease status, and disease-specific mortality), as far as we are

aware. Our study supports that both pre-surgical factors, such as male gender, presence of PBs, gross ETE, and lateral compartment LNM, as well as lymph vessel invasion, venous invasion, presence of necrosis, and incomplete surgical resection, should be taken into consideration regarding treatment and follow-up of PTC patients. Our study emphasizes the important role of complete resection margins and careful and systematic lymphadenectomy in the presence of lateral compartment LNM.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/11/3186/s1>, Table S1: Histopathological variables and considered criteria, Table S2: Clinicopathological features, Table S3: Clinicopathological features concerning recurrent/persistent disease, Table S4: Clinicopathological features concerning structural disease status, Table S5: Clinicopathological features concerning PTC-specific mortality. The graphical abstract was created with Biorender.com.

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References

- Grimm, D. Cell and Molecular Biology of Thyroid Disorders. *Int. J. Mol. Sci.* **2019**, *20*, 2895. [CrossRef] [PubMed]
- Haugen, B.R.; Alexander, E.K.; Bible, K.C.; Doherty, G.M.; Mandel, S.J.; Nikiforov, Y.E.; Pacini, F.; Randolph, G.W.; Sawka, A.M.; Schlumberger, M.; et al. American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* **2016**, *26*, 1–133. [CrossRef] [PubMed]
- Ferreira, L.B.; Lima, R.T.; Bastos, A.; Silva, A.M.; Tavares, C.; Pestana, A.; Rios, E.; Eloy, C.; Sobrinho-Simoes, M.; Gimba, E.R.P.; et al. OPNa Overexpression Is Associated with Matrix Calcification in Thyroid Cancer Cell Lines. *Int. J. Mol. Sci.* **2018**, *19*, 2990. [CrossRef] [PubMed]
- Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics. *Cancer J. Clin.* **2020**, *70*, 7–30. [CrossRef]
- Soares, P.; Celestino, R.; Gaspar da Rocha, A.; Sobrinho-Simões, M. Papillary Thyroid Microcarcinoma: How to Diagnose and Manage this Epidemic? *Int. J. Surg. Pathol.* **2014**, *22*, 113–119. [CrossRef] [PubMed]
- Gur, E.O.; Karaisli, S.; Haciyanli, S.; Kamer, E.; Genc, H.; Atahan, K.; Haciyanli, M. Multifocality related factors in papillary thyroid carcinoma. *Asian J. Surg.* **2019**, *42*, 297–302. [CrossRef] [PubMed]
- Lloyd, R.V.; Osamura, R.Y.; Klöppel, G.; Rosai, J. *WHO Classification of Tumors of Endocrine Organs*, 4th ed.; International Agency for Research on Cancer (IARC) Publications: Lyon, France, 2017; Volume 10, p. 355.
- Youngwirth, L.M.; Adam, M.A.; Scheri, R.P.; Roman, S.A.; Sosa, J.A. Extrathyroidal Extension Is Associated with Compromised Survival in Patients with Thyroid Cancer. *Thyroid* **2017**, *27*, 626–631. [CrossRef] [PubMed]
- Sugitani, I.; Kasai, N.; Fujimoto, Y.; Yanagisawa, A.J.S. A novel classification system for patients with PTC: Addition of the new variables of large (3 cm or greater) nodal metastases and reclassification during the follow-up period. *Surgery* **2004**, *135*, 139–148. [CrossRef]
- Ito, Y.; Miyauchi, A.J.E.J. Prognostic factors and therapeutic strategies for differentiated carcinomas of the thyroid. *Endocr. J.* **2009**, *56*, 177–192. [CrossRef] [PubMed]

11. Tuttle, R.M.; Tala, H.; Shah, J.; Leboeuf, R.; Ghossein, R.; Gonen, M.; Brokhin, M.; Omry, G.; Fagin, J.A.; Shaha, A. Estimating risk of recurrence in differentiated thyroid cancer after total thyroidectomy and radioactive iodine remnant ablation: Using response to therapy variables to modify the initial risk estimates predicted by the new American Thyroid Association staging system. *Thyroid* **2010**, *20*, 1341–1349. [\[CrossRef\]](#)
12. Kim, B.K.; Lee, E.M.; Kim, J.H.; Oak, S.Y.; Kwon, S.K.; Choi, Y.S.; Kim, Y.O. Relationship between ultrasonographic and pathologic calcification patterns in papillary thyroid cancer. *Medicine* **2018**, *97*, e12675. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Vaisman, F.; Shaha, A.; Fish, S.; Michael Tuttle, R.J.C.E. Initial therapy with either thyroid lobectomy or total thyroidectomy without radioactive iodine remnant ablation is associated with very low rates of structural disease recurrence in properly selected patients with differentiated thyroid cancer. *Clin. Endocrinol.* **2011**, *75*, 112–119. [\[CrossRef\]](#)
14. Liu, C.; Chen, T.; Zeng, W.; Wang, S.; Xiong, Y.; Liu, Z.; Huang, T. Reevaluating the prognostic significance of male gender for papillary thyroid carcinoma and microcarcinoma: A SEER database analysis. *Sci. Rep.* **2017**, *7*, 1–8. [\[CrossRef\]](#)
15. Guo, K.; Wang, Z. Risk factors influencing the recurrence of papillary thyroid carcinoma: A systematic review and meta-analysis. *Int. J. Clin. Exp. Pathol.* **2014**, *7*, 5393–5403.
16. Chen, S.J.; Yu, S.N.; Tzeng, J.E.; Chen, Y.T.; Chang, K.Y.; Cheng, K.S.; Hsiao, F.T.; Wei, C.K. Characterization of the major histopathological components of thyroid nodules using sonographic textural features for clinical diagnosis and management. *Ultrasound Med. Biol.* **2009**, *35*, 201–208. [\[CrossRef\]](#)
17. Bai, Y.; Zhou, G.; Nakamura, M.; Ozaki, T.; Mori, I.; Taniguchi, E.; Miyauchi, A.; Ito, Y.; Kakudo, K. Survival impact of psammoma body, stromal calcification, and bone formation in papillary thyroid carcinoma. *Mod. Pathol.* **2009**, *22*, 887–894. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Ferreira, L.B.; Gimba, E.; Vinagre, J.; Sobrinho-Simões, M.; Soares, P. Molecular Aspects of Thyroid Calcification. *Int. J. Mol. Sci.* **2020**, *21*, 7718. [\[CrossRef\]](#)
19. Johannessen, J.V.; Sobrinho-Simões, M. The origin and significance of thyroid psammoma bodies. *Lab. Investig.* **1980**, *43*, 287–296. [\[PubMed\]](#)
20. Pyo, J.-S.; Kang, G.; Kim, D.-H.; Park, C.; Kim, J.H.; Sohn, J.H. The Prognostic Relevance of Psammoma Bodies and Ultrasonographic Intratumoral Calcifications in Papillary Thyroid Carcinoma. *World J. Surg.* **2013**, *37*, 2330–2335. [\[CrossRef\]](#)
21. Xu, B.; Ghossein, R. Critical Prognostic Parameters in the Anatomic Pathology Reporting of Differentiated Follicular Cell-Derived Thyroid Carcinoma. *Cancers* **2019**, *11*, 1100. [\[CrossRef\]](#)
22. Eloy, C.; Santos, J.; Soares, P.; Sobrinho-Simões, M.J.V.A. Intratumoural lymph vessel density is related to presence of lymph node metastases and separates encapsulated from infiltrative papillary thyroid carcinoma. *Virchows Arch.* **2011**, *459*, 595–605. [\[CrossRef\]](#)
23. Ghossein, R.; Barletta, J.; Bullock, M.; Johnson, S.; Kakudo, K.; Lam, A.; Moonim, M.T.; Poller, D.; Tallini, G.; Tuttle, R.M.; et al. Data set for reporting carcinoma of the thyroid: Recommendations from the International Collaboration on Cancer Reporting. *Hum. Pathol.* **2020**. [\[CrossRef\]](#)
24. Niemeier, L.A.; Kuffner Akatsu, H.; Song, C.; Carty, S.E.; Hodak, S.P.; Yip, L.; Ferris, R.L.; Tseng, G.C.; Seethala, R.R.; Lebeau, S.O.; et al. A combined molecular-pathologic score improves risk stratification of thyroid papillary microcarcinoma. *Cancer* **2012**, *118*, 2069–2077. [\[CrossRef\]](#)
25. Wang, L.Y.; Ghossein, R.; Palmer, F.L.; Nixon, I.J.; Tuttle, R.M.; Shaha, A.R.; Shah, J.P.; Patel, S.G.; Ganly, I. Microscopic Positive Margins in Differentiated Thyroid Cancer Is Not an Independent Predictor of Local Failure. *Thyroid* **2015**, *25*, 993–998. [\[CrossRef\]](#)
26. Arora, N.; Turbendian, H.K.; Scognamiglio, T.; Wagner, P.L.; Goldsmith, S.J.; Zarnegar, R.; Fahey, T.J., III. Extrathyroidal extension is not all equal: Implications of macroscopic versus microscopic extent in papillary thyroid carcinoma. *Surgery* **2008**, *144*, 942–948. [\[CrossRef\]](#)
27. Ito, Y.; Ichihara, K.; Masuoka, H.; Fukushima, M.; Inoue, H.; Kihara, M.; Tomoda, C.; Higashiyama, T.; Takamura, Y.; Kobayashi, K.; et al. Establishment of an Intraoperative Staging System (iStage) by Improving UICC TNM Classification System for Papillary Thyroid Carcinoma. *World J. Surg.* **2010**, *34*, 2570–2580. [\[CrossRef\]](#)
28. Ito, Y.; Miyauchi, A.; Kudo, T.; Kihara, M.; Fukushima, M.; Miya, A.J.W. The effectiveness of prophylactic modified neck dissection for reducing the development of lymph node recurrence of papillary thyroid carcinoma. *World J. Surg.* **2017**, *41*, 2283–2289. [\[CrossRef\]](#)

29. Kim, H.; Kim, J.-A.; Son, E.J.; Youk, J.H.; Chung, T.-S.; Park, C.S.; Chang, H.-S. Preoperative prediction of the extrathyroidal extension of papillary thyroid carcinoma with ultrasonography versus MRI: A retrospective cohort study. *Int. J. Surg.* **2014**, *12*, 544–548. [\[CrossRef\]](#)
30. Bates, M.F.; Lamas, M.R.; Randle, R.W.; Long, K.L.; Pitt, S.C.; Schneider, D.F.; Sippel, R.S. Back so soon? Is early recurrence of papillary thyroid cancer really just persistent disease? *Surgery* **2018**, *163*, 118–123. [\[CrossRef\]](#)
31. Hundahl, S.A.; Cady, B.; Cunningham, M.P.; Mazzaferri, E.; McKee, R.F.; Rosai, J.; Shah, J.P.; Fremgen, A.M.; Stewart, A.K.; Hölzer, S. Initial results from a prospective cohort study of 5583 cases of thyroid carcinoma treated in the United States during 1996. *Cancer* **2000**, *89*, 202–217. [\[CrossRef\]](#)
32. Gorostis, S.; Ragun, T.; Schneegans, O.; Takeda, C.; Debry, C.; Dupret-Bories, A. Incidental thyroid papillary microcarcinoma: Survival and follow-up. *Laryngoscope* **2019**, *129*, 1722–1726. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Ito, Y.; Miyauchi, A.; Masuoka, H.; Fukushima, M.; Kihara, M.; Miya, A. Excellent prognosis of central lymph node recurrence-free survival for cN0M0 papillary thyroid carcinoma patients who underwent routine prophylactic central node dissection. *World J. Surg.* **2018**, *42*, 2462–2468. [\[CrossRef\]](#)
34. Viola, D.; Materazzi, G.; Valerio, L.; Molinaro, E.; Agate, L.; Faviana, P.; Seccia, V.; Sensi, E.; Romei, C.; Piaggi, P.; et al. Prophylactic Central Compartment Lymph Node Dissection in Papillary Thyroid Carcinoma: Clinical Implications Derived From the First Prospective Randomized Controlled Single Institution Study. *J. Clin. Endocrinol. Metab.* **2015**, *100*, 1316–1324. [\[CrossRef\]](#)
35. Amin, M.B.; Edge, S.; Greene, F.; Byrd, D.R.; Brookland, R.K.; Washington, M.K.; Gershenwald, J.E.; Compton, C.C.; Hess, K.R.; Sullivan, D.C.; et al. (Eds.) *AJCC Cancer Staging Manual*, 8th ed.; Springer International Publishing: Basel, Switzerland, 2017.
36. Momesso, D.; Vaisman, F.; Yang, S.P.; Bulzico, D.; Corbo, R.; Vaisman, M.; Tuttle, R. Dynamic risk stratification in differentiated thyroid cancer patients treated without radioactive iodine. *J. Clin. Endocrinol. Metab.* **2016**, *101*, 2692–2700. [\[CrossRef\]](#)

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Supplementary Materials: Clinicopathological Features as Prognostic Predictors of Poor Outcome in Papillary Thyroid Carcinoma

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Table S1. Histopathological variables and considered criteria.

Analyzed Category	Criteria	Evaluation
Poorly differentiated component	percentage within the primary tumor	%
Oncocytic component	polygonal shape cells, with large (three times the nucleus) eosinophilic and granular cytoplasm	%
Solid component	compact cell distribution or in trabecular disposition, without follicle formation	%
Papillae	papillary architecture, with neoplastic cells covering fibrovascular stalks	%
Inflammatory infiltrate	may range from only few isolated lymphocytes within the tumor or in its periphery, always less than 10 cells aggregated, to large lymphocytes groups that occupy at least 20% of the tumor	Absent/present
Fibrosis	all kinds: sclerosing, desmoplastic, hyaline tracts, central post ischemic, central hyalinization, wide fibrovascular stalks	%
Psammoma bodies	when present, even if it's just one	Absent/present
Dystrophic calcification	irregular shape calcification, whose size is bigger than psammoma bodies, no spherical or concentric shape	Absent/present
Necrosis	presence of focal ischemic or lytic necrosis	Absent/present
Tumor peripheric location	a. A–tumor is close (1–3 mm) to thyroid capsule without reaching it and 50% of the tumor is located more than 10mm of the thyroid capsule	A
	b. B–tumor contacts thyroid capsule or is close to it (1–3mm) and 50% of the tumor is located less than 10 mm of the thyroid capsule	B
	c. No–tumor is located at more than 3 mm of thyroid capsule	C
Tumor capsule invasion	tumor cells beyond tumor capsule a. Minimal–or less invasive foci	Minimal/Extensive

b. Extensive—more than 3 invasive foci		
Analyzed category	Criteria	Evaluation
Tumor growth	a. Expansive—more than 95% of the tumor has regular limit	Expansive/Infiltrative
	b. Infiltrative—more than 5% of the tumor has infiltrative border	
Extra-thyroidal extension	tumor cells beyond thyroid capsule	Minimal/ Gross
	a. Minimal—affects fibroadipose tissue with prominent vessels or nerves surrounding thyroid gland	
	b. Gross—affects strap muscles	
Lymphatic invasion	a. Yes—mesothelial cover of the invaded vessel	Yes
	b. Doubtful—round structure apart from the tumor, in relation with artery or vein, or that forms a longitudinal path inside the thyroid gland apart from the tumor	No
	c. No?	Doubtful
Venous invasion	1. venous invasion of tumor capsule vessels or outside the thyroid capsule	Absent/present
NIFTP	capsulated or well delimited tumor, follicular patterned, with papillary-like nucleus, without capsular or vascular invasion and without solid/trabecular/insular pattern	Absent/present



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Full Supplementary Tables S1-S5 are available in Excel format (XLSX.) at <https://www.mdpi.com/2072-6694/12/11/3186/s1>

4.2. ARTICLE II

After completion of **ARTICLE I**, we aimed to search the potential application of molecular markers as prognostic tools in TC treatment. Therefore, molecular analyses of *BRAF*, *RAS* and *TERT promoter* genes were screened in a consecutive series of patients. The main goal of this study was to evaluate the association between molecular markers and patient status, namely, recurrent/persistent disease, structural disease and disease-specific mortality, as well to understand if there was a polygenic effect on disease progression of PTC patients.

Article

Genetic Determinants for Prediction of Outcome of Patients with Papillary Thyroid Carcinoma

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Simple Summary: Aggressive metastatic disease is rare in papillary thyroid carcinoma (PTC), a neoplasia that usually carries an excellent prognosis. BRAF, RAS, and TERT promoter (TERTp) genes are altered in PTC, and their impact on patient outcomes remains controversial. We performed Sanger sequencing on a series of 241 PTCs to determine the role of genetic mutations (BRAF, RAS, and TERTp) in PTC patient outcomes. The implication of RAS^{mut} tumors remain uncertain in clinical terms. BRAF^{mut}/TERTp^{wt} tumors were prone to be associated with local aggressiveness (recurrent, persistent/disease), whereas TERTp^{mut} tumors were predisposed to recurrent/persistent structural disease, and disease-specific mortality. Our results indicate that different molecular markers play a distinct role in predicting PTC patient outcomes.

Abstract: Papillary thyroid carcinoma (PTC) usually presents an excellent prognosis, but some patients present with aggressive metastatic disease. BRAF, RAS, and TERT promoter (TERTp) genes are altered in PTC, and their impact on patient outcomes remains controversial. We aimed to determine the role of genetic alterations in PTC patient outcomes (recurrent/persistent disease, structural disease, and disease-specific mortality (DSM)). The series included 241 PTC patients submitted to surgery, between 2002–2015, in a single hospital. DNA was extracted from tissue samples of 287 lesions (primary tumors and metastases). Molecular alterations were detected by Sanger sequencing. Primary tumors presented 143 BRAF, 16 TERTp, and 13 RAS mutations. Isolated TERTp^{mut} showed increased risk of structural disease (HR = 7.0, $p < 0.001$) and DSM (HR = 10.1, $p = 0.001$). Combined genotypes, BRAF^{wt}/TERTp^{mut} (HR = 6.8, $p = 0.003$), BRAF^{mut}/TERTp^{wt} (HR = 3.2, $p = 0.056$) and BRAF^{mut}/TERTp^{wt} (HR = 2.2, $p = 0.023$) showed increased risk of recurrent/persistent disease. Patients with tumors BRAF^{wt}/TERTp^{mut} (HR = 24.2, $p < 0.001$) and BRAF^{mut}/TERTp^{mut} (HR = 11.5, $p = 0.002$) showed increased risk of structural disease. DSM was significantly increased in patients with TERTp^{mut} regardless of BRAF status (BRAF^{mut}/TERTp^{mut}, log-rank $p < 0.001$; BRAF^{wt}/TERTp^{mut}, log-rank $p < 0.001$). Our results indicate that molecular markers may have a role in predicting PTC patients' outcome. BRAF^{mut}/TERTp^{wt} tumors were prone to associate with

local aggressiveness (recurrent/persistent disease), whereas TERT^{mut} tumors were predisposed to recurrent structural disease and DSM.

Keywords: thyroid cancer; papillary thyroid carcinoma; prognosis; BRAF; RAS; TERT; patient outcome; recurrent/persistent disease; structural disease; PTC-specific mortality

1. Introduction

Thyroid cancer (TC) is the most common endocrine malignancy worldwide, and its incidence has been remarkably increasing over the past three decades, particularly in developed countries [1–4]. According to GLOBOCAN 2018, TC accounts for 3.1% of all diagnosed cancer worldwide [5]. TC incidence was 18.6/100,000 in women and 4.1/100,000 in men in the same period [6], mainly due to one TC histotype, papillary thyroid carcinoma (PTC) [7,8]. The overall mortality rate for both genders was about 0.30/100,000 [6] (less than 5% at 10-year follow-up [9]), which remains low despite increasing TC incidence. While usually presenting an excellent prognosis, some PTC patients experience persistence/recurrence or metastatic disease [10,11], and patients with distant metastases (DM) display 45% disease-specific survival (DSS) at 10-year follow-up [12]. Presurgical prognostication is practically nonexistent [13]. Fine-needle aspiration biopsy (FNAB) is an important tool for TC diagnosis [13]. FNAB presents limitations, with 10–40% of cases being classified as indeterminate without providing any prognostic indication [14,15]. Several factors (such as gender and age, tumor invasion, namely angioinvasion and extrathyroidal extension, presence of metastases, response to treatment) have proven prognostic value in PTC [16]. Over the last years, initiation and progression of TC have been steadily associated with genetic/epigenetic events that lead to the activation of cellular signaling pathways, namely MAPK and PI3K-AKT [17–19]. Several studies have advanced the detection of alterations of genes or gene panels for PTC diagnosis and/or prognosis evaluation and patient management [20]. Mutations in BRAF, RAS, and TERT promoter (TERTp) genes have been described as altered in PTC by our group [10,21,22] and by others [20,23,24]. Yet, discordance exists on how these genes impact tumor behavior and patient outcome. A BRAF^{V600E} point mutation on exon 15 is frequently detected in PTC, accounting for more than 90% of all BRAF^{mut} in TC. BRAF^{mut} is present in about 45% PTCs [25] and has been associated, in some studies, with features of clinical aggressiveness such as older age, larger tumors, extrathyroidal extension (ETE), lymph node metastases (LNM), higher stage, and poorer prognosis [26,27]. The impact of BRAF^{mut} in PTC DM, lack of response to radioiodine (RAI) therapy, and mortality are still controversial [10,26,28–30]. The genetic significance of RAS^{mut} in TC remains an open question since point mutations are detected in all subtypes of thyroid nodules, from benign to anaplastic lesions [31,32]. Studies on RAS^{mut} and TC prognostication have shown controversial results so far. Some studies showed no association of RAS^{mut} with poorer prognosis [33], while others have reported an association with DM and poorer survival [34,35]. TERTp^{mut} was found in approximately 10% of PTC cases [4], being more frequent in aggressive PTC variants [22]. In TC, TERTp^{mut} has been associated with older age, larger tumors, ETE, higher tumor stage, DM, RAI therapy resistance, and patient mortality, by our group [10,21,22] and by others [36–38].

Beyond their utility for diagnostic purposes, further data are needed in order to use molecular markers as prognostic tools in TC. In this study, we decided to evaluate BRAF, RAS (NRAS, HRAS, and KRAS), and TERTp molecular status in a consecutive series of PTC patients in an attempt to assess the impact of gene status on patients' outcome, namely recurrent/persistent disease, structural disease, and patient survival.

2. Materials and Methods

2.1. Patient Samples

The study was performed in a consecutive series of patients submitted to thyroid surgery at a single hospital (Centro Hospitalar de Vila Nova de Gaia e Espinho (CHVNG/E)), from January 2002 to December 2015 and diagnosed as PTC. Formalin-fixed, paraffin-embedded (FFPE) tissues were collected from institutional files. All tumor samples were reviewed by a single pathologist (MRB), according to the fourth edition of the World Health Organization classification of tumors of endocrine organs [39]. Inclusion criteria were PTC diagnosis in patients older than 18 years, followed for a minimum of two years (unless recurrence or disease-specific mortality (DSM) has occurred earlier), in which there were thyroid samples for histological re-evaluation. Following these criteria, 241 patients were included in the study. A total of 287 lesions were evaluated: besides 238 primary tumors, we reviewed 31 LNM at diagnosis, 16 locoregional recurrences (LR), and two DM, along with clinical information that was revised by a single physician (AAP).

The histological diagnosis of the 241 patients was the following: classical PTC (CPTC), follicular variant PTC (FVPTC), oncocytic variant PTC (OVPTC: Warthin-like PTC, classical oncocytic PTC, and follicular patterned oncocytic PTC) and aggressive variants of PTC (APTC: tall cell, hobnail, diffuse sclerosing and solid trabecular).

All procedures described in this study were in accordance with national and institutional ethical standards. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of CHVNG/E (Project investigation 30/2016, 28 January 2016, Comissão de Ética do Centro Hospitalar de Vila Nova de Gaia/Espinho). According to Portuguese law, informed consent is not required for retrospective studies.

2.2. Patient Follow-Up and Risk Stratification

Patients were staged using the eighth edition of the American Joint Cancer Committee/Union for International Cancer Control (AJCC/UICC) staging system [40]. Risk stratification at the second year and at the end of follow-up was evaluated according to the 2015 American Thyroid Association (ATA) guidelines [11] for patients submitted to total thyroidectomy followed by RAI therapy. For patients that were not submitted to RAI or less than total thyroidectomy, risk stratification was performed using the system published by Momesso et al. [41].

According to 2015 ATA guidelines, no clinical evidence of disease (NED) at final follow-up was established if patients had thyroglobulin (Tg) levels that fit excellent response [11], no detectable Tg antibodies (TgAb), and no structural evidence of disease. Patients were classified as persistent disease whenever Tg values fit indeterminate or incomplete response (elevated basal or stimulated Tg values alone, without structural correlation), or there was any evidence of disease on cross-sectional imaging (ultrasonography (US), computed tomography (CT) scan), functional imaging (RAI scintigraphy or 2-[¹⁸F]fluoro-2-deoxy-d-glucose positron-emission tomography (2-¹⁸F-FDG-PET) scan), or biopsy-proven disease (cytology or histology). Several Tg assays were used with different functional sensitivities, reflecting the long study period. For the sake of simplification, the two patients who had positive TgAb, were included in the group of incomplete biochemical responses. Recurrence was defined if new biochemical, structural, and/or functional evidence of disease was detected following any period of NED.

Patients were considered to have a positive structural disease status if any of the following conditions were met: (1) positive cytology/histology, (2) highly suspicious lymph nodes or thyroid bed nodules in the neck US (hypervascularity, cystic areas, heterogeneous content, rounded shape, or enlargement on the follow-up), (3) findings in RAI scintigraphy, ¹⁸F-FDG-PET scans, or other cross-sectional imaging highly suspicious for metastatic disease. Recurrent/persistent disease ($n = 57$) was considered if patients had an indeterminate or incomplete response to treatment, whether it was only biochemical disease ($n = 32$) or biochemical and structural disease ($n = 25$). DSM was also an endpoint (patients dying

of unrelated conditions had the final status determined based on data available before their demise).

2.3. DNA Extraction

DNA from FFPE tissues was retrieved from 10 µm sections after H&E guided careful microdissection. DNA extraction was performed using the GRS Genomic DNA Kit BroadRange (GRiSP Research Solutions, Porto, Portugal) following the manufacturer's instructions. Quantitative and qualitative analysis of all samples was then performed by spectrophotometry using Nanodrop N-1000 Spectrophotometer for microvolume UV-Vis measurements (Thermo Scientific, Waltham, MA, USA).

2.4. PCR and Sanger Sequencing Analysis

Genetic characterization of the series of tumors regarding BRAF, RAS (NRAS, HRAS, and KRAS), and TERTp mutations was performed as previously reported [9,19,27]. Primer design was performed accounting for the most frequent regions mutated in PTC, namely, BRAF codon 600, NRAS codon 61, HRAS and KRAS codons 12, 13, and 61, and TERTp-124, and –146 regions.

Amplification of genomic DNA (25–50 ng) was achieved using the QIAGEN multiplex PCR kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The annealing temperature of 61 °C was established after protocol optimization for BRAF, NRAS, and TERTp segments amplification in the same reaction. HRAS and KRAS were screened separately by a touchdown PCR using the MyTaq HS Mix 2× Bioline PCR Kit (Meridian Bioscience, Cincinnati, OH, USA) following the manufacturer's instructions. PCR amplification was confirmed in 1–2% agarose gel electrophoresis (GRS Agarose LE, GRiSP, Oporto, Portugal) and followed by PCR product purification. All tested hotspot mutations were sequenced by Sanger sequencing using the ABI Prism Big Dye Terminator kit v3.1 Cycle Sequencing (Fisher Scientific Applied Biosystems®, Portsmouth, NH, USA). After sequencing product precipitation, fragments were analyzed by capillary electrophoresis using the Applied Biosystems 3130/3130 ×1 Genetic Analyzers (Foster city, CA, USA). For all genes, all detected mutations were validated by performing a new and independent analysis.

2.5. Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics version 25 (IBM, New York, NY, USA). Results were expressed in absolute frequency, percentage, mean ± standard deviation (Std), and median ± interquartile range (IR). Distribution analyses were performed using crosstabs analyses. Unpaired *t*-test and Mann–Whitney tests were applied whenever adequate. Disease-free survival (DFS) and DSM were assessed by Kaplan–Meier and log-rank tests. Age- and gender-adjusted models were created to evaluate the mutational status impact of evaluated genes in the outcomes: recurrent/persistent disease, structural disease, and DSM. Hazard ratios (HR) were assessed by Cox proportional hazard models. Statistical significance was accepted with a two-tailed *p*-value < 0.05.

3. Results

3.1. Description of the Patients

Out of 241 patients, 202 (83.8%) were female. The mean age at diagnosis was 52 ± 15.2 years (18–86 years), with 56.0% of the patients being <55 years. Median tumor size was 12 ± 10.0 mm (2–70 mm), with 97 (40.2%) primary tumors presenting a size ≤10 mm (microcarcinomas). The mean follow-up time was 7 ± 2.9 years (0.2–16.8 years). In three patients, the primary tumors were not available, and just their LNM was analyzed.

Characteristics of the patients and tumors are summarized in Table 1.

Table 1. Series description: patient characteristics, tumor histology, staging, lymph node metastases, and patient outcome.

Patient Characteristics		<i>n</i>	(%)	<i>n</i> (Total)	Staging	<i>n</i>	(%)	<i>n</i> (Total)
Age				241	T stage			241
	<55 years	135	(56.0%)		T1a	96	(39.8%)	
	≥55 years	106	(44.0%)		T1b	97	(40.2%)	
Gender				241	T2	31	(12.9%)	
	Female	202	(83.8%)		T3a	7	(2.9%)	
	Male	39	(16.2%)		T3b	9	(3.7%)	
Histology		<i>n</i>	(%)	<i>n</i> (total)	T4a	1	(0.4%)	
Nodule size	Median ± IR	12.0 ± 10.00		238 ^a	N stage			241
Histological subtype				238 ^a	N0	207	(85.9%)	
	CPTC	156	(65.5%)		N1	34	(14.1%)	
	FVPTC	55	(23.1%)		M stage			241
	OVPTC	18	(7.6%)		M0	237	(98.3%)	
	AVPTC	9	(3.8%)		M1	4	(1.7%)	
Necrosis				237	Stage			241
	Absent	232	(97.9%)		SI	225	(93.4%)	
	Present	5	(2.1%)		SII	12	(5.0%)	
Psmmomas				237	SIV	4	(1.7%)	
	Absent	155	(65.4%)		Lymph node invasion and extranodal extension		<i>n</i>	(%)
	Present	82	(34.6%)		Lateral compartment			<i>n</i> (total)
Focality				241	Lymph node invasion			241
	Unifocal	151	(62.7%)		Absent	225	(93.4%)	
	Multifocal	90	(37.3%)		Present	16	(6.6%)	
Extrathyroidal extension				240	Lateral compartment			241
	Absent / minimal	230	(95.8%)		Extranodal extension			
	Major	10	(4.2%)		Absent	236	(97.9%)	
Lymphatic invasion				237	Present	5	(2.1%)	
	Absent	190	(80.2%)		Patient outcome		<i>n</i>	(%)
	Present	47	(19.8%)		Recurrent/persistent disease			<i>n</i> (total)
Venous invasion				239	Absent	184	(76.3%)	
	Absent	223	(93.3%)		Present	57	(23.7%)	
	Present	16	(6.7%)		Structural disease			241
Resection margins				240	PTC related death			241
	R0	209	(87.1%)		Alive or deceased by other causes			
	R1	31	(12.9%)		Deceased of PTC	9	(3.7%)	

CPTC: classical PTC; FVPTC: follicular variant PTC; OVPTC: oncocytic variant PTC (includes Warthin-like, classical oncocytic PTC, and follicular patterned oncocytic PTC); AVPTC: aggressive variants of PTC (includes tall cell, hobnail, diffuse sclerosing, and solid/trabecular variants). ^a Three primary tumors were not available, only lymph node metastases.

Two hundred twenty-five patients (93.4%) were at stage I, 12 patients (5.0%) were at stage II, and four patients (1.7%) were at stage IV.

During follow-up, 57 patients (23.7%) had recurrent/persistent disease, 25 patients (10.4%) had structural disease (LNM and/or DM) and nine patients (3.7%) had DSM.

3.2. Primary Tumors' Genetic Characterization

The study of primary tumors demonstrated the presence of BRAF^{mut} in 143 (62.7%), TERTp^{mut} in 16 (7.3%), and RAS^{mut} in 13 (5.6%) patients.

All BRAF^{mut} cases presented with p.Val600Glu, which was present in all four histological subtypes, but more frequent in CPTC (Table 2). Three different TERTp^{mut} were found, −124 G > A mutation was detected in 12 cases (5.5%) from all four histological variants (Table 2), −146 G > A mutation was found in three PTC (1.4%), all CPTC, and one tandem −124/−125 G > A mutation was detected in an FVPTC. Of the 16 TERTp^{mut} tumors, 11 (5.1%) were concomitantly mutated for BRAF (BRAF^{mut}/TERTp^{mut}), and four (1.9%) were only TERTp^{mut} (BRAF^{wt}/TERTp^{mut}). One hundred and twenty (56.1%) tumors were BRAF^{mut} but not TERTp (BRAF^{mut}/TERTp^{wt}). A total of 13 (5.6%) RAS^{mut} were detected in primary tumors. p.Gln61Arg mutation was detected in all RAS genes (NRAS, HRAS, and KRAS) with a frequency of 3.5%, 2.3%, and 1.7%, respectively, mostly in follicular patterned tumors. One KRAS p.Gln61Arg mutation was concomitantly present with a −124 G > A TERTp^{mut}.

Table 2. BRAF, TERTp, and RAS molecular status in primary tumors and distribution in the different PTC variants.

Gene(s)	Status	All <i>n</i> (%)	Histological Variants of Primary Tumors			
			CPTC	FVPTC	OVPTC	AVPTC
		<i>n</i> = 238 ^a	<i>n</i> = 156	<i>n</i> = 55	<i>n</i> = 18	<i>n</i> = 9
BRAF	wt	85	39	33	9	4
	p.Val600Glu	143 (62.7%)	110 (73.8%)	20 (37.7%)	8 (47.1%)	5 (55.6%)
TERTp	wt	202	133	47	14	8
	−124 G > A	12 (5.5%)	8 (5.6%)	2 (4.0%)	1 (6.7%)	1 (11.1%)
	−146 G > A	3 (1.4%)	3 (2.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	−124/−125 G > A	1 (0.5%)	0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)
BRAF/TERTp	BRAF ^{wt} /TERTp ^{wt}	79	36	31	8	4
	BRAF ^{mut} /TERTp ^{wt}	120 (56.1%)	94 (67.1%)	16 (32.0%)	6 (40.0%)	4 (44.4%)
	BRAF ^{wt} /TERTp ^{mut}	4 (1.9%)	2 (1.4%)	1 (2.0%)	1 (6.7%)	0 (0.0%)
	BRAF ^{mut} /TERTp ^{mut}	11 (5.1%)	8 (5.7%)	2 (4.0%)	0 (0.0%)	1 (11.1%)
NRAS	wt	223	152	47	15	9
	p.Gln61Arg	8 (3.5%)	1 (0.7%)	5 (9.6%)	2 (11.8%)	0 (0.0%)
HRAS	wt	127	75	36	10	6
	p.Gln61Arg	3 (2.3%)	0 (0.0%)	3 (7.7%)	0 (0.0%)	0 (0.0%)
KRAS	wt	117	67	35	10	5
	p.Gln61Arg	2 (1.7%)	1 (1.5%)	1 (2.8%)	0 (0.0%)	0 (0.0%)
RAS ^b	wt	218	151	43	15	9
	RAS ^{mut}	13 (5.6%)	2 (1.3%)	9 (17.3%)	2 (11.8%)	0 (0.0%)

CPTC: classical PTC; FVPTC: follicular variant PTC; OVPTC: oncocytic variant PTC (includes Warthin-like, classical oncocytic PTC, and follicular patterned oncocytic PTC); AVPTC: aggressive variants of PTC (includes tall cell, hobnail, diffuse sclerosing, and solid/trabecular variants). ^a Three primary tumors were not available, only lymph node metastases. ^b Represents RAS status if at least one of the RAS genes was sequenced; wt: wild-type; mut: mutated.

The detailed univariate analysis of clinicopathological features in relation to combined gene mutations is presented in Supplementary Table S1.

3.3. Metastatic Lesions' Genetic Characterization and Molecular Profile Concordance

The study of 31 LNM that were present at diagnosis demonstrated the presence of BRAF^{mut} in 17 LNM (54.8%) and TERTp^{mut} in three LNM (10.0%). Of those, two LNM (6.7%) were concomitantly BRAF^{mut}/TERTp^{mut}. The study of 16 cervical recurrences (14 recurrent LNM and two thyroid bed recurrences) revealed BRAF^{mut} in 12 lesions (75.0%), TERTp^{mut} in five lesions (33.3%), and being in three lesions concomitant BRAF^{mut}/TERTp^{mut} (20.0%). The study of two DM demonstrated the presence of NRAS^{wt}

in one lesion and one concomitant BRAF^{mut}/TERTp^{mut} in the other lesion. Detailed information of metastatic lesions molecular profile is presented in Supplementary Table S2.

3.4. Molecular Alterations in Recurrent/Persistent Disease

At 10-year evaluation, recurrent/persistent DFS was significantly lower in patients with TERTp^{mut} tumors (41.7%, $p = 0.034$). Patients with TERTp^{mut} tumors presented 2.3 times increased risk of recurrent/persistent disease ($p = 0.04$), but this association was lost when the model was adjusted for age and gender ($p = 0.062$) (Table 3). Recurrent/persistent DFS was not significantly different in patients with BRAF^{mut} or RAS^{mut} tumors from patients with BRAF^{wt} or RAS^{wt} tumors (Figure 1A–C; Table 2). Considering concomitant mutations, at 10-year evaluation, recurrent/persistent DFS was significantly lower in patients with BRAF^{mut}/TERTp^{wt} (log-rank $p = 0.021$), BRAF^{mut}/TERTp^{mut} (log-rank $p = 0.035$) and BRAF^{wt}/TERTp^{mut} (log-rank $p = 0.001$) tumors than in patients with BRAF^{wt}/TERTp^{wt} tumors (Figure 1D). The aforementioned concomitant mutations significantly increased the risk of recurrent/persistent disease in comparison to BRAF^{wt}/TERTp^{wt} tumors (Table 3).

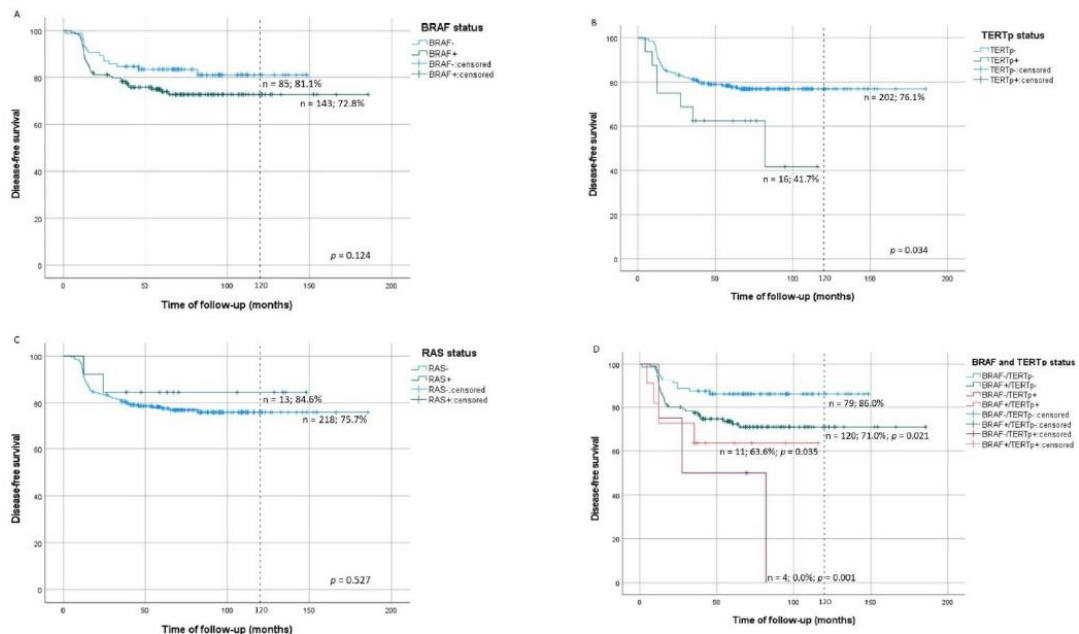


Figure 1. Kaplan–Meier curves of PTC of recurrent/persistent disease-free survival by tumor molecular status in comparison with wild type tumors; (A) BRAF status, (B) TERTp status, (C) RAS status, (D) combined BRAF and TERTp status in comparison to wild-type tumors; wt: wild-type; mut: mutated.

Table 3. Analysis of molecular status in the risk of recurrent/persistent disease.

Molecular Status	n	Recurrent/ Persistent Disease Absent	Recurrent/ Persistent Disease Present	Unadjusted HR (95% CI)	p-Value	Adjusted HR (95% CI)	p-Value *
BRAF status	228						
BRAF ^{wt}	85	70 (39.8%)	15 (28.8%)	1 (Reference)		1 (Reference)	
BRAF ^{mut}	143	106 (60.2%)	37 (71.2%)	1.6 (0.88–2.91)	0.128	—	—
TERTp status	218						
TERTp ^{wt}	202	157 (94.6%)	45 (86.5%)	1		1	
TERTp ^{mut}	16	9 (5.4%)	7 (13.5%)	2.3 (1.04–5.12)	0.040	2.2 (0.96–5.01)	0.062
RAS status	231						
RAS ^{wt}	218	168 (93.9%)	50 (96.2%)	1		1	
RAS ^{mut}	13	11 (6.1%)	2 (3.8%)	0.6 (0.16–2.61)	0.530	—	—
BRAF and TERTp status	214						
BRAF ^{wt} /TERTp ^{wt}	79	68 (41.7%)	11 (21.6%)	1		1	
BRAF ^{mut} /TERTp ^{wt}	120	87 (53.4%)	33 (64.7%)	2.2 (1.11–4.34)	0.024	2.2 (1.11–4.36)	0.023
BRAF ^{wt} /TERTp ^{mut}	4	1 (0.6%)	3 (5.9%)	6.5 (1.82–23.42)	0.004	6.8 (1.89–24.55)	0.003
BRAF ^{mut} /TERTp ^{mut}	11	7 (4.3%)	4 (7.8%)	3.4 (1.10–10.82)	0.043	3.2 (0.97–10.33)	0.056

n: number of cases; HR: Hazard ratio calculated by Cox Regression analysis; CI: Confidence interval; * Age- and gender-adjusted; p-value; wt: wild-type; mut: mutated.

3.5. Molecular Alterations in Structural Disease

Patients with TERTp^{mut} tumors had significantly lower structural disease-free survival (SDFS) (log-rank $p < 0.001$) than patients without TERTp^{mut} (Figure 2B) and presented significantly increased risk of structural disease, even when adjusted for age and gender (HR = 7.0, $p < 0.001$) (Table 4). SDFS was not significantly influenced by BRAF^{mut} or RAS^{mut} tumors (Figure 2A,C). Considering concomitant mutations, at 10-year evaluation, SDFS was significantly lower in patients with BRAF^{mut}/TERTp^{mut} (log-rank $p < 0.001$) and BRAF^{wt}/TERTp^{mut} (log-rank $p < 0.001$) tumors than in patients with BRAF^{wt}/TERTp^{wt} tumors (Figure 2D). The aforementioned concomitant mutations significantly increased the risk of structural disease status both in unadjusted and adjusted models (Table 4). Patients with BRAF^{mut}/TERTp^{wt} tumors did not display a significantly lower SDFS than BRAF^{wt}/TERTp^{wt} tumors (log-rank $p = 0.103$) (Figure 2D).

Table 4. Analysis of molecular status in the risk of structural disease.

Molecular Status	n	Structural Disease Absent	Structural Disease Present	Unadjusted HR (95% CI)	p-Value	Adjusted HR (95% CI)	p-Value *
BRAF status	228						
BRAF ^{wt}	85	79 (38.3%)	6 (27.3%)	1 (Reference)		1 (Reference)	
BRAF ^{mut}	143	127 (61.7%)	16 (72.7%)	1.7 (0.65–4.26)	0.286	—	—
TERTp status	218						
TERTp ^{wt}	202	187 (95.4%)	15 (68.2%)	1		1	
TERTp ^{mut}	16	9 (4.6%)	7 (31.8%)	7.4 (3.01–18.23)	<0.001	7.0 (2.67–18.54)	<0.001
RAS status	231						
RAS ^{wt}	218	198 (94.3%)	20 (95.2%)	1		1	
RAS ^{mut}	13	12 (5.7%)	1 (4.8%)	0.9 (0.11–6.35)	0.875	—	—
BRAF and TERTp status	214						
BRAF ^{wt} /TERTp ^{wt}	79	76 (39.6%)	3 (13.6%)	1		1	
BRAF ^{mut} /TERTp ^{wt}	120	108 (56.3%)	12 (54.5%)	2.8 (0.79–9.90)	0.112	—	—
BRAF ^{wt} /TERTp ^{mut}	4	1 (0.5%)	3 (13.6%)	24.3 (4.89–120.80)	<0.001	24.2 (4.80–122.05)	<0.001
BRAF ^{mut} /TERTp ^{mut}	11	7 (3.6%)	4 (18.2%)	13.2 (2.93–59.05)	0.001	11.5 (2.40–55.60)	0.002

n: number of cases; HR: Hazard ratio calculated by Cox Regression analysis; CI: Confidence interval; * Age- and gender-adjusted; p-value; wt: wild-type; mut: mutated.

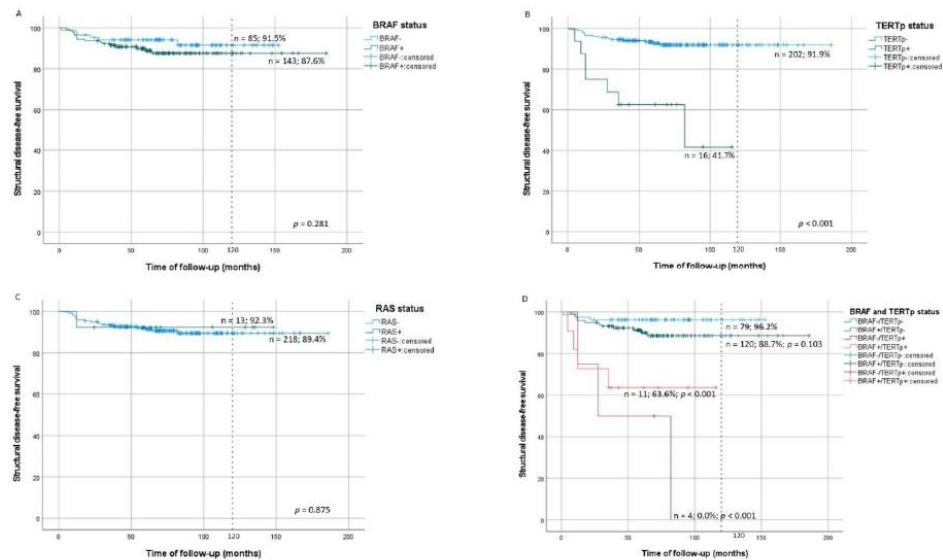


Figure 2. Kaplan–Meier survival curves of PTC structural disease-free survival (SDFS) by tumor molecular status in comparison with wild type tumors; (A) BRAF status, (B) TERTp status, (C) RAS status, (D) combined BRAF and TERTp status in comparison to wild-type tumors; wt: wild-type; mut: mutated.

3.6. Molecular Alterations in Disease-Specific Mortality

DSM was significantly increased in patients with TERTp^{mut} tumors (log-rank $p < 0.001$) in comparison with patients without TERTp^{mut} (Figure 3B). When adjusted for gender and age at diagnosis, TERTp^{mut} significantly increased the risk of DSM (HR = 10.1, $p = 0.010$) (Table 5). DSM was not significantly increased in patients with BRAF^{mut} or RAS^{mut} tumors (Figure 3A,C). DSM was increased in patients with BRAF^{mut}/TERTp^{mut} (log-rank $p < 0.001$) and BRAF^{wt}/TERTp^{mut} (log-rank $p = 0.035$) tumors in comparison with patients with BRAF^{wt}/TERTp^{wt} tumors (Figure 3D). There were no DSM events in patients with BRAF^{mut}/TERTp^{wt} tumors (Table 5).

Table 5. Analysis of molecular status in the risk of disease-specific mortality.

Molecular Status	n	Disease-Specific Mortality Absent	Disease-Specific Mortality Present	Unadjusted HR (95% CI)	p-Value	Adjusted HR (95% CI)	p-Value *
BRAF status	228						
BRAF ^{wt}	85	81 (36.8%)	4 (50.0%)	1 (Reference)		1 (Reference)	
BRAF ^{mut}	143	139 (63.2%)	4 (50.0%)	0.6 (0.15–2.39)	0.466	-	-
TERTp status	218						
TERTp ^{wt}	202	199 (94.8%)	3 (37.5%)	1		1	
TERTp ^{mut}	16	11 (5.2%)	5 (62.5%)	23.9 (5.70–100.23)	<0.001	10.1 (1.76–58.41)	0.010
RAS status	231						
RAS ^{wt}	218	212 (94.6%)	6 (85.7%)	1		1	
RAS ^{mut}	13	12 (5.4%)	1 (14.3%)	2.4 (0.28–20.36)	0.429	-	-
BRAF and TERTp status	214						
BRAF ^{wt} /TERTp ^{wt}	79	76 (36.9%)	3 (37.5%)	1		1	
BRAF ^{mut} /TERTp ^{wt}	120	120 (58.3%)	0 (0.0%)	n.a.	n.a.	-	-
BRAF ^{wt} /TERTp ^{mut}	4	3 (1.5%)	1 (25.0%)	6.5 (0.67–63.31)	0.109	-	-
BRAF ^{mut} /TERTp ^{mut}	11	7 (3.4%)	4 (50.0%)	11.6 (2.59–52.09)	0.001	n.s.	n.s.

n: number of cases; HR (Hazard ratio) calculated by Cox Regression analysis; CI: Confidence interval; * Age- and gender-adjusted; p-value; wt: wild-type; mut: mutated.

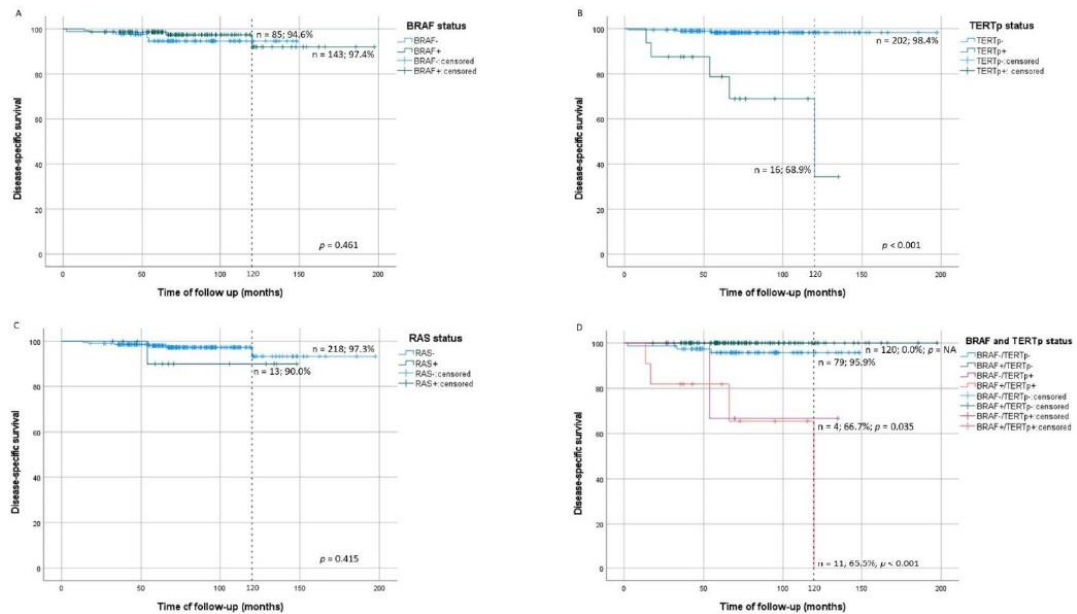


Figure 3. Kaplan–Meier survival curves of PTC disease-specific survival (DSS) by tumor molecular status in comparison with wild type tumors; (A) BRAF status, (B) TERTp status, (C) RAS status, (D) combined BRAF and TERTp status in comparison to wild-type tumors; wt: wild-type; mut: mutated.

4. Discussion

In the present study, we aimed to investigate the role played by point mutations of BRAF, TERTp, and RAS (NRAS, HRAS, and KRAS) in the outcome of PTC patients (recurrent/persistent disease, structural disease status, and DSM).

The most frequent alteration in our PTC series was, as expected, mutation of BRAF gene. BRAF^{mut} was also frequent in LNM at diagnosis and often detected in locoregional recurrences. All three RAS genes were found mutated in primary tumors, being overall the second most common alteration in PTC. The occurrence of TERTp^{mut}, although less common than alterations of the other genes, was higher in recurrent and metastatic lesions, namely distant metastases, than in primary tumors, as hitherto shown by our group [10].

The role of BRAF^{mut} in the outcome of patients is still an open question. In our study, patients whose tumors had BRAF^{mut}, regardless of the status of the other mutations, did not present a higher risk for any of the outcomes evaluated (recurrent/persistent disease, structural disease status, or DSM). In 2015, George et al. [42] reported a 92% frequency of BRAF^{mut} in a series of recurrent/persistent tumors. The increased incidence of BRAF^{mut} and the lack of a control group in this study did not allow to establish any association with recurrent/persistent disease [42]. In 2019, de Castro et al. [43] also did not find an association between BRAF^{mut} and recurrent/persistent disease, although their series contained 48% BRAF^{mut} tumors. This lack of association was corroborated by other studies [44–46]. Some studies have shown that older age and male gender are strong and independent risk factors for PTC—specific mortality in patients with BRAF^{mut} tumors, but not in patients with BRAF^{wt} tumors [27,47]. We did not find similar results in our series. The strength of BRAF^{mut} on patient outcomes may be overestimated in the aforementioned studies [27,47] since in them, no other molecular alterations, namely TERTp^{mut}, were tested. As discussed below, our results indicate that the presence of TERTp^{mut} in PTC, and not of BRAF^{mut}, indeed plays a major role in the outcome.

Several studies have demonstrated BRAF^{mut} association with PTC local invasiveness, recurrent/persistent disease, and LNM [30,48–51]; detection of BRAF^{mut} has led to advancing the idea that it may predict recurrence in low-risk CPTC patients [52]. Other studies suggested that BRAF^{mut} was associated with poorer outcomes, RAI treatment resistance, and DSM [26,29]. In contrast with this, it was suggested that isolated BRAF status has a limited role in guiding patient management [11], and other studies did not validate BRAF^{mut} impact on the outcome of patients [28,44,53,54]. In our analysis, isolated evaluation of BRAF^{mut}, regardless of the status of the other mutations, converged with the limited role of this mutation in ascertain recurrent/persistent disease, structural disease, or DSM.

We detected mutations in all RAS genes. The most commonly described p.Gln61Arg mutation [55] was present in 13 primary tumors. It was shown that RAS^{mut} is present in all stages of thyroid neoplasia [55] ruling out the utilization of RAS^{mut} as a marker of malignancy. In a few studies, RAS^{mut} has been correlated with DM and poorer outcomes [34,56]. In our series, RAS^{mut} was not associated with a poorer prognosis; patients with RAS^{mut} had a stage I diagnosis, usually presenting excellent responses to therapy and were free of disease at the end of follow-up, except for one patient who had concomitant TERTp^{mut} and died from PTC. We can hypothesize that, in this patient, TERTp^{mut} was determinant in the outcome rather than the presence of KRAS^{mut}. Of note, no RAS^{mut} was detected in locoregional metastases nor DM (Supplementary Table S2).

TERTp^{mut} has been consistently associated with poorer outcomes in PTC patients [57]. Some authors showed that recurrent/persistent disease was four times more frequent in patients with TERTp^{mut} than in patients with TERTp^{wt} tumors [58,59]. Whereas in another study, no significant increased risk of recurrent/persistent disease in patients with TERTp^{mut} tumors was reported [60]. The former finding fits with our own results; in the unadjusted analysis, we observed that patients with TERTp^{mut} tumors had twice the risk of recurrent/persistent disease; upon age and gender adjustment, there is still a suggested association ($p = 0.062$; Table 3).

In our study, TERTp^{mut} tumors were associated with significantly lower SDFS and significantly increased DSM. Furthermore, patients with TERTp^{mut} tumors presented a significantly increased risk of structural disease and of DSM, after adjustment for age and gender, suggesting that TERTp^{mut} may contribute to worse prognosis in PTC patients, as previously shown by our group [21]. Several studies have consistently associated TERTp^{mut} with DSM [21,22,42,46,57–59,61,62].

Given previous studies showing a frequent concomitant BRAF^{mut} and TERTp^{mut} in thyroid cancer [13,37,46,63–65], we tested the possible relationship of molecular status combinations and different outcomes. This analysis also aimed to evaluate a more trustworthy effect of each mutation separately.

Comparing with BRAF^{wt}/TERTp^{wt} tumors, we observed that BRAF^{mut}/TERTp^{wt} tumors were associated with an increased risk of recurrent/persistent disease. BRAF^{wt}/TERTp^{mut} tumors were associated with an increased risk of recurrent/persistent disease, structural disease, and DSM. We are aware that the latter group (DSM) had few cases, and therefore these results should be interpreted with caution. Nevertheless, taking into account that TERTp^{mut} is far less common than BRAF^{mut} in PTC (less than 10% vs. 45%) [4,25] and that TERTp^{mut} is consistently associated with more aggressive forms of PTC and with increased risk of disease [22,53], we think that our results support the determinant role TERTp^{mut} in the outcome of PTC patients.

In our series, 11/16 TERTp^{mut} cases presented concomitant BRAF^{mut}. We observed that BRAF^{mut}/TERTp^{mut} significantly increased the risk of recurrent/persistent disease, structural disease, and DSM in comparison to BRAF^{wt}/TERTp^{wt} tumors. In accordance with this, Kim et al. [61] showed that the presence of TERTp^{mut} was associated with increased mortality in PTC patients and further demonstrated that BRAF^{mut}/TERTp^{mut} tumors were associated with worsened DSM in PTC patients in comparison to patients with isolated BRAF^{mut} tumors.

It was suggested that BRAF^{mut}/TERTp^{mut} tumors presented a higher risk of worse outcomes in comparison with BRAF^{wt}/TERTp^{mut} tumors [24,66]. In our study, we observed that DSM was similar in cases with BRAF^{wt}/TERTp^{mut} tumors and BRAF^{mut}/TERTp^{mut} tumors. Our results differ from those reported by Vuong et al. [67]; in the latter metanalysis, tumor aggressiveness varies according to isolated BRAF^{mut}, isolated TERTp^{mut}, or concomitant BRAF^{mut}/TERTp^{mut}. Vuong et al. [67] stratified PTC tumors' aggressiveness in four groups with decreasing aggressiveness: BRAF^{mut}/TERTp^{mut} > BRAF^{wt}/TERTp^{mut} = BRAF^{mut}/TERTp^{wt} > BRAF^{wt}/TERTp^{wt}. Xing et al. [24] proposed a synergistic role of concomitant BRAF^{mut}/TERTp^{mut} on patients' prognosis; in their series, BRAF^{mut}/TERTp^{mut} tumors were associated with increased disease recurrence, higher than the sum of the two mutations alone, even when performing multivariable adjustments for the classical clinicopathologic risk factors. Xing et al. [24] hypothesized that a possible synergistic effect between the two mutations might occur, advancing that coexistence of the two mutations might be associated with increased expression of the TERT mRNA in PTC [24,64–66,68].

We observed that BRAF^{wt}/TERTp^{mut} tumors had an increased risk of recurrent/persistent disease and structural disease in comparison with BRAF^{mut}/TERTp^{mut} tumors. These results may indicate that TERTp^{mut}, rather than BRAF^{mut}, is the molecular alteration that confers higher aggressiveness to PTC. These results fit with our previous studies, in which we showed that PTC patients with TERTp^{mut} tumors had a significantly lower survival [10,21] at variance with the results obtained regarding BRAF^{mut}. Similar findings were reported by Vuong et al. [53], who concluded that TERTp^{mut} was associated with unfavorable DSS and DFS, whereas BRAF^{mut} revealed an association with increased risk of recurrence but not with mortality, thus concluding that BRAF^{mut} usefulness to evaluate patient prognosis must be cautiously considered [53]. Gandolfi et al. [37] reported decreased survival in tumors TERTp^{mut} and BRAF^{mut}/TERTp^{mut}, but no association between BRAF^{mut} and DM or decreased survival was detected. BRAF^{mut} appears associated with local aggressiveness, while TERTp^{mut} was associated with distant metastasis that confers a dismal prognosis [10]. In accordance with our series, no significant differences in recurrent/persistent disease, structural disease, or DSM were observed when comparing patients with BRAF^{mut}/TERTp^{mut} tumors and patients whose tumors were BRAF^{wt}/TERTp^{mut} (Supplementary Figures S1–S3). The low number of tumors with BRAF^{wt}/TERTp^{mut} genotype in our series indicates that a larger series is necessary to confirm our results.

5. Conclusions

We realize that our series has advantages and disadvantages; the positive aspect resides in the fact that it is a consecutive real-life series obtained from a single hospital and not a selected series. On the other hand, we are aware of the consequent limitations: few patients with DM and few patients with PTC-related death. As an example, having only nine patients dying from PTC disease makes the multivariate analysis for such output rather limited. These results should be evaluated with caution also due to the low number of patients with some genotypes.

Summing up, our results indicate that molecular markers can play a role in predicting the outcome of PTC patients. We have data supporting the importance of searching BRAF^{mut}/TERTp^{wt} in terms of putative association with local aggressiveness (recurrent/persistent disease). Furthermore, we have observed a much more important result regarding the prognosis of patients with tumors presenting TERTp point mutations, given their increased risk to develop structural disease and DSM.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/cancers13092048/s1>, Figure S1: Kaplan–Meier curves of recurrent/persistent disease-free survival by concomitant BRAF and TERT promoter mutations in comparison to tumors mutated only for BRAF (E) and only for TERTp (F). Figure S2: Kaplan–Meier curves of structural disease-free survival by concomitant BRAF and TERT promoter mutations in comparison to tumors mutated only for BRAF (E) and only for TERTp (F). Figure S3: Kaplan–Meier curves of disease-specific survival by concomitant BRAF and TERT promoter mutations in comparison to tumors mutated only

for BRAF (E) and only for TERTp (F). Table S1: Univariate analysis of clinicopathological features and combined gene mutations. Table S2: BRAF, TERTp, and RAS molecular status in lymph node metastases at diagnosis, locoregional recurrences, and distant metastases and distribution in the different PTC variants.

Author Contributions: Conceptualization, A.A.P. and P.S.; methodology, A.A.P., E.T., and M.R.B.-C.; software, A.A.P. and E.T.; validation, A.A.P., E.T., and P.S.; formal analysis, A.A.P., M.R.B.-C., and E.T.; investigation, A.A.P., E.T., R.B., A.P., T.A., and M.P.; resources, P.S.; data curation, A.A.P. and E.T.; writing—original draft preparation, A.A.P. and E.T.; writing—review and editing, M.M., M.S.-S., J.M., and P.S.; supervision, P.S. and J.M.; project administration, 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: The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of CHVNG/E (Project investigation 30/2016, 28 January 2016, Comissão de Ética do Centro Hospitalar de Vila Nova de Gaia/Espinho).

Informed Consent Statement: It was an anonymized retrospective study that was exempted from the informed consent according to DR n.º 18/2005, Série I-A de 2005-01-26.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Raposo, L.; Morais, S.; Oliveira, M.J.; Marques, A.P.; Bento, M.J.; Lunet, N. Trends in thyroid cancer incidence and mortality in Portugal. *Eur. J. Cancer Prev.* **2017**, *26*, 135–143. [\[CrossRef\]](#)
2. Olson, E.; Wintheiser, G.; Wolfe, K.M.; Droessler, J.; Silberstein, P.T. Epidemiology of Thyroid Cancer: A Review of the National Cancer Database, 2000–2013. *Cureus* **2019**, *11*, e4127. [\[CrossRef\]](#)
3. Maso, L.D.; Tavilla, A.; Pacini, F.; Serraino, D.; Van Dijk, B.; Chirlaque, M.; Capocaccia, R.; Larrañaga, N.; Colonna, M.; Agius, D.; et al. Survival of 86,690 patients with thyroid cancer: A population-based study in 29 European countries from EURO-CARE-5. *Eur. J. Cancer* **2017**, *77*, 140–152. [\[CrossRef\]](#)
4. Agrawal, N.; Akbani, R.; Aksoy, B.A.; Ally, A.; Arachchi, H.; Asa, S.L.; Auman, J.T.; Balasundaram, M.; Balu, S.; Baylin, S.B.; et al. Integrated Genomic Characterization of Papillary Thyroid Carcinoma. *Cell* **2014**, *159*, 676–690. [\[CrossRef\]](#)
5. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca Cancer J. Clin.* **2018**, *68*, 394–424. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Global Cancer Observatory. Available online: <https://gco.iarc.fr> (accessed on 30 December 2020).
7. Enewold, L.; Zhu, K.; Ron, E.; Marrogi, A.J.; Stojadinovic, A.; Peoples, G.E.; Devesa, S.S. Rising Thyroid Cancer Incidence in the United States by Demographic and Tumor Characteristics, 1980–2005. *Cancer Epidemiol. Biomark. Prev.* **2009**, *18*, 784–791. [\[CrossRef\]](#)
8. Kitahara, C.M.; Sosa, J.A. The changing incidence of thyroid cancer. *Nat. Rev. Endocrinol.* **2016**, *12*, 646–653. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Ito, Y.; Miyauchi, A.; Kihara, M.; Fukushima, M.; Higashiyama, T.; Miya, A. Overall Survival of Papillary Thyroid Carcinoma Patients: A Single-Institution Long-Term Follow-Up of 5897 Patients. *World J. Surg.* **2018**, *42*, 615–622. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Melo, M.; Da Rocha, A.G.; Batista, R.; Vinagre, J.; Martins, M.J.; Costa, G.; Ribeiro, C.; Carrilho, F.; Leite, V.; Lobo, C.; et al. TERT, BRAF, and NRAS in Primary Thyroid Cancer and Metastatic Disease. *J. Clin. Endocrinol. Metab.* **2017**, *102*, 1898–1907. [\[CrossRef\]](#) [\[PubMed\]](#)

11. Haugen, B.R.; Alexander, E.K.; Bible, K.C.; Doherty, G.M.; Mandel, S.J.; Nikiforov, Y.E.; Pacini, F.; Randolph, G.W.; Sawka, A.M.; Schlumberger, M.; et al. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* **2016**, *26*, 1–133. [\[CrossRef\]](#)
12. Sugitani, I.; Fujimoto, Y.; Yamamoto, N. Papillary thyroid carcinoma with distant metastases: Survival predictors and the importance of local control. *Surgery* **2008**, *143*, 35–42. [\[CrossRef\]](#)
13. Giorgenon, T.M.V.; Carrijo, F.T.; Arruda, M.A.; Cerqueira, T.L.O.; Barreto, H.R.; Cabral, J.B.; Da Silva, T.M.; Magalhães, P.K.R.; Maciel, L.M.Z.; Ramos, H.E. Preoperative detection of TERT promoter and BRAFV600E mutations in papillary thyroid carcinoma in high-risk thyroid nodules. *Arch. Endocrinol. Metab.* **2019**, *63*, 107–112. [\[CrossRef\]](#)
14. Nikiforov, Y.E.; Steward, D.L.; Robinson-Smith, T.M.; Haugen, B.R.; Kloppe, J.P.; Zhu, Z.; Fagin, J.A.; Falciglia, M.; Weber, K.; Nikiforova, M.N. Molecular Testing for Mutations in Improving the Fine-Needle Aspiration Diagnosis of Thyroid Nodules. *J. Clin. Endocrinol. Metab.* **2009**, *94*, 2092–2098. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Baloch, Z.W.; Sack, M.J.; Yu, G.H.; Livolsi, V.A.; Gupta, P.K. Fine-Needle Aspiration of Thyroid: An Institutional Experience. *Thyroid* **1998**, *8*, 565–569. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Sipos, J.; Mazzaferri, E. Thyroid Cancer Epidemiology and Prognostic Variables. *Clin. Oncol.* **2010**, *22*, 395–404. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Castro, L.; Alves, S.; Chaves, S.R.; Costa, J.L.; Soares, P.; Preto, A. RAF-1 promotes survival of thyroid cancer cells harboring RET/PTC1 rearrangement independently of ERK activation. *Mol. Cell. Endocrinol.* **2015**, *415*, 64–75. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Sobrinho-Simões, M.; Máximo, V.; Rocha, A.S.; Trovisco, V.; Castro, P.; Preto, A.; Lima, J.; Soares, P. Intragenic Mutations in Thyroid Cancer. *Endocrinol. Metab. Clin. N. Am.* **2008**, *37*, 333–362. [\[CrossRef\]](#)
19. Nikiforov, Y.E. Thyroid carcinoma: Molecular pathways and therapeutic targets. *Mod. Pathol.* **2008**, *21*, S37–S43. [\[CrossRef\]](#)
20. Panebianco, F.; Mazzanti, C.; Tomei, S.; Aretini, P.; Franceschi, S.; Lessi, F.; Di Coscio, G.; Bevilacqua, G.; Marchetti, I. The combination of four molecular markers improves thyroid cancer cytologic diagnosis and patient management. *Bmc Cancer* **2015**, *15*, 918. [\[CrossRef\]](#)
21. Melo, M.; Da Rocha, A.G.; Vinagre, J.; Batista, R.; Peixoto, J.; Tavares, C.; Celestino, R.; Almeida, A.; Salgado, C.; Eloy, C.; et al. TERT Promoter Mutations Are a Major Indicator of Poor Outcome in Differentiated Thyroid Carcinomas. *J. Clin. Endocrinol. Metab.* **2014**, *99*, E754–E765. [\[CrossRef\]](#)
22. Penna, G.C.; Pestana, A.; Cameselle, J.M.; Momesso, D.; De Andrade, F.A.; Vidal, A.P.A.; Junior, M.L.A.; Melo, M.; Fernandes, P.V.; Corbo, R.; et al. TERTp mutation is associated with a shorter progression free survival in patients with aggressive histology subtypes of follicular-cell derived thyroid carcinoma. *Endocrine* **2018**, *61*, 489–498. [\[CrossRef\]](#)
23. Censi, S.; Cavedon, E.; Bertazza, L.; Galuppini, F.; Watutantrige-Fernando, S.; De Lazzari, P.; Nacamulli, D.; Pennelli, G.; Fassina, A.; Iacobone, M.; et al. Frequency and Significance of Ras, Tert Promoter, and Braf Mutations in Cytologically Indeterminate Thyroid Nodules: A Monocentric Case Series at a Tertiary-Level Endocrinology Unit. *Front. Endocrinol.* **2017**, *8*, 273. [\[CrossRef\]](#)
24. Xing, M.; Liu, R.; Liu, X.; Murugan, A.K.; Zhu, G.; Zeiger, M.A.; Pai, S.; Bishop, J. BRAF V600E and TERT Promoter Mutations Cooperatively Identify the Most Aggressive Papillary Thyroid Cancer With Highest Recurrence. *J. Clin. Oncol.* **2014**, *32*, 2718–2726. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Cantwell-Dorris, E.R.; O’Leary, J.J.; Sheils, O.M. BRAFV600E: Implications for Carcinogenesis and Molecular Therapy. *Mol. Cancer Ther.* **2011**, *10*, 385–394. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Xing, M.; Westra, W.H.; Tufano, R.P.; Cohen, Y.; Rosenbaum, E.; Rhoden, K.J.; Carson, K.A.; Vasko, V.; Larin, A.; Tallini, G.; et al. BRAF Mutation Predicts a Poorer Clinical Prognosis for Papillary Thyroid Cancer. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 6373–6379. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Shen, X.; Zhu, G.; Liu, R.; Viola, D.; Elisei, R.; Puxeddu, E.; Fugazzola, L.; Colombo, C.; Jarzab, B.; Czarniecka, A.; et al. Patient Age-Associated Mortality Risk Is Differentiated by BRAF V600E Status in Papillary Thyroid Cancer. *J. Clin. Oncol.* **2018**, *36*, 438–445. [\[CrossRef\]](#)
28. Trovisco, V.; Soares, P.; Preto, A.; De Castro, I.V.; Lima, J.; Castro, P.; Máximo, V.; Botelho, T.; Moreira, S.; Meireles, A.M.; et al. Type and prevalence of BRAF mutations are closely associated with papillary thyroid carcinoma histotype and patients’ age but not with tumour aggressiveness. *Virchows Arch.* **2005**, *446*, 589–595. [\[CrossRef\]](#)
29. Xing, M.; Alzahrani, A.S.; Carson, K.A.; Viola, D.; Elisei, R.; Bendlova, B.; Yip, L.; Mian, C.; Vianello, F.; Tuttle, R.M.; et al. Association between BRAF V600E Mutation and Mortality in Patients With Papillary Thyroid Cancer. *JAMA* **2013**, *309*, 1493–1501. [\[CrossRef\]](#)
30. Xing, M.; Alzahrani, A.S.; Carson, K.A.; Shong, Y.K.; Kim, T.Y.; Viola, D.; Elisei, R.; Bendlová, B.; Yip, L.; Mian, C. Association between BRAF V600E mutation and recurrence of papillary thyroid cancer. *J. Clin. Oncol.* **2015**, *33*, 42. [\[CrossRef\]](#)
31. Lemoine, N.R.M.E.; Wyllie, F.S.; Williams, E.D.; Goyns, M.; Stringer, B.; Wynford-Thomas, D. High frequency of ras oncogene activation in all stages of human thyroid tumorigenesis. *Oncogene* **1989**, *4*, 149–164.
32. Schulten, H.-J.; Salama, S.; Al-Ahmadi, A.; Al-Mansouri, Z.; Mirza, Z.; Al-Ghamdi, K.; Al-Hamour, O.A.; Huwait, E.; Gari, M.; Al-Qahtani, M.H.; et al. Comprehensive survey of HRAS, KRAS, and NRAS mutations in proliferative thyroid lesions from an ethnically diverse population. *Anticancer. Res.* **2013**, *33*, 4779–4784. [\[PubMed\]](#)
33. Medici, M.; Kwong, N.; Angell, T.E.; Marqusee, E.; Kim, M.I.; Frates, M.C.; Benson, C.B.; Cibas, E.S.; Barletta, J.A.; Krane, J.F.; et al. The variable phenotype and low-risk nature of RAS-positive thyroid nodules. *BMC Med.* **2015**, *13*, 184. [\[CrossRef\]](#) [\[PubMed\]](#)

34. Fukahori, M.; Yoshida, A.; Hayashi, H.; Yoshihara, M.; Matsukuma, S.; Sakuma, Y.; Koizume, S.; Okamoto, N.; Kondo, T.; Masuda, M.; et al. The association between RAS gene mutations and clinical characteristics in follicular thyroid tumors: New insights from a single center and a large patient cohort. *Thyroid* **2012**, *22*, 683–689. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Jang, E.K.; Song, D.E.; Sim, S.Y.; Kwon, H.; Choi, Y.M.; Jeon, M.J.; Han, J.M.; Kim, W.G.; Kim, T.Y.; Shong, Y.K.; et al. NRAS Codon 61 Mutation Is Associated with Distant Metastasis in Patients with Follicular Thyroid Carcinoma. *Thyroid* **2014**, *24*, 1275–1281. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Jin, A.; Xu, J.; Wang, Y. The role of TERT promoter mutations in postoperative and preoperative diagnosis and prognosis in thyroid cancer. *Medicine* **2018**, *97*, e11548. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Gandolfi, G.; Ragazzi, M.; Frasoldati, A.; Piana, S.; Ciarrocchi, A.; Sancisi, V. TERT promoter mutations are associated with distant metastases in papillary thyroid carcinoma. *Eur. J. Endocrinol.* **2015**, *172*, 403–413. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Liu, R.; Xing, M. TERT promoter mutations in thyroid cancer. *Endocr. Relat. Cancer* **2016**, *23*, R143–R155. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Lloyd, R.V.; Osamura, R.Y.; Klöppel, G.; Rosai, J. *WHO Classification of Tumours of Endocrine Organs*, 4th ed.; International Agency for Research on Cancer (IARC): Lyon, France, 2017.
40. Tuttle, R.M.; Haugen, B.; Perrier, N.D. Updated American Joint Committee on Cancer/Tumor-Node-Metastasis Staging System for Differentiated and Anaplastic Thyroid Cancer (Eighth Edition): What Changed and Why? *Thyroid* **2017**, *27*, 751–756. [\[CrossRef\]](#)
41. Momesso, D.P.; Vaisman, F.; Yang, S.P.; Bulzico, D.A.; Corbo, R.; Vaisman, M.; Tuttle, R.M. Dynamic Risk Stratification in Patients with Differentiated Thyroid Cancer Treated Without Radioactive Iodine. *J. Clin. Endocrinol. Metab.* **2016**, *101*, 2692–2700. [\[CrossRef\]](#) [\[PubMed\]](#)
42. George, J.R.; Henderson, Y.C.; Williams, M.D.; Roberts, D.B.; Hei, H.; Lai, S.Y.; Clayman, G.L. Association of TERT Promoter Mutation, But Not BRAF Mutation, with Increased Mortality in PTC. *J. Clin. Endocrinol. Metab.* **2015**, *100*, E1550–E1559. [\[CrossRef\]](#)
43. De Castro, T.P.; Penha, R.C.C.; Buexm, L.A.; De Carvalho, F.N.; Oliveira, R.D.V.C.; Agarez, F.V.; Pinto, L.W.; Carvalho, D.P. Molecular Predictors for Advanced Papillary Thyroid Carcinoma Recurrence. *Front. Endocrinol.* **2019**, *10*, 839. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Yan, C.; Huang, M.L.; Li, X.; Wang, T.; Ling, R. Relationship between BRAFV600E and clinical features in papillary thyroid carcinoma. *Endocr. Connect.* **2019**, *8*, 988–996. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Garre, M.C.; Casares, M.L.D.L.T.; Massare, P.B.; Nevot, M.; Ángel, L.; Del-Moral, J.V.; Pérez, N.M.; Joya, R.V.; Ramírez, R.M.; Elvira, J.M.L. Presencia de la mutación BRAFT1799A en el tumor primario como indicador de riesgo, recidiva o persistencia de carcinoma papilar de tiroides. *Endocrinol. Y Nutr.* **2011**, *58*, 175–184. [\[CrossRef\]](#)
46. Shen, X.; Liu, R.; Xing, M. A six-genotype genetic prognostic model for papillary thyroid cancer. *Endocr. Relat. Cancer* **2017**, *24*, 41–52. [\[CrossRef\]](#)
47. Wang, F.; Zhao, S.; Shen, X.; Zhu, G.; Liu, R.; Viola, D.; Elisei, R.; Puxeddu, E.; Fugazzola, L.; Colombo, C.; et al. BRAF V600E Confers Male Sex Disease-Specific Mortality Risk in Patients With Papillary Thyroid Cancer. *J. Clin. Oncol.* **2018**, *36*, 2787–2795. [\[CrossRef\]](#)
48. Howell, G.M.; Nikiforova, M.N.; Carty, S.E.; Armstrong, M.J.; Hodak, S.P.; Stang, M.T.; McCoy, K.L.; Nikiforov, Y.E.; Yip, L. BRAF V600E Mutation Independently Predicts Central Compartment Lymph Node Metastasis in Patients with Papillary Thyroid Cancer. *Ann. Surg. Oncol.* **2012**, *20*, 47–52. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Kim, S.-J.; Lee, K.E.; Myong, J.P.; Park, J.-H.; Jeon, Y.K.; Min, H.S.; Park, S.Y.; Jung, K.C.; Koo, D.H.; Youn, Y.-K. BRAFV600E Mutation is Associated with Tumor Aggressiveness in Papillary Thyroid Cancer. *World J. Surg.* **2012**, *36*, 310–317. [\[CrossRef\]](#)
50. O'Neill, C.J.; Bullock, M.; Chou, A.; Sidhu, S.B.; Delbridge, L.W.; Robinson, B.G.; Gill, A.J.; Learoyd, D.L.; Clifton-Bligh, R.; Sywak, M.S. BRAFV600E mutation is associated with an increased risk of nodal recurrence requiring reoperative surgery in patients with papillary thyroid cancer. *Surgery* **2010**, *148*, 1139–1146. [\[CrossRef\]](#)
51. Tufano, R.P.; Bishop, J.; Wu, G. Reoperative central compartment dissection for patients with recurrent/persistent papillary thyroid cancer: Efficacy, safety, and the association of the BRAF mutation. *Laryngoscope* **2012**, *122*, 1634–1640. [\[CrossRef\]](#)
52. Kim, T.Y.; Kim, W.B.; Rhee, Y.S.; Song, J.Y.; Kim, J.M.; Gong, G.; Lee, S.; Kim, S.Y.; Kim, S.C.; Hong, S.J.; et al. The BRAF mutation is useful for prediction of clinical recurrence in low-risk patients with conventional papillary thyroid carcinoma. *Clin. Endocrinol.* **2006**, *65*, 364–368. [\[CrossRef\]](#)
53. Vuong, H.G.; Duong, U.N.; Altibi, A.M.; Ngo, H.T.; Pham, T.Q.; Tran, H.M.; Gandolfi, G.; Hassell, L. A meta-analysis of prognostic roles of molecular markers in papillary thyroid carcinoma. *Endocr. Connect.* **2017**, *6*, R8–R17. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Zheng, X.; Wei, S.; Han, Y.; Li, Y.; Yu, Y.; Yun, X.; Ren, X.; Gao, M. Papillary Microcarcinoma of the Thyroid: Clinical Characteristics and BRAFV600E Mutational Status of 977 Cases. *Ann. Surg. Oncol.* **2013**, *20*, 2266–2273. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Howell, G.M.; Hodak, S.P.; Yip, L. RAS Mutations in Thyroid Cancer. *Oncology* **2013**, *18*, 926–932. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Garcia-Rostan, G.; Zhao, H.; Camp, R.L.; Pollan, M.; Herrero, A.; Pardo, J.; Wu, R.; Carcangiu, M.L.; Costa, J.; Tallini, G. ras Mutations Are Associated With Aggressive Tumor Phenotypes and Poor Prognosis in Thyroid Cancer. *J. Clin. Oncol.* **2003**, *21*, 3226–3235. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Bullock, M.; Ren, Y.; O'Neill, C.J.; Gill, A.J.; Aniss, A.; Sywak, M.S.; Sidhu, S.B.; Delbridge, L.; Learoyd, D.L.; De Vathaire, F.; et al. TERT promoter mutations are a major indicator of recurrence and death due to papillary thyroid carcinomas. *Clin. Endocrinol.* **2016**, *85*, 283–290. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Liu, C.; Liu, Z.; Chen, T.; Zeng, W.; Guo, Y.; Huang, T. TERT promoter Mutation and Its Association with Clinicopathological Features and Prognosis of Papillary Thyroid Cancer: A Meta-analysis. *Sci. Rep.* **2016**, *6*, 36990. [\[CrossRef\]](#)

59. Yin, D.-T.; Mengyuan, L.; Lu, R.-Q.; Li, X.; Xu, J.; Lei, M.; Li, H.; Wang, Y.; Liu, Z. Clinicopathological significance of TERT promoter mutation in papillary thyroid carcinomas: A systematic review and meta-analysis. *Clin. Endocrinol.* **2016**, *85*, 299–305. [\[CrossRef\]](#)
60. Myung, J.K.; Kwak, B.K.; Lim, J.A.; Lee, M.-C.; Kim, M.J. TERT Promoter Mutations and Tumor Persistence/Recurrence in Papillary Thyroid Cancer. *Cancer Res. Treat.* **2016**, *48*, 942–947. [\[CrossRef\]](#)
61. Kim, T.H.; Kim, Y.-E.; Ahn, S.; Kim, J.-Y.; Ki, C.-S.; Oh, Y.L.; Kim, K.; Yun, J.W.; Park, W.-Y.; Choe, J.-H.; et al. TERT promoter mutations and long-term survival in patients with thyroid cancer. *Endocr. Relat. Cancer* **2016**, *23*, 813–823. [\[CrossRef\]](#)
62. Yang, J.; Gong, Y.; Yan, S.; Chen, H.; Qin, S.; Gong, R. Association between TERT promoter mutations and clinical behaviors in differentiated thyroid carcinoma: A systematic review and meta-analysis. *Endocrine* **2019**, *67*, 44–57. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Rusinek, D.; Pfeifer, A.; Krajewska, J.; Oczko-Wojciechowska, M.; Handkiewicz-Junak, D.; Pawlaczek, A.; Zebracka-Gala, J.; Kowalska, M.; Cyplinska, R.; Zembala-Nozynska, E.; et al. Coexistence of TERT Promoter Mutations and the BRAF V600E Alteration and Its Impact on Histopathological Features of Papillary Thyroid Carcinoma in a Selected Series of Polish Patients. *Int. J. Mol. Sci.* **2018**, *19*, 2647. [\[CrossRef\]](#)
64. Jin, L.; Chen, E.; Dong, S.; Cai, Y.; Zhang, X.; Zhou, Y.; Zeng, R.; Yang, F.; Pan, C.; Liu, Y.; et al. BRAF and TERT promoter mutations in the aggressiveness of papillary thyroid carcinoma: A study of 653 patients. *Oncotarget* **2016**, *7*, 18346–18355. [\[CrossRef\]](#)
65. Vinagre, J.; Almeida, A.; Pópulo, H.; Batista, R.; Lyra, J.; Pinto, V.; Coelho, R.; Celestino, R.; Prazeres, H.; Lima, L.; et al. Frequency of TERT promoter mutations in human cancers. *Nat. Commun.* **2013**, *4*, 2185. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Trybek, T.; Walczyk, A.; Gąsior-Perczak, D.; Pałyga, I.; Mikina, E.; Kowalik, A.; Hińcza, K.; Kopczyński, J.; Chrapek, M.; Góźdz, S.; et al. Impact of BRAF V600E and TERT Promoter Mutations on Response to Therapy in Papillary Thyroid Cancer. *Endocrinology* **2019**, *160*, 2328–2338. [\[CrossRef\]](#)
67. Vuong, H.G.; Altibi, A.M.; Duong, U.N.; Hassell, L. Prognostic implication of BRAF and TERT promoter mutation combination in papillary thyroid carcinoma-A meta-analysis. *Clin. Endocrinol.* **2017**, *87*, 411–417. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Liu, R.; Bishop, J.; Zhu, G.; Zhang, T.; Ladenson, P.W.; Xing, M. Mortality Risk Stratification by Combining BRAF V600E and TERT Promoter Mutations in Papillary Thyroid Cancer. *Jama Oncol.* **2017**, *3*, 202–208. [\[CrossRef\]](#) [\[PubMed\]](#)

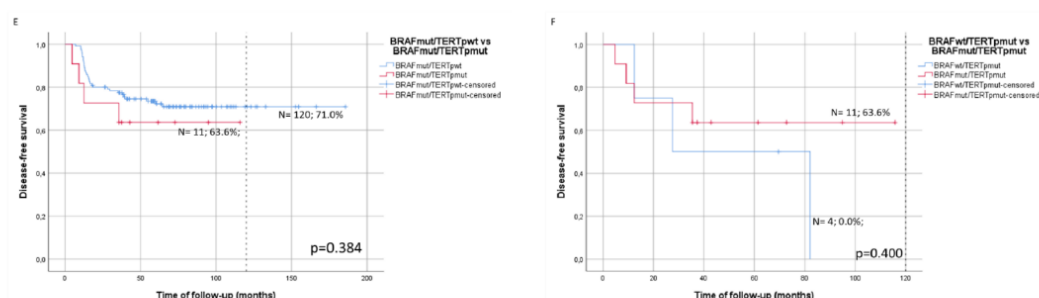
Supplementary Table S1: Univariate analysis of clinicopathological features and combined gene mutations

Clinicopathological features		A	B	C	D	p-values			
		BRAFwt/TERTpwt n = 79 n (%)	BRAFmut/TERTpwt n = 120 n (%)	BRAFwt/TERTpmt n= 4 n (%)	BRAFmut/TERTpmt n= 11 n (%)	A... vs .B...	A... vs ...C...	A... vs ...D...	.B... vs ...D
Patient characteristics									
Age	Mean ± Std	51.1 ± 14.3	50.2 ± 15.2	55.8 ± 16.3	67.7 ± 14.6	0.691	0.610	0.004	0.003
Gender	Female	68 (86.1%)	102 (85%)	3 (75%)	7 (63.6%)	0.833	0.539	0.082	0.089
	Male	11 (13.9%)	18 (15%)	1 (25%)	4 (36.4%)				
Histological characteristics									
Nodule size	Median ± IR	12.0 ± 9.0	11.0 ± 9.0	26.5 ± 17.0	20.0 ± 16.0	0.158	0.041	0.267	0.057
Solid component	Absent	66 (83.5%)	117 (97.5%)	3 (75%)	9 (81.8%)	<0.001	0.530	1.000	0.056
	Present	13 (16.5%)	3 (2.5%)	1 (25%)	2 (18.2%)				
Inflammatory infiltrate	Absent	18 (22.8%)	6 (5%)	1 (25%)	0 (0%)	<0.001	1.000	0.112	1.000
	Present	61 (77.2%)	114 (95%)	3 (75%)	11 (100%)				
Fibrosis	Absent	11 (13.9%)	5 (4.2%)	0 (0%)	0 (0%)	0.013	1.000	0.347	1.000
	Present	68 (86.1%)	115 (95.8%)	4 (100%)	11 (100%)				
Venous invasion	Absent	70 (88.6%)	116 (96.7%)	3 (75%)	9 (81.8%)	0.024	0.408	0.619	0.080
	Present	9 (11.4%)	4 (3.3%)	1 (25%)	2 (18.2%)				
Lymph vessel invasion	Absent	69 (87.3%)	94 (78.3%)	2 (50%)	6 (54.5%)	0.106	0.098	0.017	0.130
	Present	10 (12.7%)	26 (21.7%)	2 (50%)	5 (45.5%)				
Nodule growth pattern	Expansive	59 (74.7%)	54 (45%)	4 (100%)	2 (18.2%)	<0.001	0.568	<0.001	0.115
	Infiltrative	20 (25.3%)	66 (55%)	0 (0%)	9 (81.8%)				
Minimal extrathyroidal extension	Absent	61 (78.2%)	52 (45.6%)	2 (50%)	3 (37.5%)	<0.001	0.228	0.024	0.729
	Minimal	17 (21.8%)	62 (54.4%)	2 (50%)	5 (62.5%)				
Gross extrathyroidal extension	Absent/ minimal	78 (98.7%)	114 (95%)	4 (100%)	8 (72.7%)	0.162	1.000	0.005	0.028
	Gross	1 (1.3%)	6 (5%)	0 (0%)	3 (27.3%)				
Resection margins	R0	72 (91.1%)	104 (86.7%)	3 (75%)	8 (72.7%)	0.334	0.339	0.102	0.200
	R1/R2	7 (8.9%)	16 (13.3%)	1 (25%)	3 (27.3%)				
Central lymph node metastasis	Absent	78 (98.7%)	101 (84.2%)	4 (100%)	8 (72.7%)	0.001	1.000	0.005	0.394
	Present	1 (1.3%)	19 (15.8%)	0 (0%)	3 (27.3%)				
Extranodal extension of central lymph node metastasis	Absent	79 (100%)	116 (96.7%)	4 (100%)	8 (72.7%)	0.153	-	0.001	0.013
	Present	0 (0%)	4 (3.3%)	0 (0%)	3 (27.3%)				
Lateral lymph node metastasis	Absent	74 (93.7%)	112 (93.3%)	4 (100%)	8 (72.7%)	0.153	1.000	0.055	0.050
	Present	5 (6.3%)	8 (6.7%)	0 (0%)	3 (27.3%)				
Extranodal extension of lateral lymph node metastasis	Absent	77 (97.5%)	120 (100%)	4 (100%)	8 (72.7%)	0.156	1.000	0.012	<0.001
	Present	2 (2.5%)	0 (0%)	0 (0%)	3 (27.3%)				
Staging									
T stage	T1-T2	73 (92.4%)	112 (93.3%)	4 (100%)	8 (72.7%)	0.802	1.000	0.077	0.050
	T3-T4	6 (7.6%)	8 (6.7%)	0 (0%)	3 (27.3%)				
N stage	N0	73 (92.4%)	100 (83.3%)	4 (100%)	8 (72.7%)	0.063	1.000	0.077	0.407
	N1	6 (7.6%)	20 (16.7%)	0 (0%)	3 (27.3%)				
M stage	M0	78 (98.7%)	120 (100%)	3 (75%)	9 (81.8%)	0.397	0.095	0.038	0.006
	M1	1 (1.3%)	0 (0%)	1 (25%)	2 (18.2%)				
Stage	SI	76 (96.2%)	114 (95%)	3 (75%)	6 (54.5%)	0.355	0.182	<0.001	<0.001
	SII	2 (2.5%)	6 (5%)	0 (0%)	3 (27.3%)				
	SIVB	1 (1.3%)	0 (0%)	1 (25%)	2 (18.2%)				
RAI treatment									
RAI number of treatments	1	53 (93%)	79 (85.9%)	2 (50%)	6 (66.7%)	0.184	0.045	0.048	0.151
	>1	4 (7%)	13 (14.1%)	2 (50%)	3 (33.3%)				
Risk stratification									
Risk stratification (RAI treated)	Low	32 (56.1%)	31 (33.7%)	0 (0%)	1 (11.1%)	0.024	0.036	0.002	0.007
	Intermediate	22 (38.6%)	55 (59.8%)	3 (75%)	4 (44.4%)				
	High	3 (5.3%)	6 (6.5%)	1 (25%)	4 (44.4%)				
Risk stratification (non-RAI treated)	Low	19 (86.4%)	18 (64.3%)	0 (0%)	1 (50%)	0.108	-	0.094	0.067
	Intermediate	3 (13.6%)	10 (35.7%)	0 (0%)	0 (0%)				
	High	0 (0%)	0 (0%)	0 (0%)	1 (50%)				
Global dynamic risk stratification (RAI treated)	Excelent	52 (91.2%)	90 (97.8%)	2 (50%)	5 (55.6%)	0.107	0.061	0.016	<0.001
	Incomplete	5 (8.8%)	2 (2.2%)	2 (50%)	4 (44.4%)				
Global dynamic risk stratification (non-RAI treated)	Excelent	21 (95.5%)	27 (96.4%)	0 (0%)	2 (100%)	1.000	-	1.000	1.000
	Incomplete	1 (4.5%)	1 (3.6%)	0 (0%)	0 (0%)				

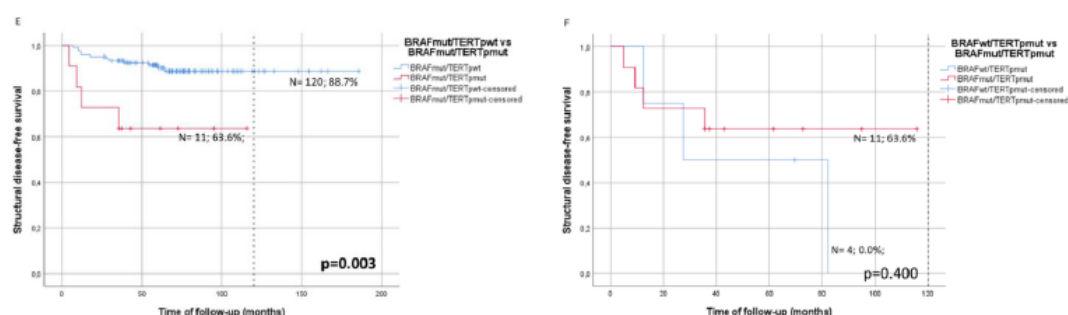
Supplementary Table S2 – BRAF, TERTp and RAS molecular status in lymph node metastases at diagnosis, locoregional recurrences and distant metastases and distribution in the different PTC variants.

Gene(s)	Status	Lymph node metastases	Locoregional recurrences	Distant metastases
		N=31	N=16	N=2 [#]
BRAF	wt	14	4	0
	p.Val600Glu	17 (54.8%)	12 (75.0%)	1 (n.a.)
TERTp	wt	27	10	0
	-124 G>A	2 (6.7%)	3 (20.0%)	1 (n.a.)
	-146 G>A	1 (3.3%)	2 (13.3%)	-
	-124/-125 G>A	0 (0.0%)	0 (0.0%)	-
BRAF/TERTp	BRAF ^{wt} /TERTp ^{wt}	13	2	0
	BRAF ^{mut} /TERTp ^{wt}	14 (46.7%)	8 (53.3%)	-
	BRAF ^{wt} /TERTp ^{mut}	1 (3.3%)	2 (13.3%)	-
	BRAF ^{mut} /TERTp ^{mut}	2 (6.7%)	3 (20.0%)	1 (n.a.)
RAS	wt	31	15	2 [#]
	RAS ^{mut}	0 (0.0%)	0 (0.0%)	(n.a.)

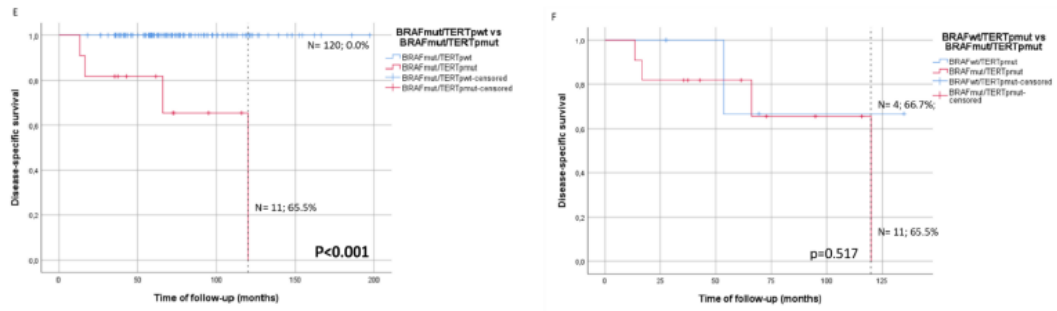
[#]one distant metastasis only amplified for NRAS; wt: wild-type; mut: mutated; n.a.: Not applicable.



Supplementary Figure S1 – Kaplan-Meier curves of recurrent/persistent disease-free survival by concomitant BRAF and TERT promoter mutations in comparison to tumors mutated only for BRAF (E) and only for TERTp (F). wt: wild-type; mut: mutated.



Supplementary Figure S2 – Kaplan-Meier curves of structural disease-free survival by concomitant BRAF and TERT promoter mutations in comparison to tumors mutated only for BRAF (E) and only for TERTp (F). wt: wild-type; mut: mutated.



Supplementary Figure S3– Kaplan-Meier curves of disease-specific survival by concomitant BRAF and TERT promoter mutations in comparison to tumors mutated only for BRAF (E) and only for TERTp (F). wt: wild-type; mut: mutated.

4.3. ARTICLE III

ARTICLE I investigates clinicopathological factors associated with thyroid cancer prognosis. In this study, we perform an evaluation of a consecutive case series of tumors regardless of their size. In **ARTICLE III** we highlight an issue that has been enigmatical to investigators and clinicians, which is how to appropriately handle subcentimetric papillary tumors (≤ 1 cm). Our team posed the question: which are the clinicopathological characteristics of indolent subcentimetric papillary tumors and those that require tight vigilance and aggressive treatment? To answer this, we hereby present a study in which we compare the outcome of subcentimetric tumors that were clinically diagnosed with tumors that were incidental findings (overdiagnosed).

Subcentimetric Papillary Thyroid Carcinoma: Does the Diagnosis Kind Impact Prognosis?

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Abstract

Introduction: Subcentimetric papillary thyroid carcinoma (SPTC) (papillary thyroid carcinoma with less than 10 mm in size) usually presents an excellent prognosis, with few aggressive reported cases. Given the globally increased incidence of SPTC, physicians are struggling with the need to identify prognostic factors to stratify SPTC. The aim was to compare clinicopathological variables and prognosis between clinically and incidentally diagnosed SPTC.

Materials and methods: This is a retrospective observational study on patients with SPTC who underwent thyroidectomy between 2002 and 2015. Two groups were considered: G1 (n=60 (61.9%)), clinical diagnosis (Bethesda III-VI cytology in the thyroid tumor/in cervical lymphadenopathies) and G2 (n=37 (38.1%)), incidental diagnosis (thyroidectomy for benign thyroid pathology). The histological material was reviewed, and molecular analysis of the *BRAF*, *RAS*, and *TERT* promoter (*TERTp*) genes was performed.

Results: Ninety-seven individuals were included, 60 (61.9%) of which were from G1, with a predominance of female sex (n=83 (85.6%)). Individuals of G1 were younger (53.0±14.2 versus 59.3±13.9 years; p=0.035), were more frequently treated with 131-iodine (39.2% versus 13.4%; p=0.007), had the largest diameter (8 (p25-p75: 7-9) versus 5 (p25-p75: 4-6.5) mm; p<0.001), and higher frequency of minimal extracapsular invasion (45% versus 24.3%; p=0.041). Increased tumor size was the only independent predictor of a clinical diagnosis (p<0.001).

Conclusions: Clinically and incidentally diagnosed SPTC showed excellent medium- to long-term prognosis. A larger SPTC was more likely a driver of clinical detection than a marker of tumor aggressiveness, but caution should be taken as contradictory data persists.

Categories: Endocrinology/Diabetes/Metabolism, Pathology, General Surgery

Keywords: disease-free survival, recurrence, prognosis, papillary thyroid microcarcinoma, thyroid neoplasms

Introduction

In 1989, the World Health Organization (WHO) introduced the term "papillary thyroid microcarcinoma" for papillary thyroid carcinomas (PTC) that measure a maximum of 10 mm in size and later redefined it as subcentimetric papillary thyroid carcinoma (SPTC) [1].

The incidence of SPTC has been increasing widely in the last decade, mainly at the expense of its incidental detection, in histopathological samples of patients submitted to thyroidectomy for presumably benign thyroid conditions [2,3]. The widespread use of high-resolution neck ultrasound has also contributed to the rise of clinical diagnosis of SPTC, due to increased detection of small thyroid nodules, greater accuracy in assessing malignant suspicious ultrasound features, and more optimized guidance of fine needle aspiration biopsy (FNAB), which enhances its diagnostic sensitivity [4]. SPTC accounts for >50% of thyroid malignancies [5]. Some authors consider the term "carcinoma" as too disproportionate to SPTC that present usually an excellent prognosis, with rare aggressive reported cases, inducing unnecessary anxiety in patients and families [6,7]. Regarding SPTC prognosis, Choi et al. [8] stated that there were no clinical, biochemical, or pathological factors that easily discriminate indolent from aggressive SPTC, while other authors converge on affirming clinical lymph node metastasis (LNM) at diagnosis as a poor prognostic factor [9-11].

Given the global increase in SPTC incidence, thyroidologists and oncologists are struggling with the need to identify prognostic factors to stratify SPTC. Depending on the type of diagnosis (incidental or clinical), the therapeutic approach of SPTC may range from active surveillance to surgical intervention, which may impact the natural history of SPTC (persistence, recurrence, and disease-related mortality).

The aim of this study was to compare clinicopathological variables and medium- to long-term prognosis

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between clinically and incidentally diagnosed SPTC.

Materials And Methods

Patient cohort and tissue samples

An observational retrospective study was performed by reviewing electronic records for the attainment of clinical and demographic data of 1,652 consecutive individuals submitted to thyroid surgery at Centro Hospitalar de Vila Nova de Gaia/Espinho between 2002 and 2015. Thyroid cancer was diagnosed in 461 (27.9%) individuals. From these, 97 (21%) were confirmed as SPTC on histopathological analysis and followed at endocrinology consultation. Inclusion criteria were the diagnosis of SPTC in patients older than 18 years, followed for a minimum of two years (unless recurrence or disease-specific mortality occurred earlier), in which there were thyroid specimens for histological re-evaluation.

Formalin-fixed paraffin-embedded (FFPE) material was collected, and all tumor samples were reviewed by a single pathologist (MRB) according to the WHO classification, fourth edition [12]. All doubtful tumor samples were reviewed by two senior pathologists (MSS and MRB) for consensual diagnosis. Each patient was staged according to the eighth edition of the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) staging system [13].

All procedures described in this study were in accordance with national and institutional ethical standards. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the local ethics committee (project investigation 30/2016, 2016-01-28, Comissão de Ética do Centro Hospitalar de Vila Nova de Gaia/Espinho). According to Portuguese law, informed consent is not required for retrospective studies.

Patient follow-up

Patients underwent surgery with or without subsequent radioiodine (RAI) treatment. Surgical options depended on thyroid pathology and tumor characteristics (extrathyroidal extension (ETE), clinically identified lymph node metastasis (LNM), and distant metastasis (DM)).

Lymphadenectomy was performed whenever LNMs were found before surgery or intraoperatively. RAI treatment was dependent on the type of surgery performed (total versus lobectomy and isthmectomy and surgery completeness), presence of DM, and post-surgery pathological findings (ETE, lymph vessel and venous invasion, and LNM).

RAI therapy was given four weeks after levothyroxine (LT4) withdrawal or using recombinant thyroid-stimulating hormone (rhTSH) preparation. This treatment was followed by post-therapy scintigraphy, seven days later. Stimulated thyroglobulin (sTg) was evaluated at the time of RAI therapy, and it was defined as the thyroglobulin (Tg) level measured when serum TSH was >50 mIU/L. The post-ablation Tg level was assessed approximately 12 months after surgery. During the first follow-up year, patients were followed every three months and, in subsequent years, twice a year. Serum biochemical evaluation (TSH, free T4, Tg, and anti-Tg antibodies (TgAb)) and neck ultrasonography (US) were routinely performed. Computed tomography (CT) scans, 18-fluorodeoxyglucose positron emission tomography (18-FDG-PET) scans, or RAI thyroid scintigraphy were performed only if previous examinations were doubtful or suspicious. During follow-up, we used the 2015 American Thyroid Association (ATA) risk stratification system [14] to predict the risk of recurrent or persistent disease in patients who were treated with RAI. In non-RAI-treated patients, we used the risk stratification system for patients treated without RAI published by Momesso et al. [15]. All patients were stratified as having four levels of possible response to treatment: excellent, indeterminate, biochemically incomplete, and structurally incomplete.

Structural disease status was detected by appropriate imaging modalities and, whenever possible, confirmed by cytology. Additional surgery was performed whenever feasible. If disease was unresectable, patients received RAI therapy.

Two groups were considered, according to the type of SPTC diagnosis: clinical diagnosis, if thyroid nodule or cervical lymphadenopathy cytology was diagnosed or suggested (Bethesda III-VI) malignancy (G1, n=60 (61.9%)), and incidental diagnosis, if SPTC was diagnosed in thyroid specimens removed for benign thyroid disease (G2, n=37 (38.1%)).

Clinical endpoints

The following clinical and demographic data were evaluated: age at diagnosis, sex, family history of thyroid cancer, thyroid-stimulating hormone (TSH) level at diagnosis of SPTC, thyroid function status (euthyroid, hypothyroidism, and thyrotoxicosis), type of thyroid surgery (total thyroidectomy and hemithyroidectomy), cervical lymphadenectomy, SPTC staging according to the eighth edition of the AJCC/UICC staging system (I, II, and IVb) [13], largest tumor size, focality (unifocal and multifocal), laterality (unilateral and bilateral), histological subtype, major extrathyroidal invasion, minimal ETE (mETE), surgical resection margins (R0

and >R1), lymph node metastases and cervical location (central and lateral compartments), lymphocytic thyroiditis, RAI therapy, mutations in the *TERT* (*TERT* promoter (*TERTp*)), *BRAF* (*BRAFV600E*) and *RAS* genes, persistent and/or recurrent disease, disease-free survival period, and all-cause and SPTC-related mortality.

Persistence was defined as any clinical, biochemical, and/or radiological (cervical ultrasound, computed tomography (CT), scintigraphy with ¹³¹Iodine, or positron emission tomography-CT) suspicion or evidence of malignancy (indeterminate or incomplete response to treatment), and recurrence as any evidence of disease after a disease-free period (excellent response to treatment). The disease-free survival period extended from the time of thyroidectomy to the persistence and/or recurrence date or to the last consultation date, if no persistence or recurrence event occurred.

DNA extraction

Histological material was reviewed, and molecular analysis of *BRAF*, *RAS*, and *TERT* gene status was performed (partially reported by Póvoa et al. [16]). DNA from FFPE tissues was retrieved from 10 µm sections after hematoxylin and eosin (H&E)-guided careful microdissection. DNA extraction was performed using the GRS Genomic DNA Kit BroadRange (GRiSP Research Solutions, Porto, Portugal) following the manufacturer's instructions. Quantitative and qualitative analysis of all samples was then performed by spectrophotometry using NanoDrop ND-1000 Spectrophotometer for microvolume UV-Vis measurements (Thermo Scientific, Waltham, MA).

PCR and Sanger sequencing analysis

Genetic characterization of the series of tumors involving *BRAF*, *RAS* (*NRAS*, *HRAS*, and *KRAS*), and *TERTp* mutations was performed as previously reported [17,18]. Primer design was performed accounting for the most frequent regions mutated in PTC, namely, *BRAF* codon 600, *NRAS* codon 61, *HRAS* and *KRAS* codons 12, 13, and 61, and *TERTp*-124, and *TERTp*-146 regions. Amplification of genomic DNA (25-50 ng) was achieved using the QIAGEN multiplex PCR kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The annealing temperature of 61°C was established after protocol optimization for *BRAF*, *NRAS*, and *TERTp* amplification in the same reaction. *HRAS* and *KRAS* were screened separately by a touchdown PCR using the MyTaq HS Mix 2× Bioline PCR Kit (Meridian Bioscience, Cincinnati, OH) following the manufacturer's instructions.

PCR amplification was confirmed in 1%-2% agarose gel electrophoresis (GRS Agarose LE, GRiSP, Oporto, Portugal) followed by PCR product purification. All tested hotspot mutations were sequenced using the ABI Prism Big Dye Terminator kit v3.1 Cycle Sequencing (Fisher Scientific Applied Biosystems®, Portsmouth, NH). After sequencing product precipitation, fragments were analyzed by capillary electrophoresis using the Applied Biosystems 3130/3130×1 Genetic Analyzers (Foster City, CA). For all genes, all detected mutations were validated by performing a new and independent analysis.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess the normality of quantitative variables. Age at diagnosis of SPTC and the disease-free survival period were described as means and standard deviations (SD). The remaining descriptive statistics for continuous variables were reported using medians (first quartile and third quartile) as they were not normally distributed. Categorical variables were expressed as absolute and relative proportions. Univariate analysis was performed to compare variables among the two groups using Fisher's exact test, unpaired Student's t-test, and Mann-Whitney U test when they were categorical, normally, and non-normally distributed continuous variables, respectively.

A multivariate logistic regression model was conducted to assess the influence of SPTC-related clinical and sociodemographic factors that were statistically significant in the univariate analysis on the clinical diagnosis of SPTC.

Receiver operating characteristic (ROC) curves were designed to determine the most accurate cut-off values of continuous independent predictors for the clinical diagnosis of SPTC.

Statistical Package for the Social Sciences (SPSS) software (version 27.0) (IBM SPSS Statistics, Armonk, NY) [19] was used, and $p < 0.05$ was considered statistically significant.

Results

A total of 97 patients were included, with 60 (61.9%) diagnosed clinically (G1) and 37 (38.1%) identified incidentally (G2). Female sex was predominant ($n=83$ (85.6%)), and the mean age at diagnosis was 55.4 ± 14.3 years. Patients of G1 were younger ($p=0.055$), were more frequently treated with RAI ($p=0.007$), had bigger tumors ($p<0.001$), and had a higher frequency of mETE ($p=0.041$) (Table 1).

Variables	CD (n=60 (81.9%))	I (n=37 (38.1%))	p
Clinical and sociodemographic			
Age at diagnosis (years) (mean±SD)	53.0±14.2	59.3±13.9	0.035*
Age at diagnosis (years) (number (%))			0.185
<55	31 (51.7)	14 (37.8)	
≥55	29 (48.3)	23 (62.2)	
Sex (number (%))			0.695
Male	8 (13.3)	6 (16.2)	
Female	52 (86.7)	31 (83.8)	
Family history of thyroid cancer (number (%))			0.796
Yes	7 (11.7)	5 (13.5)	
No	14 (23.3)	12 (32.4)	
Unknown	39 (65)	20 (54.1)	
TSH (μIU/mL) at diagnosis (median (p25, p75)) ^K	0.98 (0.23, 4.22)	2.51 (0.29, 5.68)	0.069
Thyroid function status (number (%)) ^K			
Euthyroidism	14 (23.3)	7 (18.9)	0.903
Hypothyroidism	13 (21.7)	12 (32.4)	0.281
Thyrotoxicosis	21 (35)	6 (16.2)	0.057
Staging and treatments			
131-iodine therapy (number (%))	38 (63.3)	13 (35.1)	0.007*
Surgery (number (%))			0.133
Total thyroidectomy	56 (93.3)	31 (83.8)	
Hemithyroidectomy	4 (6.7)	6 (16.2)	
Cervical lymphadenectomy (number (%))	6 (10)	1 (2.7)	0.177
Stage (AJCC eighth edition ^[13]) (number (%))			0.618
I	58 (96.6)	35 (94.6)	
II	1 (1.7)	2 (5.4)	
IVB	1 (1.7)	0 (0)	
Histopathological			
Largest tumor size (mm) (median (p25-p75))	8 (7-9)	5 (4-6.5)	<0.001*
Focality (number (%))			0.082
Unifocal	35 (58.3)	28 (75.7)	
Multifocal	25 (41.7)	9 (24.3)	
Laterality (number (%))			0.229
Unilateral	50 (83.3)	34 (91.9)	
Bilateral	10 (16.7)	3 (8.1)	
Histological subtype (number (%))			0.124
Classical	47 (78.2)	20 (54.1)	
Follicular	10 (16.7)	13 (35.1)	
Tail cell	1 (1.7)	1 (2.7)	

Oncocytic	1 (1.7)	3 (8.1)	
Wartin-like	1 (1.7)	0 (0)	
Major extrathyroidal extension (number (%))	1 (1.7)	0 (0)	0.430
Minimal extrathyroidal extension (number (%))	27 (45)	9 (24.3)	0.041*
Surgical resection margins (number (%))			0.117
R0	94 (90)	36 (97.3)	
≥R1	6 (10)	1 (2.7)	
Lymph node metastases (number (%))	7 (11.7)	2 (5.4)	0.276
Central compartment	3 (5)	2 (5.4)	
Lateral compartment	4 (6.7)	0 (0)	
Lymphocytic thyroiditis (number (%))	26 (43.3)	13 (35.1)	0.426
Molecular (mutations)			
TERTp (number (%))	4 (7.4)	0 (0)	0.115
BRAF ^{V600E} (number (%))	40 (69)	23 (63.9)	0.611
TERTp and BRAF ^{V600E} (number (%))	3 (5)	0 (0)	0.112
Prognosis			
Persistence (number (%))	8 (13.3)	8 (21.6)	0.286
Recurrence (number (%))	5 (8.3)	0 (0)	0.071
Disease-free survival period (months) (mean±SD)	70.7±33.6	62.0±40.0	0.797
All-cause mortality (number (%))	3 (5)	3 (8.1)	0.537
Attributable to SPTC	2 (3.3)	0 (0)	0.262

TABLE 1: Univariate analysis stratified by the type of subcentimetric papillary thyroid carcinoma diagnosis.

SPTC: subcentimetric papillary thyroid carcinoma, SD: standard deviation, CD: clinical diagnosis, I: incidental diagnosis, TSH: thyroid-stimulating hormone, AJCC: American Joint Committee on Cancer

*Statistically significant

‡24 (24.7%) cases of omitted data

G1 also showed a tendentially higher frequency of multifocality (G1: 41.7% versus G2: 24.3%; $p=0.082$), bilaterality (G1: 16.7% versus G2: 8.1%; $p=0.229$), positive surgical resection margins $\geq R1$ (G1: 10% versus G2: 2.7%; $p=0.117$), lymph node metastasis (G1: 11.7% versus G2: 5.4%; $p=0.276$), and *TERTp* mutations (G1: 7.4% versus G2: 0%; $p=0.115$), although without statistical significance.

During the follow-up period (67.4 ± 36.2 months), SPTC persistence rates and mean disease-free survival periods remained comparable between groups, although G1 evidenced a recurrence rate of 8.5% and G2 reported no cases of recurrence ($p=0.071$). Six (6.2%) patients died; in two (2.1%) cases, mortality was SPTC-attributable and were all clinically detected. No cases of SPTC-specific mortality were reported in patients with an incidental diagnosis. SPTC-specific mortality was tendentially higher in the clinically diagnosed group, although no statistical significance was found ($p=0.262$).

No statistically significant differences were reported between groups for the presence of thyroid cancer family history, TSH level at SPTC diagnosis, thyroid function status, type of thyroid surgery, presence of cervical lymphadenectomy, SPTC staging, SPTC histological subtype, presence of major ETE invasion, lymphocytic thyroiditis, and *BRAF* mutation status (Table 1). No *RAS* gene mutations were identified.

The multivariate logistic regression model showed that increased tumor size was the only independent factor predictive of a clinical diagnosis of SPTC (odds ratio (OR): 1.785, 95% confidence interval (CI): 1.368-2.329; $p<0.001$) (Table 2).

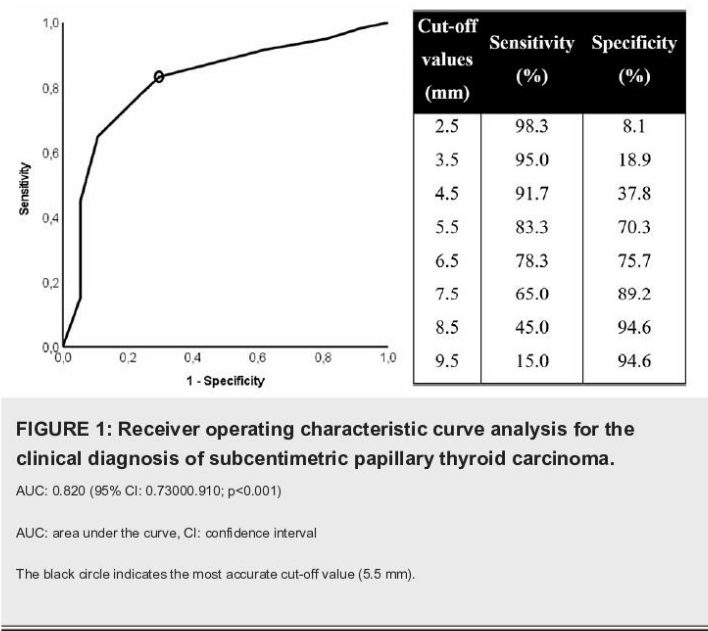
Variables	β	SE	OR (95% CI)	p
Age at diagnosis (+1 year)	0.028	0.019	1.029 (0.990-1.069)	0.145
131I-iodine therapy (yes)	0.829	0.830	2.291 (0.811-6.473)	0.118
Largest tumor size (+1 mm)	1.760	0.136	1.785 (1.368-2.329)	<0.001*
Minimal extrathyroidal extension (yes)	0.001	0.591	1.001 (0.314-3.189)	0.999

TABLE 2: Logistic regression of variables predictive of a clinical diagnosis of subcentimetric papillary thyroid carcinoma.

β : adjusted regression coefficient, SE: standard error, OR: odds ratio, 95% CI: 95% confidence interval

*Statistically significant

After ROC curve analysis, a tumor size of 5.5 mm was the most accurate cut-off value (sensitivity: 83.3%, specificity: 70.3%), demonstrating the power to discriminate clinically diagnosed SPTC (area under the curve (AUC): 0.820, 95% CI: 0.730-0.910, $p<0.001$) (Figure 1).



To assess variables of interest related to an SPTC size ≥ 5.5 mm, a subanalysis was performed. In addition to clinically diagnosed SPTC, RAI therapy, total thyroidectomy, and mETE were significantly associated with a tumor size of ≥ 5.5 mm. LNM rates and prognostic variables were similar between groups (Table 3).

Variables	≤ 5.5 mm (n=36 (37.1%))	≥ 5.5 mm (n=61 (62.9%))	p
Clinical and sociodemographic			
Age at diagnosis (years) (mean $\pm$ SD)	57.4 $\pm$ 14.1	54.2 $\pm$ 14.4	0.294

Age at diagnosis (years) (number (%))			0.473
<55	15 (41.7)	30 (49.2)	
≥55	21 (58.3)	31 (50.8)	
Sex (number (%))			0.281
Male	7 (19.4)	7 (11.5)	
Female	29 (80.6)	54 (88.5)	
Family history of thyroid cancer (number (%))			0.658
Yes	6 (16.7)	6 (9.8)	
No	11 (30.5)	15 (24.6)	
Unknown	19 (52.8)	40 (65.6)	
TSH (μU/mL) at diagnosis (median (p25, p75)) ^a	2.01 (0.20, 8.18)	0.41 (0.05, 4.26)	0.176
Thyroid function status (number (%)) ^b			
Euthyroidism	7 (19.4)	13 (21.3)	0.976
Hypothyroidism	11 (30.6)	14 (23)	0.228
Thyrotoxicosis	7 (19.4)	20 (32.8)	0.225
Staging and treatments			
131-iodine therapy (number (%))	14 (38.9)	37 (60.7)	0.038*
Surgery (number (%))			0.023*
Total thyroidectomy	29 (80.6)	58 (95.1)	
Hemithyroidectomy	7 (19.4)	3 (4.9)	
Cervical lymphadenectomy (number (%))	3 (8.3)	4 (6.6)	0.744
Stage (AJCC eighth edition ¹⁵) (number (%))			0.586
I	34 (94.4)	59 (96.7)	
II	2 (5.6)	2 (3.3)	
IVB	0 (0)	0 (0)	
Type of diagnosis (number (%))			<0.001*
Clinical	10 (27.8)	50 (82)	
Incidental	26 (72.2)	11 (18)	
Histopathological			
Focality (number (%))			0.476
Unifocal	25 (69.4)	38 (62.3)	
Multifocal	11 (30.6)	23 (37.7)	
Laterality (number (%))			0.260
Unilateral	33 (91.7)	51 (83.6)	
Bilateral	3 (8.3)	10 (16.4)	
Histological subtype (number (%))			0.483
Classical	22 (61.1)	45 (73.8)	
Follicular	10 (27.7)	13 (21.4)	
Tall cell	1 (2.8)	1 (1.6)	

Oncologic	3 (8.4)	1 (1.6)	
Warthin-like	0 (0)	1 (1.6)	
Major extrathyroidal extension (number (%))	0 (0)	1 (1.6)	0.440
Minimal extrathyroidal extension (number (%))	6 (16.7)	30 (49.2)	0.001*
Surgical resection margins (number (%))			0.135
R0	36 (100)	54 (88.5)	
≥R1	0 (0)	7 (11.5)	
Lymph node metastases (number (%))			0.501
Central compartment	2 (5.6)	2 (3.3)	
Lateral compartment	1 (2.8)	5 (8.2)	
Lymphocytic thyroiditis (number (%))	15 (38.5)	24 (39.3)	0.822
Molecular (mutations)			
TERTp (number (%))	0 (0)	4 (7.1)	0.134
BRAP ^{V600E} (number (%))	19 (63.3)	35 (67.3)	0.715
TERTp and BRAP ^{V600E} (number (%))	0 (0)	3 (4.9)	0.194
Prognosis			
Persistence (number (%))	8 (22.2)	8 (13.1)	0.243
Recurrence (number (%))	2 (5.6)	3 (4.9)	0.891
Disease-free survival period (months) (mean±SD)	61.7±36.9	70.7±35.7	0.238
All-cause mortality (number (%))	3 (8.3)	3 (4.9)	0.500
Attributable to SPTC	1 (2.8)	1 (1.6)	0.703

TABLE 3: Univariate analysis stratified by subcentimetric papillary thyroid carcinoma size.

SPTC: subcentimetric papillary thyroid carcinoma, SD: standard deviation, TSH: thyroid-stimulating hormone, AJCC: American Joint Committee on Cancer

*Statistically significant

‡24 (24.7%) cases of omitted data

In the multivariate analysis, clinically diagnosed SPTC and mETE were independently associated with an SPTC size of ≥5.5 mm (Table 4).

Variables	β	SE	OR (95% CI)	P
131I-iodine therapy (yes)	-0.088	0.576	0.916 (0.266-2.832)	0.879
Total thyroidectomy (yes)**	1.315	0.923	3.726 (0.610-22.768)	0.154
Minimal extrathyroidal extension (yes)	1.423	0.691	4.152 (1.277-13.492)	0.018*
Clinical diagnosis (yes)***	2.390	0.965	10.919 (3.750-31.788)	<0.001*

TABLE 4: Logistic regression of variables predictive of a subcentimetric papillary thyroid carcinoma size of ≥ 5.5 mm.

β : adjusted regression coefficient, SE: standard error, OR: odds ratio, 95% CI: 95% confidence interval

*Statistically significant

**Reference category: hemithyroidectomy

***Reference category: incidental diagnosis

Discussion

In our study, larger tumor size was the only independent factor for a clinical diagnosis of SPTC, specifically in tumors ≥ 5.5 mm. This observation is concordant with most published evidence [20-23], although some authors did not find any association [24,25].

Our subanalysis showed an independent association between mETE and a larger SPTC size (≥ 5.5 mm), which is supported by previous findings [26-28]. SPTC size did not affect the presence of LNM and prognosis (Table 5), contradicting the most recognizable evidence as published by other authors, at least regarding LNM, persistence/recurrence risk, and disease-free survival [28-32]. However, these studies [28-32] included all sizes of PTC and not exclusively SPTC. The eighth edition of the AJCC/UICC staging system [13] and the ATA 2015 risk stratification system [14] also do not provide specific guidance on subdifferentiation by tumor size for SPTC, focusing solely on discriminating tumor size cut-off values above 1 cm (i.e., 1, 2, and 4 cm). Therefore, we highlight the need for larger studies on SPTC to accurately assess the impact of their size on prognosis, especially regarding lymph node metastasis, persistence/recurrence, and disease-free survival.

Patients with clinically diagnosed SPTC were younger, were more often submitted to RAI therapy, and had a higher frequency of mETE. However, these associations were observed merely in the univariate analysis (Table 1), concordant with previous analyses. In addition to a heightened propensity for highly aggressive tumor behavior in earlier age cohorts, younger patients exhibited a proclivity for increased medical scrutiny and more frequent utilization of fine needle aspiration biopsy (FNAB), thereby providing a potential rationale for its correlation with a clinical diagnosis [25,24]. Once our cohort of patients was mainly treated before 2015, the higher frequency of RAI therapy in both groups might be related to the expansion of the "ATA low risk" category from the 2009 ATA guidelines [33] to the 2015 ATA guidelines (intrathyroidal, papillary microcarcinoma, and unifocal or multifocal, including BRAFV600E mutated; intrathyroidal, encapsulated follicular variant of papillary thyroid cancer) [14], which best explains a subsequent decrease in the number of cases with clear indication for RAI treatment, since 2015. Until recently, mETE was considered one prominent independent predictor of recurrence and survival [33,34]. While the 2015 ATA guidelines [14] still define mETE as a predictor of intermediate risk of recurrence, other published data have been divergent in this regard as described below. In fact, the exclusion of mETE from the eighth edition of the AJCC/UICC staging system [13] was predicated upon the absence of substantive evidence of a prognostic impact on survival. mETE weight on disease persistence/recurrence remains a matter of debate [35]. Some studies [35-37] found an association between mETE and increased overall recurrence risk, but the meta-analysis [37] included studies with divergent findings in this matter, which ultimately resulted in a nonsignificant small increase in absolute risk of recurrence (OR: 2.40, 95% CI: 0.95-6.03). Evidence is more consistent in PTC without LNM, as several studies found no association between mETE and recurrence risk [38,39]. Of note, the majority of these studies did not include histopathological subtypes and/or the presence of vascular invasion, which are major recurrence risk factors. Hence, putting together the evidence highlighted above, we can advance that isolated mETE might not add substantial risk to global survival and recurrence of SPTC without LNM, but its actual effect on the recurrence risk of SPTC with LNM remains unclear.

In our study, clinically diagnosed SPTC is more frequently associated with other histological and molecular markers of aggressive behavior (multifocality, bilaterality, incomplete surgical resection margins, lymph node metastasis, and TERTp mutations), recurrence, and SPTC-specific mortality. Although these

associations did not reach statistical significance, they are supported by robust evidence from previous studies [40,41]. A tendency of association was found for multifocality and clinically diagnosed SPTC, but it seems to be better explained by the greater ease in obtaining a clinical diagnosis with a higher number of SPTC foci than an association with greater tumor aggressiveness, as outlined by other reports [20,24,42]. Other studies stated that multifocality was not a predictive factor of a clinical diagnosis [21,22,43]. The ATA 2015 risk stratification system [14] classifies a multifocal SPTC as possessing an "intermediate risk" of recurrence (5%-20%) only if an extrathyroidal extension is concomitantly present. Accordingly, intrathyroidal SPTC is categorized as "low risk" of recurrence (<5%), independently of focality [14]. The eighth edition of the AJCC/UICC staging system [13] considers four independent prognostic factors of SPTC, namely, age, sex, tumor size, and LNM, and thus, focality is not included in the staging system. A higher frequency of LNM has been consistently reported in clinically diagnosed SPTC. Once FNAB is rarely indicated in thyroid micronodules, clinical cases were frequently diagnosed by FNAB assessment of adenopathy, which largely explains the association between a clinical diagnosis of SPTC and the presence of LNM [20-22,24,25,42]. Conversely, *TERTp* mutations have been related to more aggressive behavior of SPTC, and consequently, poor prognostic outcomes have been reported in such patients [44].

Therefore, taking those analyses into account, the association of larger tumor size with clinical diagnosis seems to be best explained by a greater ability to detect larger lesions through imaging techniques than the presence of greater intrinsic tumor aggressiveness. However, two older studies found an association between larger tumor size and greater aggressive behavior of SPTC, and thus, this needs to be clarified by more robust evidence [40,41].

Clinically diagnosed patients underwent FNAB and subsequently proceeded to surgery with a median largest tumor size of 8 mm. This decision is supported by the fact that the majority of these patients were young adults with nodules categorized as European Thyroid Imaging Reporting and Data System (EU-TIRADS) 5, nearing the threshold for FNAB, or presenting locoregional adenopathies. Despite the consistent option of active surveillance, the choice of surgical intervention was guided by the patient's personal preference. This decision was influenced by a desire to alleviate anxiety associated with maintaining active surveillance in the context of a known malignancy.

In our analysis, both groups demonstrated reasonable persistence and recurrence rates and extended mean disease-free survival time, similar to the mean follow-up period of around five years and a half. A nonsignificant higher recurrence rate was observed in clinically diagnosed SPTC when compared to incidentally diagnosed cases, which did not report any recurrence (G1: 8.3% versus G2: 0%; $p=0.071$). Published series are divergent regarding this topic (association [45,46] versus no association [21,25,42]).

Despite the reasonable persistence and recurrence rates and the potential link to histopathological and molecular markers of aggressive behavior, medium- to long-term survival prognosis was excellent in clinically diagnosed SPTC, as our study revealed only two (3.3%) deaths attributable to SPTC in clinically diagnosed cases, which is comparable to the low SPTC-related mortality rates reported in the literature for both clinically and incidentally diagnosed cases (i.e., up to 5.8% and 2.4%, respectively) [22,45]. Both patients who had disease-related death were elderly and presented with lateral compartment LNM.

However, there are some limitations that require particular attention. First, the retrospective nature of our approach, reliant on electronic records from 2002 to 2015, introduces inherent challenges, including susceptibility to selection bias, incomplete data, and the complexity of establishing causation. Second, the study's single-center focus raises concerns about the generalizability of findings, as the patient cohort may not fully represent the diverse demographic and clinical characteristics of a broader population. Third, the relatively small sample size may restrict the statistical power and overall reliability of our study. Larger series are needed to further investigate our findings. Finally, the specified follow-up duration might be insufficient to capture long-term outcomes and survival data, particularly for a cancer type such as SPTC, known for its generally indolent course. A more extended follow-up period would provide a more comprehensive understanding of the disease trajectory. These limitations should be considered when interpreting and applying the findings of our study.

Conclusions

A larger tumor size was more likely linked to the clinical detection of SPTC, rather than indicating a theoretically higher aggressiveness in these clinically diagnosed cases. While aggressive histopathological and molecular features, as well as recurrence, showed nonsignificant associations with clinically detected SPTC, it is important to note the potential influence of a small sample size bias. Notably, both clinically and incidentally diagnosed cases demonstrated excellent medium- to long-term survival outcomes, characterized by low SPTC-related mortality rates and extended disease-free survival times. However, conflicting evidence persists in the published literature, underscoring the need for additional research, particularly larger prospective studies, to provide more conclusive insights in this domain.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Disclosures

Human subjects: Consent was obtained or waived by all participants in this study. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

References

- Hedinger C, Williams ED, Sobin LH: The WHO histological classification of thyroid tumors: a commentary on the second edition. *Cancer*. 1989, 63:908-11. [10.1002/1097-0142\(19890301\)63:5<908::aid-cnrcr2820630520>3.0.co;2-i](#)
- Gürleyik E, Gurleyik G, Karapolat B, Onsal U: Incidental papillary thyroid microcarcinoma in an endemic goiter area. *J Thyroid Res*. 2016, 2016:1784397. [10.1155/2016/1784397](#)
- Kaliszewski K, Strutyńska-Karpińska M, Zubkiewicz-Kucharska A, et al.: Should the prevalence of incidental thyroid cancer determine the extent of surgery in multinodular goiter? *PLoS One*. 2016, 11:e0168654. [10.1371/journal.pone.0168654](#)
- Lew JI, Solorzano CC: Use of ultrasound in the management of thyroid cancer. *Oncologist*. 2010, 15:253-8. [10.1634/theoncologist.2009-0524](#)
- Miyauchi A, Ito Y, Oda H: Insights into the management of papillary microcarcinoma of the thyroid. *Thyroid*. 2018, 28:25-31. [10.1089/thy.2017.0227](#)
- Hong YR, Yan CX, Mo GQ, Luo ZY, Zhang Y, Wang Y, Huang PT: Conventional US, elastography, and contrast enhanced US features of papillary thyroid microcarcinoma predict central compartment lymph node metastases. *Sci Rep*. 2015, 5:7748. [10.1038/srep07748](#)
- Nickel B, Brito JP, Moynihan R, Barratt A, Jordan S, McCaffery K: Patients' experiences of diagnosis and management of papillary thyroid microcarcinoma: a qualitative study. *BMC Cancer*. 2018, 18:242. [10.1186/s12885-018-4152-9](#)
- Choi JB, Lee WK, Lee SG, et al.: Long-term oncologic outcomes of papillary thyroid microcarcinoma according to the presence of clinically apparent lymph node metastasis: a large retrospective analysis of 5,348 patients. *Cancer Manag Res*. 2018, 10:2883-91. [10.2147/CMAR.S173853](#)
- Jiang W, Wei HY, Zhang HY, Zhuo QL: Value of contrast-enhanced ultrasound combined with elastography in evaluating cervical lymph node metastasis in papillary thyroid carcinoma. *World J Clin Cases*. 2019, 7:49-57. [10.12998/wjcc.v7.i1.49](#)
- Liu LS, Liang J, Li JH, et al.: The incidence and risk factors for central lymph node metastasis in cN0 papillary thyroid microcarcinoma: a meta-analysis. *Eur Arch Otorhinolaryngol*. 2017, 274:1327-38. [10.1007/s00405-016-4502-0](#)
- Zhi J, Zhao J, Gao M, et al.: Impact of major different variants of papillary thyroid microcarcinoma on the clinicopathological characteristics: the study of 1041 cases. *Int J Clin Oncol*. 2018, 23:59-65. [10.1007/s10147-017-1170-6](#)
- International Agency for Research on Cancer: WHO classification of tumours of endocrine organs: WHO classification of tumours, 4th edition, volume 10. Lloyd RV, Osamura RY, Klöppel G, Rosai J (ed): IARC, 2017.
- Amin MB, Greene FL, Edge SB, et al.: The eighth edition AJCC cancer staging manual: continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin*. 2017, 67:93-9. [10.3322/caac.21388](#)
- Haugen BR, Alexander EK, Bible KC, et al.: 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association Guidelines Task Force on thyroid nodules and differentiated thyroid cancer. *Thyroid*. 2016, 26:1-133. [10.1089/thy.2015.0020](#)
- Momesso DP, Vaisman F, Yang SP, Bulzico DA, Corbo R, Vaisman M, Tuttle RM: Dynamic risk stratification in patients with differentiated thyroid cancer treated without radioactive iodine. *J Clin Endocrinol Metab*. 2016, 101:2692-700. [10.1210/je.2015-4290](#)
- Póvoa AA, Teixeira E, Bella-Cueto MR, et al.: Genetic determinants for prediction of outcome of patients with papillary thyroid carcinoma. *Cancers (Basel)*. 2021, 13:10.3390/cancers13092048

17. Shen X, Zhu G, Liu R, et al.: Patient age-associated mortality risk is differentiated by BRAF V600E status in papillary thyroid cancer. *J Clin Oncol*. 2018, 36:438-45. [10.1200/JCO.2017.74.5497](#)
18. Ito Y, Miyauchi A, Kihara M, Fukushima M, Higashiyama T, Miya A: Overall survival of papillary thyroid carcinoma patients: a single-institution long-term follow-up of 5897 patients. *World J Surg*. 2018, 42:615-22. [10.1007/s00268-018-4479-z](#)
19. IBM SPSS Statistics for Windows. (2020). <https://www.ibm.com/support/pages/how-cite-ibm-spss-statistics-or-earlier-versions-spss>.
20. Elliott MS, Gao K, Gupta R, Chua EL, Gargya A, Clark J: Management of incidental and non-incidental papillary thyroid microcarcinoma. *J Laryngol Otol*. 2013, 127 Suppl 2:S17-23. [10.1017/S0022215113000455](#)
21. Vasileiadis I, Karatzas T, Vasileiadis D, Kapetanakis S, Charitoudis G, Karakostas E, Kouraklis G: Clinical and pathological characteristics of incidental and nonincidental papillary thyroid microcarcinoma in 339 patients. *Head Neck*. 2014, 36:564-70. [10.1002/hed.23333](#)
22. Mehanna H, Al-Maqbili T, Carter B, et al.: Differences in the recurrence and mortality outcomes rates of incidental and nonincidental papillary thyroid microcarcinoma: a systematic review and meta-analysis of 21 329 person-years of follow-up. *J Clin Endocrinol Metab*. 2014, 99:2834-43. [10.1210/jc.2013-2118](#)
23. Ruiz J, Ríos A, Rodríguez JM, et al.: Incidental versus clinical diagnosis of papillary thyroid microcarcinoma. Long-term prognosis. *Endocrinol Diabetes Nutr (Engl Ed)*. 2020, 67:517-25. [10.1016/j.endinu.2019.09.012](#)
24. Roti E, Rossi R, Trasforini G, et al.: Clinical and histological characteristics of papillary thyroid microcarcinoma: results of a retrospective study in 243 patients. *J Clin Endocrinol Metab*. 2006, 91:2171-8. [10.1210/jc.2005-2372](#)
25. Ortiz S, Rodríguez JM, Torregrosa N, Balsalobre M, Ríos A, Parrilla P: [Relation between clinical presentation and prognosis of patients with papillary thyroid microcarcinoma] (Article in Spanish). *Med Clin (Barc)*. 2003, 120:773-4. [10.1016/s0025-7755\(05\)73841-x](#)
26. Liu L, Oh C, Heo JH, et al.: Clinical significance of extrathyroidal extension according to primary tumor size in papillary thyroid carcinoma. *Eur J Surg Oncol*. 2018, 44:1754-9. [10.1016/j.ejso.2018.05.009](#)
27. Kim JW, Roh JL, Gong G, Cho KJ, Choi SH, Nam SY, Kim SY: Extent of extrathyroidal extension as a significant predictor of nodal metastasis and extranodal extension in patients with papillary thyroid carcinoma. *Ann Surg Oncol*. 2017, 24:460-8. [10.1245/s10434-016-5594-4](#)
28. Shin CH, Roh JL, Song DE, Cho KJ, Choi SH, Nam SY, Kim SY: Prognostic value of tumor size and minimal extrathyroidal extension in papillary thyroid carcinoma. *Am J Surg*. 2020, 220:925-31. [10.1016/j.amjsurg.2020.02.020](#)
29. Kim JW, Roh JL, Gong G, Cho KJ, Choi SH, Nam SY, Kim SY: Recurrence in patients with clinically early-stage papillary thyroid carcinoma according to tumor size and surgical extent. *Am J Surg*. 2016, 212:419-425.e1. [10.1016/j.amjsurg.2015.12.015](#)
30. Tam S, Amit M, Boonsripitayanon M, et al.: Effect of tumor size and minimal extrathyroidal extension in patients with differentiated thyroid cancer. *Thyroid*. 2018, 28:982-90. [10.1089/thy.2017.0513](#)
31. Ito Y, Fukushima M, Higashiyama T, et al.: Tumor size is the strongest predictor of microscopic lymph node metastasis and lymph node recurrence of N0 papillary thyroid carcinoma. *Endocr J*. 2013, 60:113-7. [10.1507/endocr.je12-0511](#)
32. Ito Y, Fukushima M, Kihara M, Takamura Y, Kobayashi K, Miya A, Miyauchi A: Investigation of the prognosis of patients with papillary thyroid carcinoma by tumor size. *Endocr J*. 2012, 59:457-64. [10.1507/endocr.je12-0013](#)
33. Cooper DS, Doherty GM, Haugen BR, et al.: Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. *Thyroid*. 2009, 19:1167-214. [10.1089/thy.2009.0110](#)
34. Edge SB, Compton CC: The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol*. 2010, 17:1471-4. [10.1245/s10434-010-0985-4](#)
35. Hay ID, Johnson TR, Thompson GB, Sebo TJ, Reinalda MS: Minimal extrathyroidal extension in papillary thyroid carcinoma does not result in increased rates of either cause-specific mortality or postoperative tumor recurrence. *Surgery*. 2016, 159:11-9. [10.1016/j.surg.2015.05.046](#)
36. Danilovic DL, Castroneves LA, Suemoto CK, et al.: Is there a difference between minimal and gross extension into the strap muscles for the risk of recurrence in papillary thyroid carcinomas?. *Thyroid*. 2020, 30:1008-16. [10.1089/thy.2019.0753](#)
37. Diker-Cohen T, Hirsch D, Shimon I, Bachar G, Akirov A, Duskin-Bitan H, Robenshtok E: Impact of minimal extra-thyroid extension in differentiated thyroid cancer: systematic review and meta-analysis. *J Clin Endocrinol Metab*. 2018, [10.1210/jc.2018-00081](#)
38. Ahn D, Sohn JH, Jeon JH, Jeong JY: Clinical impact of microscopic extrathyroidal extension in patients with papillary thyroid microcarcinoma treated with hemithyroidectomy. *J Endocrinol Invest*. 2014, 37:167-73. [10.1007/s40618-013-0025-x](#)
39. Rivera M, Ricarte-Filho J, Tuttle RM, et al.: Molecular, morphologic, and outcome analysis of thyroid carcinomas according to degree of extrathyroid extension. *Thyroid*. 2010, 20:1085-93. [10.1089/thy.2010.0174](#)
40. Lee KJ, Cho YJ, Kim SJ, Lee SC, Kim JG, Ahn CJ, Lee DH: Analysis of the clinicopathologic features of papillary thyroid microcarcinoma based on 7-mm tumor size. *World J Surg*. 2011, 35:318-23. [10.1007/s00268-010-0886-5](#)
41. Zhang L, Wei WJ, Ji QH, et al.: Risk factors for neck nodal metastasis in papillary thyroid microcarcinoma: a study of 1066 patients. *J Clin Endocrinol Metab*. 2012, 97:1250-7. [10.1210/jc.2011-1546](#)
42. Pisanu A, Recchia I, Nardello O, Ucheddu A: Risk factors for nodal metastasis and recurrence among patients with papillary thyroid microcarcinoma: differences in clinical relevance between nonincidental and incidental tumors. *World J Surg*. 2009, 33:460-8. [10.1007/s00268-008-9870-8](#)
43. Lin JD, Kuo SF, Chao TC, Hsueh C: Incidental and nonincidental papillary thyroid microcarcinoma. *Ann Surg Oncol*. 2008, 15:2287-92. [10.1245/s10434-008-9958-2](#)
44. Rusinek D, Pfeifer A, Cieslicka M, et al.: TERT promoter mutations and their impact on gene expression profile in papillary thyroid carcinoma. *Cancers (Basel)*. 2020, 12:10.3390/cancers12061597

45. Ríos A, Rodríguez JM, Navas D, Cepero A, Torregrosa NM, Balsalobre MD, Parrilla P: Family screening in familial papillary carcinoma: the early detection of thyroid disease. *Ann Surg Oncol*. 2016, 23:2564-70. [10.1245/s10434-016-5149-8](https://doi.org/10.1245/s10434-016-5149-8)
46. Gschwandtner E, Klatte T, Swietek N, et al.: Increase of papillary thyroid microcarcinoma and a plea for restrictive treatment: a retrospective study of 1,391 prospective documented patients. *Surgery*. 2016, 159:503-11. [10.1016/j.surg.2015.06.015](https://doi.org/10.1016/j.surg.2015.06.015)

4.4. ARTICLE IV

During the course of our studies on **ARTICLE I** and **ARTICLE III**, we detected one atypical PTC case that called our attention. A patient of the series, diagnosed with subcentimetric PTC (a 2mm FVPTC) died of PTC-specific mortality. Subcentimetric PTC often present good prognosis and indolent behavior, however, this patient presented many large LNM and further developed brain metastases eight years after diagnosis. In order to understand what contributed to disease progression, we performed an evaluation of the molecular status of the developed LNM, and brain metastases surgically removed over the course of 10 years of follow up and correlated those results with patient outcome.

Subcentimetric papillary thyroid carcinoma with extensive lymph node and brain metastasis: case report and review of literature

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Summary

We report a 61-year-old male patient without personal history of thyroid carcinoma or radiation exposure. In 2011, he presented with a cervical mass whose biopsy diagnosed a papillary thyroid carcinoma (PTC) in a lymph node metastasis (LNM). Total thyroidectomy with lymphadenectomy of central and ipsilateral compartment was performed. Histopathology identified a 2 mm follicular variant of PTC and LNM in 25/25 lymph nodes. The patient was treated with 150 mCi of radioactive iodine (RAI), followed by levothyroxine suppressive therapy. In 2016, a retrotracheal mass was diagnosed, suggesting local recurrence; patient was submitted to surgical excision and RAI therapy (120 mCi). Due to seizures, in 2019, a brain CT was performed that diagnosed brain metastases. The patient underwent debulking of the main lesion. Histopathology analysis confirmed a metastatic lesion with variated morphology: classical PTC and follicular pattern and hobnail and tall cell features. Molecular analysis revealed BRAFV600E in LNM at presentation and BRAFV600E and TERT promoter (TERTp) mutations in the recurrent LNM and brain metastasis. Based upon this experience we review the reported cases of subcentimetric PTC with brain metastases and discuss the molecular progression of the present case.

Learning points

- Papillary microcarcinoma (PMCs) usually have very good prognosis with low impact on patient survival.
- PMCs presenting in elderly patients with LNM at diagnosis may carry a guarded outcome.
- Brain metastasis although rare indicate aggressive phenotypic features.
- Patient risk stratification of PMCs based on histopathological analysis and genetic testing may have a significant impact on prognosis providing therapeutic markers, that may predict disease progression and overall outcome.

Background

Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy (80–90% of thyroid cancer) (1, 2).

Generally, this tumor is characterized by an indolent behavior. It carries good prognosis with survival rates at 10 years exceeding 90–95% (3). PTC measuring 10 mm or less in diameter was defined as papillary thyroid



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microcarcinoma (PMC) and an active debate questioning if size '*per se*' could be considered a diagnostic feature is ongoing. In the upcoming 5th edition of the World Health Organization classification of thyroid neoplasms this distinction will be eliminated (2, 4). For the sake of simplicity along this work, we will keep the designation PMC for <1 cm PTC. The incidence of PMCs is increasing and represents approximately 50% of PTC, with a mortality rate of nearly 0.1% at 10 years (5, 6). Aggressive disease develops in approximately 3.8% of patients (5). The presence of metastatic disease at initial presentation is known to carry worse prognosis (7). Lungs and bones are typical locations of distant metastases (8). Brain metastases are very rare (0.8–1.3%) and suggest a more aggressive behavior (9).

In PMCs, histological subtyping of primary tumor or lymph node metastases (LNM) is rarely evaluated, due to PMC's low clinical aggressiveness and its uncertain prognostic meaning (5). This size-defined variant comprises all histological PTC subtypes, and a wide molecular and biological heterogeneity (6, 10). Ghossein *et al.* pointed out the importance of LNM subtyping in PMC patients' prognosis regarding a more accurate risk stratification due to known aggressive variants such as tall cell and hobnail variants of PTC (11). Concerning molecular status, PMCs do not evidence a substantial difference in prevalence of BRAF mutation and RET/PTC rearrangement, in comparison to PTC >1 cm (6). It was advanced that nearly 0.05% of PMCs with BRAFV600E progress to aggressive disease (10). TERT promoter (TERTp) mutations may be present in <10% of PMCs and relate with unfavorable prognosis (12). It was advanced that molecular markers such as BRAF and TERTp mutations combined with histological analysis can have a predictive value for distant metastasization and overall prognosis (12, 13, 14, 15). The occurrence and clinical relevance of the observed histological and molecular alteration in PMCs remain unclear.

Case presentation

A 61-year-old male patient without personal history of thyroid carcinoma or previous radiation exposure presented, in 2011, with a dull cervical mass adherent to muscle planes and without other associated symptoms. The patient was submitted to a cervical lesion excisional biopsy that showed PTC LNM. Total thyroidectomy and lymph node dissection of central and ipsilateral cervical compartments was performed. Histopathology analysis diagnosed a 2 mm partially encapsulated PTC displaying

a predominantly follicular pattern. The PMC was localized in the central part of the lobe and there were no signs of vascular invasion. Metastases were detected in 25 out of 25 lymph nodes (LN) harvested, the largest LN measured 35 mm and displayed extranodal extension. The tumor was staged as pT1aN1bM0, according to the 8th AJCC edition (16) and high risk, according to the ATA stratification (17). The patient received 150 mCi of radioactive iodine (RAI) and TSH was suppressed below 0.1 ng/mL. At first-year evaluation, the patient had an excellent response to treatment. At second year of follow-up, thyroglobulin (Tg) levels started to increase (Fig. 1). From 2013 until 2016 patient had rising TGB levels fulfilling indeterminate biochemical response (TGB <10 ng/mL in stimulation and no evidence of structural disease in cervical US). In 2016, TGB rise to incomplete biochemical response (16.02 ng/mL in stimulation) and since there was still no evidence of structural disease in cervical US, an FDG PET/CT scan was performed. A retrotracheal mass was identified in the PET-CT scan, suggesting local recurrence. Cervical ultrasound did not evidence any structural disease. A cervical and thoracic CT scan was performed to better characterize the aforementioned lesion (Figs 2A and B).

The patient was submitted to surgical excision of the cervical lesion, followed by therapy with 120 mCi RAI. The post-RAI scintigraphy showed no focal abnormal uptake and stimulated serum TGB level was undetectable (0.83 ng/mL) (Fig. 1). Histological analysis found metastatic tissue extensively occupying the LN. In 2017, the patient started

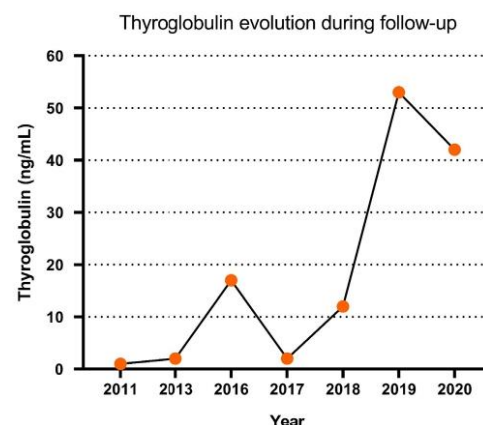


Figure 1
Patient thyroglobulin (ng/mL) evolution during follow-up.

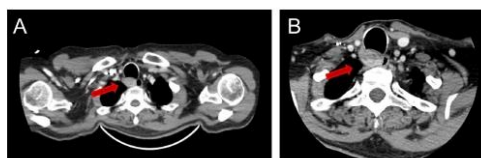


Figure 2
Detection of disease recurrence; A) Thoracic and B) Cervical CT demonstrating a retro-tracheal lymph node of 3 cm.

to experience asthenia and lack of strength in the lower limbs. An FDG-PET/CT scan revealed focal pathological uptake in lungs and dorsal lumbar spine (data not shown). A magnetic resonance imaging (MRI) exhibited infiltrative lesions in D9, D11, S1, and S2, highly suggestive of distant metastases. The patient received 190 mCi of RAI. In this period, suppressed TGB levels increased from 0.83 to 8 mg/dL (Fig. 1). In November 2019, patient presented seizures at the Emergency Room and underwent a brain CT scan and MRI that diagnosed multiple brain lesions (Figs. 3A and B). At this time, the suppressed TGB level was 42 mg/dL (Fig. 1) with negative anti-thyroglobulin antibodies.

The patient underwent debulking of the main lesion. Histopathological analysis confirmed PTC metastases. He underwent whole-brain radiotherapy. In May 2021, the patient deceased, due to disease progression.

Investigation

Histological and molecular characterization of retrieved specimens was performed to ascertain lesions' histological subtyping and genetic analysis. There was no remaining material available from the subcentimetric primary PMC.

Histopathological analysis of collected LNM at diagnosis revealed the presence of classical PTC, follicular variant of PTC, and hobnail variant of PTC, as depicted

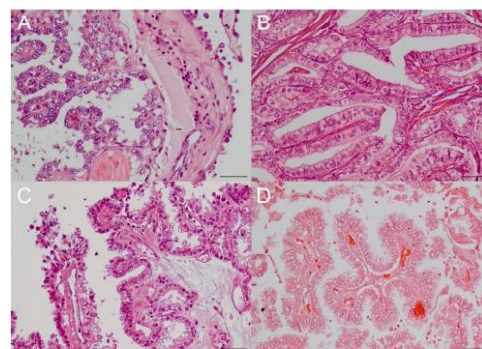


Figure 4
Microscopic analysis of LNM (A, B, C) and brain metastasis (D) (H&E-stained slides). (A) Micropapillary structures (left) and tail like/tear-drop/hobnail shape malignant thyrocytes (right) revealed the areas of hobnail variant PTC in LNM metastasis present at diagnosis. (B) Thin papillary structures lined by tall cell (3:1 or 2:1 ratio) indicating tall cell variant PTC emerging in the retrotracheal metastases (recurrence). (C) Brain metastasis depicting hobnail component. (D) Brain metastasis – papillary structures lined by malignant thyrocytes characterized by clear cell cytoplasm and PTC nuclear features. Scale bars: 50 µm.

in Fig. 4A. In the retrotracheal recurrence, tall cell variant PTC was also detected along with the previous aspects (Fig. 4B). In the brain metastasis samples, it was detected papillae lined by malignant thyrocytes characterized by a hobnail component (Fig. 4C), as well as a component with clear cell cytoplasm and PTC nuclear features (Fig. 4D).

Samples retrieved from LNM at first diagnosis (2011), first recurrence (2016), and brain distant metastases (2019) were studied for p53 expression, Ki-67 index and the most frequent molecular alterations associated with thyroid carcinoma: BRAF, RAS (NRAS, HRAS, and KRAS), and TERTp mutations (Table 1).

Table 1 Molecular analysis of the retrieved specimens.

Lesion/Year	Samples, n	Status	TERTp [#]	BRAF [#]
LNM/2011	13	WT	12	1
		Mut	0	12
LNM/2016	6	WT	0	0
		Mut*	6	5
BM/2019	7	WT	0	0
		Mut**	7	7

No RAS (N-, H-, K-RAS) mutations were detected in any lesion.

*5 out of 6 samples presented BRAFV600E and -124G>ATERTp simultaneous mutation; **7 out of 7 samples presented BRAFV600E and -124G>ATERTp simultaneous mutation; #Not all the samples gave result for the genetic analysis.

BM, brain metastases; LNM, lymph node metastases; Mut, mutated; WT, wild-type.

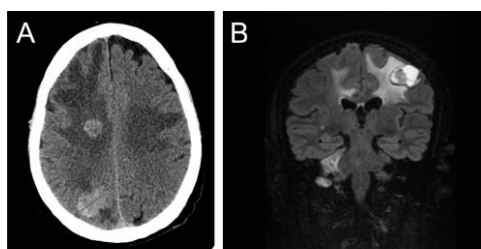


Figure 3
Brain metastasis detection in 2019. (A) Brain CT scan showing brain metastases. (B) Brain MRI showing brain main metastasis.

In LNM present at diagnosis, p53 staining was faint and present in rare and disperse cells, with strong and more extensive reactivity in the focus of hobnail component (Fig. 5A). The overall Ki-67 index was very low (<0.5%) (Fig. 5B). Molecular analysis of the DNA extracted from these samples demonstrated the presence of BRAFV600E mutation. No alterations were detected in RAS genes or TERTp.

Retrotracheal cervical recurrence revealed the presence of tall cell variant of PTC in addition to the other morphologic manifestations found at diagnosis (Fig. 4B). The p53 staining of this lesion was faint and disperse; however, the Ki-67 index was higher (~5%), when comparing to the first LNM, and was mostly localized in the periphery of tumor rather than in the center. Molecular analyses demonstrated the coexistence of BRAFV600E and TERTp mutations in all the successful analyzed samples.

Brain metastases were mainly constituted by hobnail variant of PTC (with a minority of classical features) and also showed areas with clear cells (Fig. 4D). p53 expression was faint and disperse (Fig. 5C). Ki-67 index was higher (< 10%) and more widespread than in the previous lesions (Fig. 5D). Like the retrotracheal lesions, brain metastases showed coexistent BRAFV600E and TERTp mutations. No RAS mutations were detected.

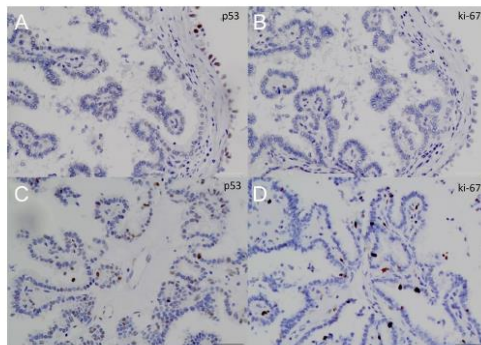


Figure 5
Immunohistochemical analysis of p53 expression and Ki-67 index in LNM present at diagnosis (A and B, respectively) and in brain metastases (C and D, respectively). (A) p53 staining was present in the hobnail thyrocytes that lined the early LNM while being negative in the remaining of the lesion. (B) The hobnail variant presented to be negative for Ki-67 staining. (C) p53 staining was weak and rather disperse in the brain metastasis; however, some areas with more frequent p53-positive cells were found, as the one depicted. (D) Brain metastases showed higher Ki-67 index, in which <10% Ki-67-positive cells were homogeneously present in all the lesion.

Discussion

According to the existing literature, PMCs have mild biological behavior and good prognosis in most patients (5). It is important to distinguish asymptomatic (or incidental) PMC, that carry an excellent prognosis and may undergo active surveillance, from clinically recognized PMCs with LNM at diagnosis, whose prognosis is similar to PTC >1 cm and are not indicated for active surveillance (5). Clinical features that associate with increased risk of adverse outcome remain controversial. Size larger than 5–7 mm has been associated with increased rate of LNM and increased recurrence (11). The presence of clinical LNM at initial presentation is recognized as an important prognostic factor that may lead to a more aggressive course (5). In PMCs, distant metastases are rarely reported (7), and only a minority of that correspond to brain metastases (0.8–1.3%) that carry a worse outcome (9).

Nearly 25% of PMCs develop local LNM recurrence, but rarely develop distant metastases (0.5%) (7). Therefore, it is of major importance to maintain follow-up of patients who present tumors with aggressive features. TGB is used in thyroid carcinoma surveillance as the primary biochemical marker for patient monitoring (17). TGB serum level is high in almost all metastatic thyroid disease, helping in cancer progression detection (18). Sometimes TGB level is the initial sign indicating secondary disease and leading to local or distant metastasis diagnosis (Fig. 1). In this case the patient did not evidence clinical or imaging disease, at the first year evaluation after surgery. At this time, stimulated TGB was <1 mg/dL (excellent response), leading toward a favorable outcome (17). At the second year of follow-up, TGB levels started to increase. In 2016, TGB level reached incomplete biochemical response level, which led to a cervical mass diagnosis in PET-CT scan, confirming the presence of locoregional recurrence. According to ATA classification (17), disease recurrence is identified in 67–75% of high-risk patients and high-risk features may harbor a recurrence risk more than 10 years after initial treatment (17, 19). According to existing studies, persistence is more frequent and associated with a worse outcome than recurrence (17, 19, 20).

The primary tumor in this case was a central parenchymal 2 mm follicular variant of PTC, which presented together some high-risk features, namely, age at diagnosis (61 years), male gender, extensive LNM at presentation with largest LNM >30 mm, and extranodal extension. Central parenchymal PMCs <5 mm have been considered as the most indolent ones. It has been described that the LNM size has a better correlation with the outcome than primary tumor size (5).

We consider that histological subtyping of primary tumor and LNM is important, since aggressive variants have worse prognosis (21). The heterogeneous histology demonstrating classic, hobnail features along with follicular variant of PTC present in the initial LNM may highlight the potential locoregional recurrence and distant metastasization. PMCs with distant metastasis are rarely reported. We performed a literature search regarding this subject since 2010 and realized that distant metastasis in PMCs was reported in 24 patients and brain metastases was reported in only five patients (Table 2).

Particularly, some biological commonalities have been shown in both poorly differentiated thyroid carcinoma and hobnail variant of PTC, owing to a shared morphological formation 'hobnail pattern' in both types of tumors. This supports the idea of previous studies, that presence of this pattern inside the tumor might facilitate 'epithelial-to-mesenchymal transition' mechanism, which in turn leads to tumor progression, local invasion, and distant metastasis, as in the current case (33), underlined by the recently published literature (3, 21).

Some authors propose the use of Ki-67 index and/or p53 immunostaining as an indicator of high-risk thyroid carcinomas, like in other cancers (34). In our

results, p53 presented a faint and limited expression, compatible with a wild-type status, except in the hobnail component where we noted a more intense and widespread expression of p53, as previously described (34). The Ki-67 index was very low in synchronous LNM, increasing in recurrent lesions. It was advanced that the combination of TERT promoter/BRAFV600E mutations and Ki-67 may be clinically useful predicting PTC recurrence (34).

Molecular analysis in PMCs is still under debate. Nevertheless, aggressive tumor behavior can be associated with recently investigated biological characteristics. The alteration more often presents in PMC, associated with locoregional recurrence, is BRAFV600E mutation (14). BRAF mutations are detected in nearly 50% of PTC, but only 10% of these cases have unfavorable clinical outcomes (13, 14). This mutation itself does not contribute to the increase in risk mortality and should be considered in the context of high-risk features (17). BRAFV600E mutation, associated with some clinicopathological risk factors, has shown to increase the risk of disease recurrence in both classical PTCs and PMCs (17). More recently described, the presence of TERTp mutation confers an unfavorable outcome in thyroid cancer (12). TERTp mutations are more frequent

Table 2 Clinicopathological features of PMC distant metastasis reported cases.

No	Study	Age (years), gender	Tumor size (cm)	Histological variant	pN	Metastatic site
1	Lecumberi <i>et al.</i> (22)	65, F	0.2	Sclerosing	–	Brain
2	Xu <i>et al.</i> (23)	46, F	0.3	Follicular	+	Brain, lung
3	Saito <i>et al.</i> (24)	70, F	0.8	NA	–	Lung
4	Kozu <i>et al.</i> (25)	70, M	NA	Classical	NA	Lung
5	Zheng <i>et al.</i> (26)	53, F	0.6	NA	–	Bone
6	Kaseda <i>et al.</i> (27)	66, F	NA	Infiltrative follicular	NA	Lung
7	Kawai <i>et al.</i> (28)	70, M	1	NA	+	Lung
8	Jeon <i>et al.</i> 2016 (29)	51, F	0.8	Classical	+	Lung
9	Jeon <i>et al.</i> (29)	31, F	0.9	Infiltrative follicular	+	Lung
10	Jeon <i>et al.</i> (29)	55, F	0.9	Tall cell	+	Bone, lung
11	Jeon <i>et al.</i> (29)	59, F	1	Classical	+	Bone, lung
12	Jeon <i>et al.</i> (29)	73, F	1	Classical	+	Lung
13	Jeon <i>et al.</i> (29)	54, F	0.8	Classical	+	Lung
14	Jeon <i>et al.</i> (29)	63, F	0.8	Classical	+	Bone, lung
15	Jeon <i>et al.</i> (29)	46, F	0.7	Infiltrative follicular	+	Bone, lung
16	Jeon <i>et al.</i> (29)	65, M	0.6	Columnar cell	+	Brain, lung
17	Jeon <i>et al.</i> (29)	58, F	0.6	Solid	+	Brain, lung
18	Jeon <i>et al.</i> (29)	60, F	0.8	Classical	+	Bone, lung
19	Jeon <i>et al.</i> (29)	63, F	0.9	Classical	+	Bone, lung
20	Hu <i>et al.</i> (30)	70, F	0.8	NA	–	Lung
21	Hu <i>et al.</i> (30)	29, F	0.9	NA	–	Lung
22	Hitu <i>et al.</i> (31)	58, F	NA	Classical	–	Muscle
23	Shawky <i>et al.</i> (32)	59, M	0.3	Follicular	–	Brain
24	Shimizu <i>et al.</i> (7)	64, M	0.9	Classical	–	Lung

NA, not available; pN, pathological cervical lymph node metastases.

in older, male patients with local recurrent disease and presenting distant metastases and have been defined as a late event in thyroid tumorigenesis adding a functional advance toward PMC aggressiveness (12). In the present case, it was not possible to analyze the primary tumor. However, we verified that the LNM samples retrieved at diagnosis only presented BRAFV600E mutation. Notably recurrent LNM and brain metastasis acquired TERTp mutation, in line with the role of TERTp in thyroid cancer progression. There are data regarding an association between the coexistence of these two mutations and clinically aggressive disease (14, 15). The prevalence of BRAF and TERTp mutations coexistence is 7.7% and has revealed to increase the risk of locoregional recurrence and development of distant metastases (13, 35). Currently, there are no risk stratification systems that encompass tumors' molecular profile (17). Thus, the presence of TERTp mutation is emerging as an independent predictor of mortality (12, 17). In this case, the coexistence of BRAFV600E and TERTp mutations added a worse prognosis that is consistent with recent published studies (12, 13, 14, 15, 35, 36).

The present case highlights the important role of several high-risk factors paradoxically in a very small PTC: man, age >55, extensive large and locally invasive LNM at presentation, coexistence of PTC variants associated to guarded prognosis in LNM and brain metastases and TERTp mutation. Altogether, the aforementioned factors highlight the importance of patient stratification even when the primary tumor is a PMC. Patients with high recurrence risk may benefit from analysis of oncogenic driver mutation (namely, BRAFV600E and TERTp mutation) as an adjunct to recognize disease recurrence earlier and initiate systemic therapy. This case provides evidence to stress the interest of studying morphologically, immunohistochemically and molecularly subcentimetric PTCs in order to progress in the prognosis of challenging cases.

Declaration of interest

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

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Patient consent

Every effort was made to contact the next of kin of the deceased patient to obtain consent but in vain. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of CHVNG/E (Project investigation 30/2016, January 28, 2016, Comissão de Ética do Centro Hospitalar de Vila Nova de Gaia/Espinho).

Author contribution statement

A Amado: conceptualization, writing (original draft preparation); E Teixeira: methodology, formal analysis, investigation; S Canberk: methodology, formal analysis, investigation; S Macedo: methodology, formal analysis; B Castro: data curation; H Pereira: data curation; J Varanda: data curation; S Graça: named physician of the patient, supervision; A Tavares: supervision; C Soares: named physician of the patient, supervision; M J Oliveira: named physician of the patient, supervision; M Oliveira: supervision; P Soares: writing (review and editing, resources, project administration, funding acquisition, supervision; M S Simões: conceptualization, writing (review and editing), supervision; A A Pova: conceptualization, writing (review and editing), named physician of the patient, supervision. All authors read and agreed to the final version of the manuscript.

References

- 1 Pazaitou-Panayiotou K, Kaprara A, Chrisoulidou A, Boudina M, Georgiou E, Patakliouta F, Drimonitis A & Vainas I. Cerebellar metastasis as first metastasis from papillary thyroid carcinoma. *Endocrine Journal* 2005 **52** 653–657. (<https://doi.org/10.1507/endocrj.52.653>)
- 2 Haddad RI, Nasr C, Bischoff L, Busaidy NL, Byrd D, Callender G, Dickson P, Duh Q-Y, Ehya H & Goldner W. NCCN guidelines insights: thyroid carcinoma, version 2.2018. *Journal of the National Comprehensive Cancer Network* 2018 **16** 1429–1440. (<https://doi.org/10.6004/jnccn.2018.0089>)
- 3 Donaldson LB, Yan F, Morgan PF, Kaczmar JM, Fernandes JK, Nguyen SA, Jester RL & Day TA. Hobnail variant of papillary thyroid carcinoma: a systematic review and meta-analysis. *Endocrine* 2021 **72** 27–39. (<https://doi.org/10.1007/s12020-020-02505-z>)
- 4 Filetti S, Durante C, Hartl D, Leboulleux S, Locati LD, Newbold K, Papotti MG & Berruti A. A Thyroid cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology* 2019 **30** 1856–1883. (<https://doi.org/10.1093/annonc/mdz400>)
- 5 Miyauchi A, Ito Y & Oda H. Insights into the management of papillary microcarcinoma of the thyroid. *Thyroid* 2018 **28** 23–31. (<https://doi.org/10.1089/thy.2017.0227>)
- 6 Soares P, Celestino R, Gaspar da Rocha A & Sobrinho-Simões M. Papillary thyroid microcarcinoma: how to diagnose and manage this epidemic? *International Journal of Surgical Pathology* 2014 **22** 113–119. (<https://doi.org/10.1177/1066896913517394>)
- 7 Shimizu T, Oba T, Chino T, Soma A, Ono M, Ito T, Kanai T, Maeno K, Sato Y & Uehara T. Papillary thyroid microcarcinoma with lung metastases: a case report and review of the literature. *Thyroid Research* 2021 **14** 1–7. (<https://doi.org/10.1186/s13044-021-00106-0>)
- 8 Tsuda K, Tsurushima H, Takano S, Tsuboi K & Matsumura A. Brain metastasis from papillary thyroid carcinomas. *Molecular and Clinical Oncology* 2013 **1** 817–819. (<https://doi.org/10.3892/mco.2013.139>)
- 9 Aguiar PH, Agner C, Tavares FR & Yamaguchi N. Unusual brain metastases from papillary thyroid carcinoma: case report. *Neurosurgery* 2001 **49** 1008–1013. (<https://doi.org/10.1097/00006123-200110000-00044>)

- 10 Cunha Rodrigues AC, Penna G, Rodrigues E, Castro P, Sobrinho-Simoes M & Soares P. The genetics of papillary microcarcinomas of the thyroid: diagnostic and prognostic implications. *Current Genomics* 2017 **18** 244–254. (<https://doi.org/10.2174/1389202918666170105094459>)
- 11 Ghossein R, Ganly I, Biagini A, Robenshtok E, Rivera M & Tuttle RM. Prognostic factors in papillary microcarcinoma with emphasis on histologic subtyping: a clinicopathologic study of 148 cases. *Thyroid* 2014 **24** 245–253. (<https://doi.org/10.1089/thy.2012.0645>)
- 12 De Biase D, Gandolfi G, Ragazzi M, Eszlinger M, Sancisi V, Gugnoni M, Visani M, Pession A, Casadei G, Durante C, et al. Tert promoter mutations in papillary thyroid microcarcinomas. *Thyroid* 2015 **25** 1013–1019. (<https://doi.org/10.1089/thy.2015.0101>)
- 13 Rusinek D, Pfeifer A, Cieslicka M, Kowalska M, Pawlaczek A, Krajewska J, Szpak-Ulcok S, Tyszkiewicz T, Halczok M, Czarniecka A, et al. Tert promoter mutations and their impact on gene expression profile in papillary thyroid carcinoma. *Cancers* 2020 **12** 1597. (<https://doi.org/10.3390/cancers12061597>)
- 14 Melo M, Gaspar da Rocha A, Batista R, Vinagre J, Martins MJ, Costa G, Ribeiro C, Carrilho F, Leite V, Lobo C, et al. TERT, BRAF, and NRAS in Primary Thyroid Cancer and Metastatic Disease. *Journal of Clinical Endocrinology and Metabolism* 2017 **102** 1898–1907. (<https://doi.org/10.1210/jc.2016-2785>)
- 15 Póvoa AA, Teixeira E, Bella-Cueto MR, Batista R, Pestana A, Melo M, Alves T, Pinto M, Sobrinho-Simões M, Maciel J, et al. Genetic determinants for prediction of outcome of patients with papillary thyroid carcinoma. *Cancers* 2021 **13** 2048. (<https://doi.org/10.3390/cancers13092048>)
- 16 Kim HI, Kim K, Park SY, Choe JH, Kim JH, Kim JS, Oh YL, Hahn SY, Shin JH, Ahn HS, et al. Refining the eighth edition AJCC TNM classification and prognostic groups for papillary thyroid cancer with lateral nodal metastasis. *Onco Oncology* 2018 **78** 80–86. (<https://doi.org/10.1016/j.oncoloncology.2018.01.021>)
- 17 Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, Pacini F, Randolph GW, Sawka AM, Schlumberger M, et al. 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer. *Thyroid* 2016 **26** 1–133. (<https://doi.org/10.1089/thy.2015.0020>)
- 18 Misaki T, Iwata M, Kasagi K & Konishi J. Brain metastasis from differentiated thyroid cancer in patients treated with radioiodine for bone and lung lesions. *Annals of Nuclear Medicine* 2000 **14** 111–114. (<https://doi.org/10.1007/BF02988589>)
- 19 Dong W, Horiuchi K, Tokumitsu H, Sakamoto A, Noguchi E, Ueda Y & Okamoto T. Time-varying pattern of mortality and recurrence from papillary thyroid cancer: lessons from a long-term follow-up. *Thyroid* 2019 **29** 802–808. (<https://doi.org/10.1089/thy.2018.0128>)
- 20 Sapuppo G, Tavarelli M, Belfiore A, Vigneri R & Pellegri G. Time to separate persistent from recurrent differentiated thyroid cancer: different conditions with different outcomes. *Journal of Clinical Endocrinology and Metabolism* 2019 **104** 258–265. (<https://doi.org/10.1210/jc.2018-01383>)
- 21 Zhi J, Zhao J, Gao M, Pan Y, Wu J, Li Y, Li D, Yu Y & Zheng X. Impact of major different variants of papillary thyroid microcarcinoma on the clinicopathological characteristics: the study of 1041 cases. *International Journal of Clinical Oncology* 2018 **23** 59–65. (<https://doi.org/10.1007/s10147-017-1170-6>)
- 22 Lecumberri B, Alvarez-Escóla C, Martín-Vaquero P, Nistal M, Martín V, Riesco-Eizaguirre G, Sosa G & Pallardo LF. Solitary hemorrhagic cerebellar metastasis from occult papillary thyroid microcarcinoma. *Thyroid* 2010 **20** 563–567. (<https://doi.org/10.1089/thy.2010.0062>)
- 23 Xu YH, Song HJ, Qiu ZL & Luo QY. Brain metastases with exceptional features from papillary thyroid carcinoma: report of three cases. *Hellenic Journal of Nuclear Medicine* 2011 **14** 56–59.
- 24 Saito G, Sakaizawa T, Yamada K, Arimura T, Nishimura H & Hosaka N. A case of minimal thyroid carcinoma diagnosed by a solitary pulmonary nodule. *The Shinshu Medical Journal* 2011 **59** 89–95.
- 25 Kozu Y, Futagawa T & Suzuki K. Pulmonary metastases from colon cancer and occult thyroid cancer in the same lobe. *Kyobu Geka. Japanese Journal of Thoracic Surgery* 2014 **67** 448–451. (<https://doi.org/10.53347/rid-30460>)
- 26 Zheng W, Tan J & Zhang G. Extensive bone metastases as the initial symptom of papillary thyroid microcarcinoma: A case report. *Experimental and Therapeutic Medicine* 2015 **9** 2104–2108. (<https://doi.org/10.3892/etm.2015.2423>)
- 27 Kaseda K, Watanabe K, Sakamaki H & Kazama A. Solitary pulmonary metastasis from occult papillary thyroid carcinoma. *Thoracic Cancer* 2016 **7** 261–263. (<https://doi.org/10.1111/1759-7714.12295>)
- 28 Kawai H, Sugimoto R, Iga N, Ikeda H, Yoshida R, Waki N, Ishizaki M, Nishi H & Yamashita K. A case of minimal thyroid carcinoma diagnosed by a solitary pulmonary metastasis. *Gan To Kagaku Ryoho. Cancer and Chemotherapy* 2016 **43** 2127–2129.
- 29 Jeon MJ, Kim WG, Choi YM, Kwon H, Lee YM, Sung TY, Yoon JH, Chung KW, Hong SJ, Kim TY, et al. Features predictive of distant metastasis in papillary thyroid microcarcinomas. *Thyroid* 2016 **26** 161–168. (<https://doi.org/10.1089/thy.2015.0375>)
- 30 Hu R, Xu G, Stricker T, Li B, Weiss VL & Bischoff L. Incidental pulmonary metastases revealing subcentimeter papillary thyroid carcinoma. *AACE Clinical Case Reports* 2020 **6** e273–e278. (<https://doi.org/10.4158/ACCR-2020-0051>)
- 31 Hitt L, Cainap C, Apostu D, Gabor A, Bonci EA, Badan M, Mester A & Piciu A. Skeletal muscle metastasis in papillary thyroid microcarcinoma evaluated by F18-FDG PET/CT. *Diagnostics* 2020 **10** 100. (<https://doi.org/10.3390/diagnostics10020100>)
- 32 Shawky M, Proctor I, Kurzwinski T & Abdel-Aziz T. Papillary thyroid microcarcinoma presenting as a metastasis to the brain. *Annals of the Royal College of Surgeons of England* 2020 **102** e107–e110. (<https://doi.org/10.1308/rcsann.2020.0041>)
- 33 Amacher AM, Goyal B, Lewis Jr JS, El-Mofly SK & Chernock RD. Prevalence of a hobnail pattern in papillary, poorly differentiated, and anaplastic thyroid carcinoma: a possible manifestation of high-grade transformation. *American Journal of Surgical Pathology* 2015 **39** 260–265. (<https://doi.org/10.1097/PAS.0000000000000329>)
- 34 Matsue M, Yabuta T, Saenko V, Hirokawa M, Nishihara E, Suzuki K, Yamashita S, Miyauchi A & Mitsutake N. Tert promoter mutations and Ki-67 labeling index as a prognostic marker of papillary thyroid carcinomas: combination of two independent factors. *Scientific Reports* 2017 **7** 1–8. (<https://doi.org/10.1038/srep41752>)
- 35 Xing M, Liu R, Liu X, Murugan AK, Zhu G, Zeiger MA, Pai S, Bishop J & BRAF V. BRAF V600E and tert promoter mutations cooperatively identify the most aggressive papillary thyroid cancer with highest recurrence. *Journal of Clinical Oncology* 2014 **32** 2718–2726. (<https://doi.org/10.1200/JCO.2014.55.5094>)
- 36 Liu R & Xing M. Tert promoter mutations in thyroid cancer. *Endocrine-Related Cancer* 2016 **23** R143–R155. (<https://doi.org/10.1530/ERC-15-0533>)

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The genetics behind non-syndromic FNMTC is rather heterogeneous and poorly understood. Many affected families remain without an identified genetic risk locus. The establishment of molecular markers would lead to accurate genetic counseling and the development of new therapies. Disease prevention and active surveillance are key factors for reducing aggressive treatments, high comorbidities, and invasive surgeries, besides avoiding high burden for the health care system.

Our team employed WES in two families (Family C and Family R) presenting with FNMTC. Although both families comprised the criteria for TC heredity (>3 first-degree relatives affected by the disease and diagnosis at early age), no syndromic forms of the disease were detected. Therefore, our team, aiming to explore the pathogenesis and genetics of NSFNMTC, by WES analysis, identified in these families two novel germline, possibly pathogenic, mutations in the *USP42* gene in family R and in the *KCNB2* gene in family C.

4.5. ARTICLE V

Family R is composed of a mother, diagnosed with TC at the age of 35 and two monozygotic twin daughters both diagnosed at the age of 19. Our team detected the *p.(Gly486Arg)* heterozygous missense mutation in exon 13 of the *USP42* gene that segregates within the family. Literature findings relating *USP42* and other similar *USPs* with cancer are strong. Its role in cell biology and more particularly in cell cycle regulation has been particularly noted. This data supports our hypothesis that *USP42* may be underlying cause of disease in Family R, which motivated our search for its validation through *in vitro* functional assays. To our knowledge, no research has been done on *USP42*'s biological role and expression pattern in FNMTC. The results of this study will be presented in **ARTICLE V**. Subsequent ongoing studies on *USP42* gene will be presented in section **4.6 - *USP42* gene knockout synthesis using CRISPR-RNP editing technique (in development)**.



Article

Investigating *USP42* Mutation as Underlying Cause of Familial Non-Medullary Thyroid Carcinoma

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Abstract: In a family with Familial Non-Medullary Thyroid Carcinoma (FNMTc), our investigation using Whole-Exome Sequencing (WES) uncovered a novel germline *USP42* mutation [*p.(Gly486Arg)*]. *USP42* is known for regulating p53, cell cycle arrest, and apoptosis, and for being reported as overexpressed in breast and gastric cancer patients. Recently, a *USP13* missense mutation was described in FNMTc, suggesting a potential involvement in thyroid cancer. Aiming to explore the *USP42* mutation as an underlying cause of FNMTc, our team validated the mutation in blood and tissue samples from the family. Using immunohistochemistry, the expression of *USP42*, Caspase-3, and p53 was assessed. The *USP42* gene was silenced in human thyroid Nthy-Ori 3-1 cells using siRNAs. Subsequently, expression, viability, and morphological assays were conducted. p53, Cyclin D1, p21, and p27 proteins were evaluated by Western blot. *USP42* protein was confirmed in all family members and was found to be overexpressed in tumor samples, along with an increased expression of p53 and cleaved Caspase-3. siRNA-mediated *USP42* downregulation in Nthy-Ori 3-1 cells resulted in reduced cell viability, morphological changes, and modifications in cell cycle-related proteins. Our results suggest a pivotal role of *USP42* mutation in thyroid cell biology, and this finding indicates that *USP42* may serve as a new putative target in FNMTc.

Keywords: hereditary cancer; familial thyroid cancer; Familial Non-Medullary Thyroid Carcinoma; molecular studies; ubiquitination

1. Introduction

Thyroid cancer (TC) incidence has increased substantially over the last three decades [1]. Up to 10% of TC cases encompass familial forms [2]. When arising from thyroid follicular cells, familial cases of thyroid carcinoma are designated as Familial Non-Medullary Thyroid Carcinoma (FNMTc) [3]. FNMTcs are further subdivided into syndromic FNMTc (SFNMTc) and non-syndromic FNMTc (NSFNMTc). In SFNMTc, patients exhibit a

predominance of non-thyroid tumors alongside thyroid cancer, and several genetic alterations associated with syndromic forms have been well described (e.g., APC, PTEN, and *DICER1*) [4,5]. NSFNMTc patients present with TC as the main clinical manifestation, comprising a very heterogeneous disorder, both at the phenotypic and genotypic levels, which is usually coupled with various low- to moderate-penetrance susceptibility alleles (e.g., *FOXE1* and *TTF-1*), and incomplete penetrance [6,7]. A polygenic pattern was suggested but many questions remain unanswered [5,8,9].

Our team identified a family with FNMTc. The family was composed of a mother and two monozygotic twin daughters with an early-onset diagnosis of Oncocytic Subtype of Papillary Thyroid Carcinoma (OSPTC). The family fulfilled the criteria for hereditary cancer, but no alterations associated with syndromic forms of the disease were found. Using Whole-Exome Sequencing (WES), our team identified a possible disease-causative mutation in a Ubiquitin-Specific Protease gene [*USP42* p.(Gly486Arg)].

Many USPs have been put forward as regulators of tumor formation and proliferation, mainly because they are involved in cell cycle progression, regulation of apoptosis-related factors, DNA repair activity, and other important cellular functions [10–12]. *USP42*, located on chromosome 7 (7p22.1), was shown to regulate cellular metabolism through p53–histone H2B interaction in cell lines by Hock et al. (2011) and Hock et al. (2014) [13,14].

To our knowledge, no prior studies investigating the role of *USP42* gene alterations in NSFNMTc have been performed; therefore, we decided to evaluate *USP42* mutation as an underlying cause of NSFNMTc.

2. Results

2.1. Case Report

Our team identified a family presenting with NSFNMTc. The family was composed of a mother (I), diagnosed at the age of 35, and of two monozygotic twin daughters both diagnosed at the age of 19 (II.1 and II.2) (Figure 1A). The histological lesions of the three cases were similar, all presenting the same diagnosis of OSPTC (Figure 1A). The mother, in addition to displaying OSPTC, also presented several lesions of Follicular Nodular Disease (FND) comprising oncocytic cells. FND lesions were also present in the surgical specimens from the twins.

2.2. Molecular Studies

2.2.1. *USP42* Missense Mutation Validation

In silico analysis of the normal DNA extracted from the three affected individuals was subjected to WES (Figure 1A,B), which revealed the presence of an extremely rare Single-Nucleotide Polymorphisms (SNP) affecting the *USP42* gene (Table 1). Although this is a variant of unknown significance according to ClinVar, it shows a predicted CADD score over 20, meaning that this SNP is predicted to be a damaging substitution (Table 1). After confirming the presence of this SNP on IGV (Integrative Genomics Viewer), PCR followed by Sanger sequencing was performed using DNA from blood samples and from FFPE (Formalin-Fixed Paraffin-Embedded) samples of tumoral and non-tumoral tissues (Figure 1C,D; Table 1). The *USP42* p.(Gly486Arg) mutation was present in all analyzed samples of the mother (I, proband) and of the monozygotic twin daughters (II.1 and II.2) in heterozygosity (Figure 1D; Figure S1).

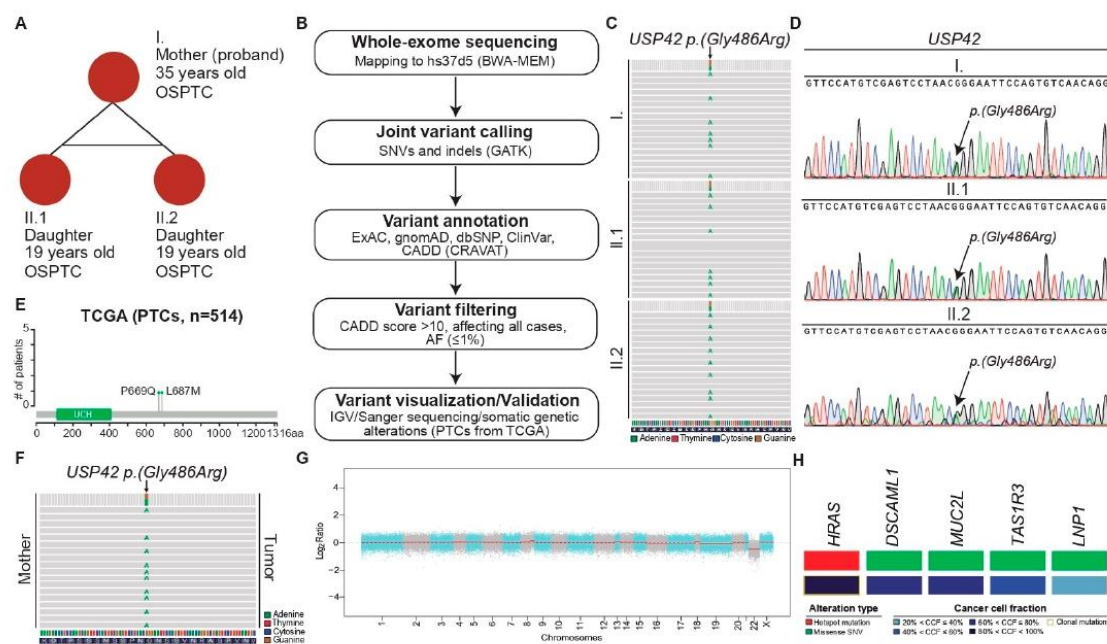


Figure 1. Case description and in silico analysis of non-syndromic FNMTc patients subjected to Whole-Exome Sequencing. **(A)** Family composed of a mother and two monozygotic twin daughters diagnosed with Oncocytic Subtype of Papillary Thyroid Carcinoma (OSPTC). Diagnosis at young age in the three elements of the family and in the absence of syndromic forms of the disease, falling into the category of Non-Syndromic Familial Non-Medullary Thyroid Carcinoma (NSFNMTc). **(B)** Sequencing analysis strategy for the detection of rare, potentially pathogenic Single-Nucleotide Polymorphisms (SNPs) affecting the three individuals included in this study. **(C)** Sequencing reads in Integrative Genomics Viewer showing the *USP42* p.(Gly486Arg) variant detected in the three individuals. **(D)** Representative Sanger electropherograms showing the G > A alteration in the normal DNA taken from the mother (top) and the twin daughters (middle and bottom). **(E)** Lollipop plots depicting the frequencies of the identified somatic mutations affecting *USP42* in 514 Papillary Thyroid Carcinomas subjected to Whole-Exome Sequencing that were selected from the TCGA database. UCH—ubiquitin carboxyl-hydrolase. **(F)** Sequencing reads in Integrative Genomics Viewer showing the *USP42* p.(Gly486Arg) variant detected in the tumor DNA of the mother. **(G)** Copy number plot of the tumor DNA of the mother. The log-ratios are plotted on the y-axis according to genomic positions (x-axis). Chromosomes are depicted by alternating blue and red bands. **(H)** Heatmaps depicting non-synonymous somatic mutations (top) and cancer cell fractions (bottom) in the tumor DNA of the mother. Mutations and cancer cell fractions are color coded according to the legend.

Table 1. Candidate SNP identified in the three affected individuals included in this study.

¹ ID	Position (Chr. 7)	Alternate Allele	Gene	Mutation Type	Amino Acid Change	gnomAD AF Total	dbSNP	CADD	ClinVar
I	6189283	G > A	<i>USP42</i>	Missense	p.(Gly486Arg)	0.0004582	rs200908439	23.6	³ US
II.1	6189283	G > A	<i>USP42</i>	Missense	p.(Gly486Arg)	0.0004582	rs200908439	23.6	US
II.2	6189283	G > A	<i>USP42</i>	Missense	p.(Gly486Arg)	0.0004582	rs200908439	23.6	US

¹ ID—Identification; ² Chr.—Chromosome; ³ US—Uncertain Significance.

When looking at mutations affecting the *USP42* gene in 514 PTCs subjected to WES that were selected from the TCGA database, two missense mutations were identified, one

of which is pathogenic [*USP42 p.(Pro669Gln)*] (Figure 1E; Figure S1; Table S1). We expanded our analysis and looked at PTC cases harboring pathogenic somatic alterations affecting any genes of the USP family. Of the 17 PTC cases found to have genetic alterations in USPs, 24% and 16% had mutations affecting *USP9X* and *USP6*, respectively. In addition, 76% of these 17 PTCs also harbored *BRAF* hotspot mutations (Figure S1; Table S1).

The tumor sample from the mother subjected to WES had a similar variant-allele frequency of the *USP42 p.(Gly486Arg)* SNP to the one observed in the matched normal sample (Figure 1F). CNA analysis revealed only a loss of chromosome 22 (Figure 1G). When looking at the somatic mutations, an *HRAS p.(Gln61Arg)* hotspot mutation was identified (Figure 1H; Table 2).

Table 2. Somatic mutations detected in the tumor DNA subjected to Whole-Exome Sequencing.

¹ ID	² Chr.	Position	Alternate Allele	Gene	Mutation Type	Amino Acid Change	Alt. Reads	Ref. Reads	Tumor ³ MAF	Tumor Depth	Cancer Cell Fraction	Clonal Status
I	11	533874	T > C	<i>HRAS</i>	Missense	<i>p.(Gln61Arg)</i>	171	68	0.2845	239	0.93	Clonal
I	3	100170600	A > T	<i>LNP1</i>	Missense	<i>p.(His78Leu)</i>	37	5	0.119	42	0.39	Subclonal
I	1	1269225	G > A	<i>TAS1R3</i>	Missense	<i>p.(Cys647Tyr)</i>	165	25	0.1316	190	0.43	Subclonal
I	6	30954414	C > G	<i>MUC21</i>	Missense	<i>p.(Asp154Glu)</i>	39	9	0.1875	48	0.61	Subclonal
I	11	117299237	G > A	<i>DSCAML1</i>	Missense	<i>p.(Pro2050Leu)</i>	24	7	0.2258	31	0.74	Subclonal

¹ ID—Identification; ² Chr.—Chromosome; ³ MAF— Minor Allele Frequency.

2.2.2. Immunohistochemistry

FFPE tissues from the affected individuals were assessed to observe tissue expression of USP42. We observed that USP42 protein was overexpressed in all tumoral tissues from all tested individuals (Figure 2B,D,F). Adjacent tissues also presented high expression of the protein, thus suggesting that *USP42* mutation may result in protein overexpression (Figure 2A,C,E).

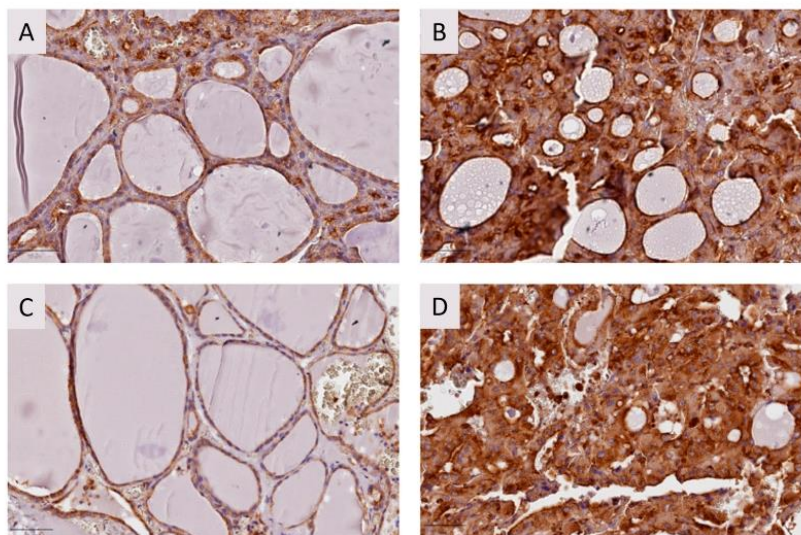


Figure 2. Cont.

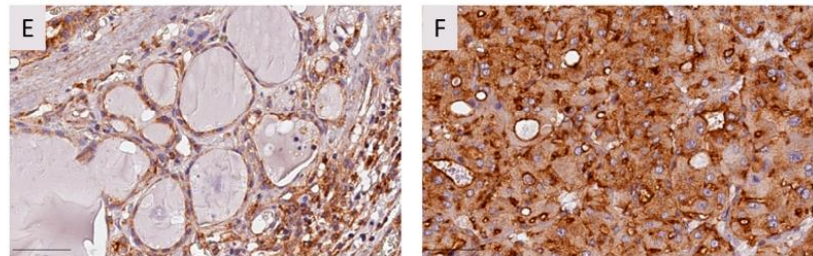


Figure 2. USP42 analysis by immunohistochemistry for FFPE tissues from the affected individuals. Adjacent (A) and tumor tissue (B) from the proband (I); adjacent (C) and tumor tissue (D) from twin II.1; and adjacent (E) and tumor tissue (F) from twin II.2. USP42 is overexpressed in the analyzed tumor tissues compared to adjacent tissue in the three individuals. Staining intensity was evaluated by semi-quantitative analysis. Bar: 50 μ m.

As for the other markers analyzed, we observed that Caspase-3 (Figure 3) and p53 (Figure 4) proteins presented tumor overexpression in all tested samples.

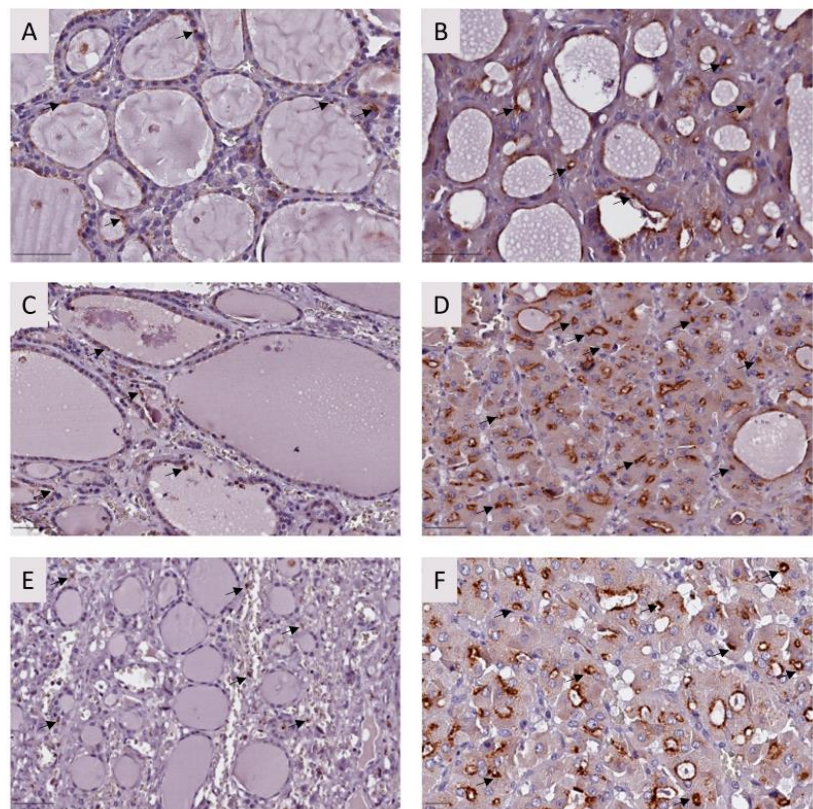


Figure 3. Caspase-3 analysis by immunohistochemistry for FFPE tissues from the affected individuals. Adjacent (A) and tumor tissue (B) from the proband (I); adjacent (C) and tumor tissue (D) from twin II.1;

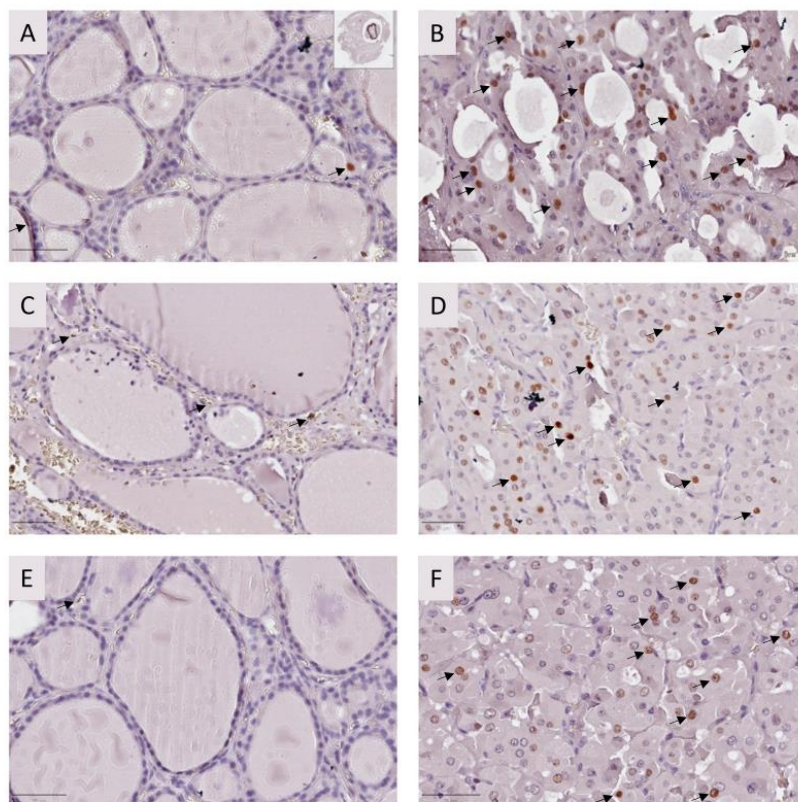


Figure 4. p53 analysis by immunohistochemistry for FFPE tissues from the affected individuals. Adjacent (A) and tumor tissue (B) from the proband (I); adjacent (C) and tumor tissue (D) from twin II.1; and adjacent (E) and tumor tissue (F) from twin II.2. p53 is overexpressed in the analyzed tumor tissues compared to adjacent tissue in the three individuals. Black arrows indicate positive cells. Bar: 50 μ m.

Quantification of p53 showed that the protein was overexpressed in the tumor tissues compared to adjacent tissues and to non-tumoral tissue from the proband (2.71% p53 positive cells) (Table 3; Figure 4). Tumor lesions of the three elements presented a range of 26.33–57.56% p53-positive cells in the available fragments, while adjacent tissue presented 0.75–10.65% p53-positive cells in the available fragments (Table 3; Figure 4).

Table 3. p53 quantification in different lesions from the three family elements.

¹ ID	Sample	Lesion	Total (N)	p53 (+)	p53 (–)	Ratio (%)
I	Sample 1	Tumor	1084	624	460	57.56
		Adjacent tissue	1026	67	959	6.53
	Sample 2	² FND	1069	29	1040	2.71
II.1	Sample 3	Tumor	1092	453	639	41.48
		Adjacent tissue	1005	107	898	10.65
	Sample 4	Tumor	1094	288	806	26.33
		Adjacent tissue	1071	8	1063	0.75
II.2	Sample 5	Tumor	1006	536	470	53.28
		Adjacent tissue	1017	14	1003	1.38
	Sample 6	Tumor	902	431	471	47.78
		Adjacent tissue	1027	48	979	4.67

At least 1000 cells were counted in each field. Quantification was performed in the areas that presented higher expression of p53. ¹ ID—Identification; ² FND—Follicular Nodular Disease.

2.2.3. USP42 Silencing and Analysis of mRNA and Protein Expression

We successfully silenced *USP42* using two different siRNA treatments, siRNA 2 and siRNA 7, which were evaluated for mRNA expression using RT-qPCR with the $2^{-\Delta\Delta Ct}$ method (Figure 5A). At the 24 h timepoint, siRNA 2 and siRNA 7 treatments each had a significant silencing effect on the gene (60%) when compared to a negative control ($p < 0.0001$ for both treatments). At the 48 h timepoint, the silencing effect (60%) was maintained for both treatments ($p = 0.0001$ and $p = 0.0003$, respectively). However, at 72 h, a recuperation of *USP42* gene expression was observed, but silencing effects of 51% and 45% were still detected for siRNA 2 ($p = 0.0002$) and siRNA 7 ($p = 0.004$), respectively (Figure 5A). *USP42* protein expression was evaluated by Western blot in these same treatments. We did not observe protein expression alterations at 24 h or 48 h timepoints (Figure 5B). However, at the 72 h timepoint, *USP42* protein silencing was significant using the siRNA 7 treatment, with a 32% reduction in protein expression compared to the negative control ($p = 0.008$). No significant changes were observed for protein expression using siRNA 2 for up to 72 h post-treatment.

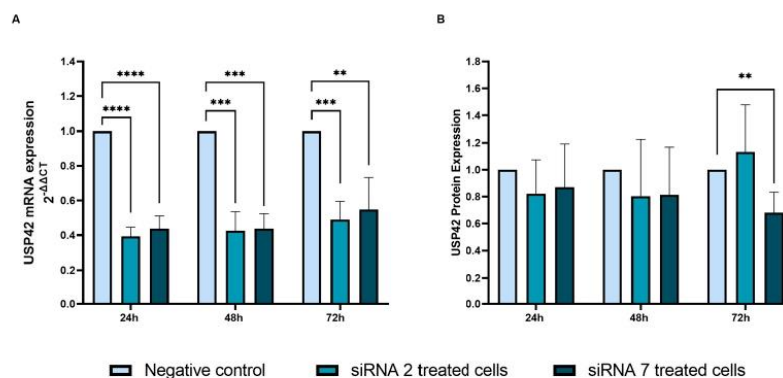


Figure 5. (A) *USP42* mRNA expression in Nthy Ori 3-1 cells after siRNA treatment. *USP42* mRNA expression was decreased with a maximal 60% silencing effect in the Nthy Ori 3-1 cell line using siRNA treatments. (B) *USP42* protein expression following siRNA treatment. Protein silencing was significant at the 72 h timepoint in the Nthy Ori 3-1 cell line following siRNA 7 treatment ($p = 0.008$). Each experiment was performed with two replicates and three independent experiments were conducted (** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

2.2.4. Cell Viability and Apoptosis Analysis

We performed flow cytometry analysis with Annexin V/PI double staining to measure cell viability/mortality following siRNA treatment (Figure 6; Figure S2). At the 24 h timepoint, the level of viable cells (Annexin V⁻/PI⁻) showed a statistically significant decrease when treated with siRNA 2 compared to the negative control ($p = 0.032$) (Figure 6A). No statistically significant changes were found between treatments at the 48 h timepoint. However, after 72 h of treatment cell viability levels decreased in cells treated with siRNA 7, compared to negative control ($p = 0.010$) (Figure 6A). It was also observed that siRNA 7 treatment resulted in lower cell viability compared to siRNA 2 treatment ($p = 0.013$) (Figure 6A).

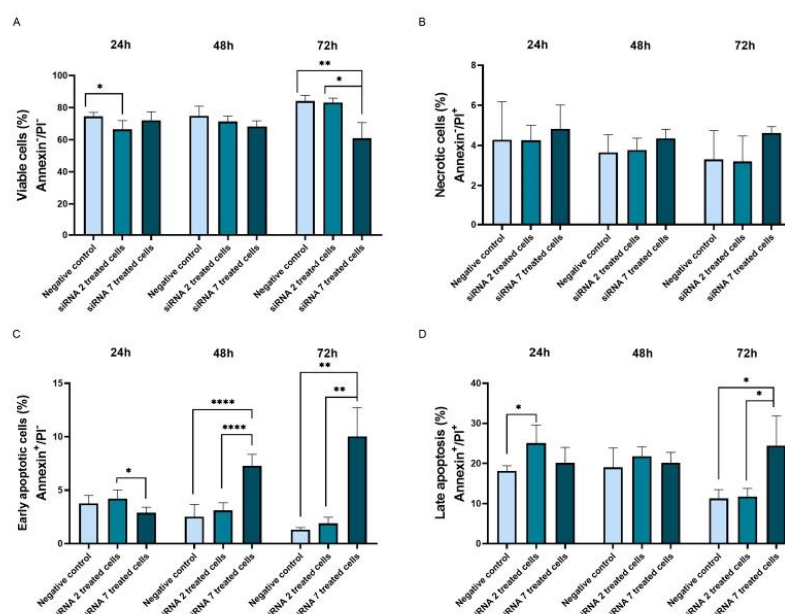


Figure 6. Cell viability and cell mortality analysis at the studied timepoints. (A) Cell viability percentage of Annexin V⁻/PI⁻ stained cells. (B) Percentage of necrotic cells (Annexin V⁻/PI⁺); (C) Percentage of early apoptotic cells (Annexin V⁺/PI⁻); (D) Percentage of late apoptotic/post-apoptotic necrotic cells (Annexin V⁺/PI⁺). Each experiment was performed in two replicates, and three independent experiments were conducted (* $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$). PI-Propidium iodide.

Necrotic cells (Annexin V⁻/PI⁺) represented the smallest population of cells. No statistically significant differences were observed between treatments or timepoints (Figure 6B). For the population of early apoptotic cells (Annexin V⁺/PI⁻), at the 24 h timepoint siRNA 2 treatment seemed to result in a higher frequency of early apoptotic cells compared to siRNA 7 treatment ($p = 0.023$) (Figure 6C). However, at the 48 h timepoint, siRNA 7 treatment showed to be more effective when compared to both negative control and siRNA 2 treatments ($p < 0.0001$, for both comparisons) (Figure 6C). The same observation was made at the 72 h timepoint ($p = 0.004$ and $p = 0.005$, respectively) (Figure 6C). Late apoptotic cells (Annexin V⁺/PI⁺) were the second most representative population of cells present in the samples (Figure 6D). The frequency of late apoptotic cells was higher following siRNA 2 treatment at the 24 h timepoint than in the negative control ($p = 0.027$) (Figure 6D). At 48 h, no differences were observed (Figure 6D). At the 72 h timepoint, siRNA 7 treatment resulted in higher percentages of late apoptotic cells compared to negative control and siRNA 2 treatment ($p = 0.032$ and $p = 0.037$, respectively) (Figure 6D).

2.2.5. USP42 Silencing Effect in Cell Proliferation

To assess if cell proliferation was affected by siRNA treatments, we performed cell counting with Trypan Blue on a Neubauer chamber. No statistically significant changes were found at 24 h and 48 h for both siRNA treatments, although a tendency for decreased proliferation was found when treating cells with siRNA 7 at 48 h ($p = 0.065$) (Figure 7). However, at the 72 h timepoint, siRNA 7-treated cells were the most affected, showing lower proliferation compared to negative control cells ($p = 0.0002$) and siRNA 2-treated cells ($p = 0.014$) (Figure 7). At the 72 h timepoint, compared to the negative control siRNA 2-treated cells proliferated two times less and siRNA 7-treated cells proliferated 7.3 times less (Figure 7).

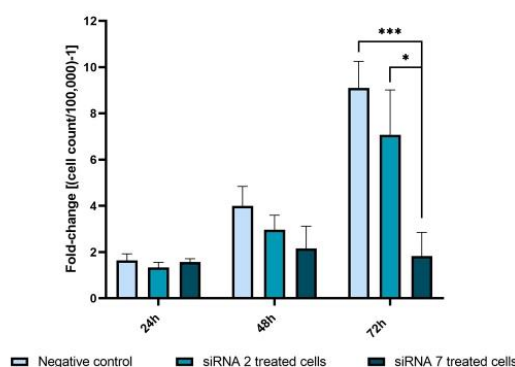


Figure 7. Nthy-Ori 3-1 cell proliferation analysis following *USP42* silencing treatment. Two to three cell counts were performed per well to establish cell proliferation rates. Fold-change was calculated from the initial number of plated cells [cell count/100,000)-1]. This evaluation was conducted using two replicates and two independent experiments were conducted (* $p \leq 0.05$; *** $p \leq 0.001$).

2.2.6. Cell Morphology Evaluation by Phalloidin Staining

Cell morphology was evaluated by immunofluorescence following 48 h treatment with siRNAs. Representative images of each condition are shown in Figure 8. Negative control-treated cells presented, as expected, an epithelial-like phenotype, higher cellular confluence, and cell-to-cell adhesion, with actin filaments being evenly distributed within the cells (Figure 8A,B). For both treatments, siRNA 2 and siRNA 7, most of the fields were composed of a significant number of cells with altered morphology. These cells were smaller and their actin filaments appeared to lose shape in the cytoplasm and to be concentrated in the cell periphery (Figure 8C–F). The described effect was more evident with siRNA 2 treatment, which presented a higher number of cells with altered morphology per field at the 48 h timepoint (Figure 8).

2.2.7. β -Actin Expression Analysis by Western Blot

To assess whether β -actin alterations were only morphological or whether siRNA treatment resulted in β -actin protein expression changes, we performed an evaluation using Western blot. At 24 h, no evident changes in β -actin expression were found. However, at 48 h we observed a reduction of 55% in β -actin expression for siRNA 2 treatment compared to the negative control ($p = 0.002$) (Figure 9). A reduction of 53% in β -actin expression was also verified using siRNA 7 treatment, although this was not statistically significant ($p = 0.065$) (Figure 9). At the 72 h timepoint, no difference was found between β -actin expression in siRNA 2-treated cells and negative control cells; however, a significant 48% reduction was found in siRNA 7-treated cells ($p = 0.010$) (Figure 9). We also detected reductions in tubulin and vinculin expression by Western blot (Figure S3).

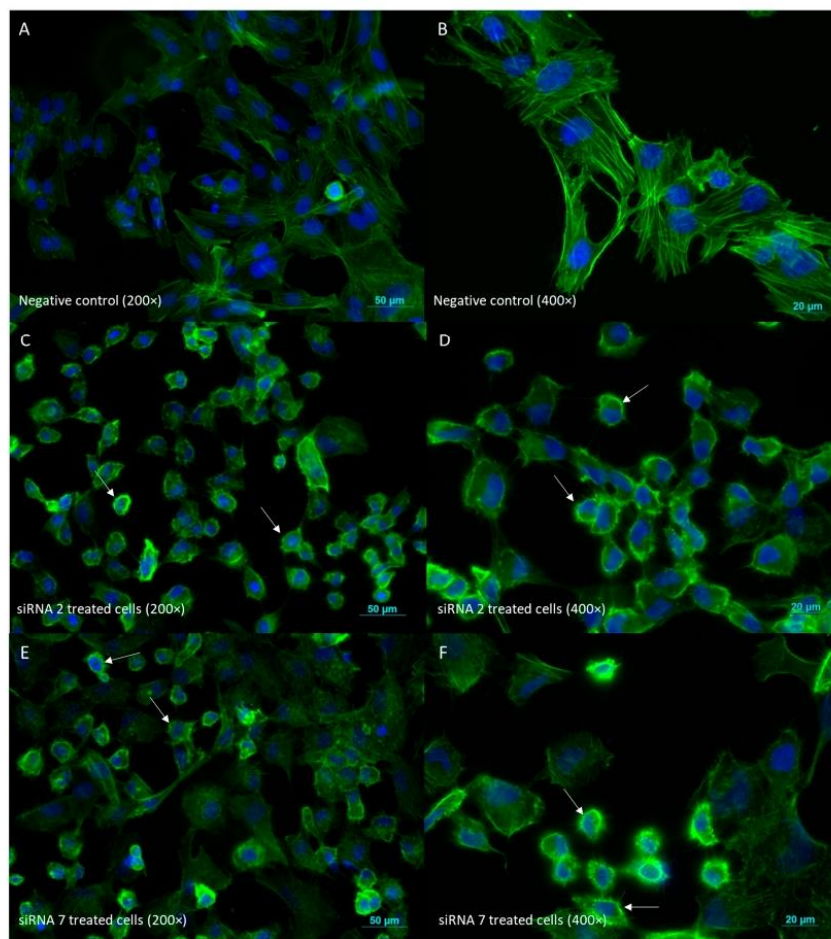


Figure 8. Immunofluorescence analysis of cell morphology by phalloidin staining of actin filaments (green) contrasted with DAPI staining of cell nuclei (blue). (**A,B**) Negative control cells under 200 $\times$ and 400 $\times$ magnification, respectively. (**C,D**) siRNA 2-treated cells under 200 $\times$ and 400 $\times$ magnification, respectively. (**E,F**) siRNA 7-treated cells under 200 $\times$ and 400 $\times$ magnification, respectively. White arrows indicate cells with altered morphology, presenting loss of shape and actin concentrated in cell periphery. Images are representative of three experiments conducted independently (each with two replicates). Bar: 50 μ m at 200 $\times$ magnification and 20 μ m at 400 $\times$ magnification.

2.2.8. Cell Cycle-Related Proteins Analyzed by Western Blot

As USP42 has been described to regulate cell cycle and apoptosis [13], protein expression of cell cycle-related proteins (p53, Cyclin D1, p27, and p21) was evaluated (Figure 10).

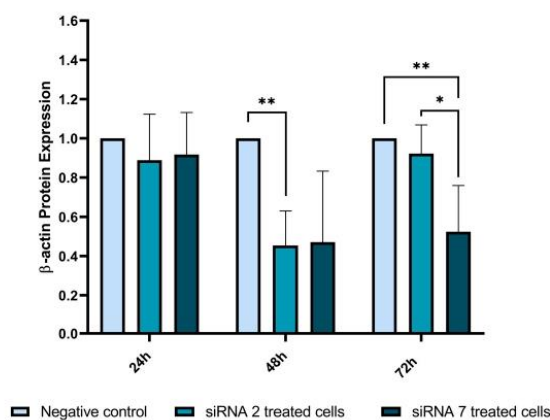


Figure 9. β -actin protein expression following siRNA treatment. siRNA 2-treated cells showed a 55% reduction in β -actin expression at 48 h ($p = 0.002$) and siRNA 7-treated cells showed a 40% reduction 72 h post-treatment ($p = 0.010$). Each experiment was performed with two replicates and three independent experiments were conducted. (* $p \leq 0.05$; ** $p \leq 0.01$).

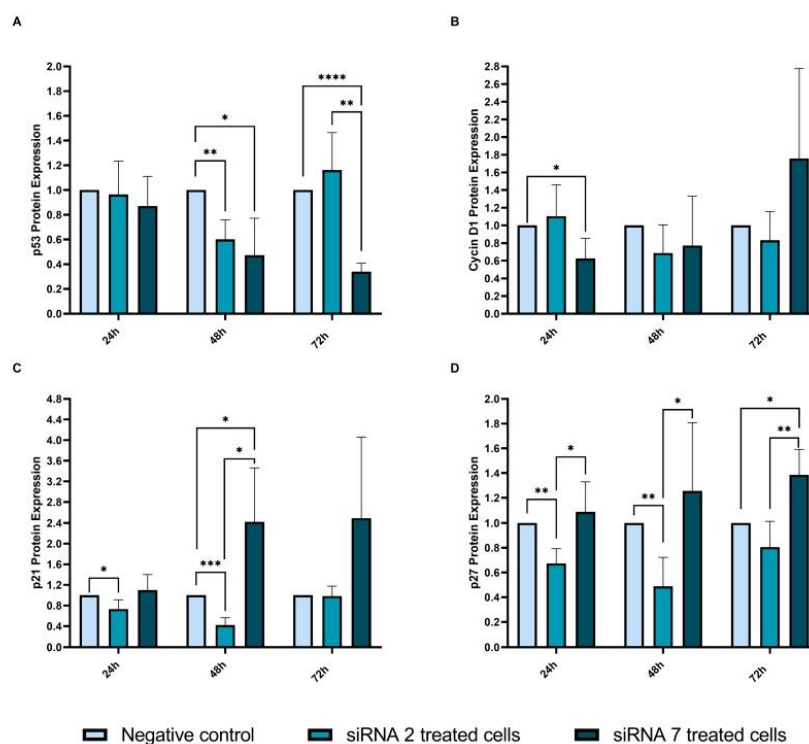


Figure 10. Cell cycle-related protein expression following siRNA treatment of Nthy-Ori 3-1 cells. (A) p53 protein expression; (B) Cyclin D1 protein expression; (C) p21 protein expression; and (D) p27 protein expression. Each experiment was performed with two replicates and three independent experiments were conducted (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

siRNAs had not altered USP42 protein expression at 48 h post-treatment; however, p53 expression was decreased at this timepoint (Figure 10A). Compared to the negative control, siRNA 2 treatment led to a 40% reduction in p53 expression ($p = 0.004$) and siRNA 7 treatment led to a 53% reduction ($p = 0.037$) (Figure 10A). After 72 h, siRNA 2 treatment did not give a statistically significant change compared to the negative control, but p53 expression in siRNA 2-treated cells presented a large discrepancy compared to siRNA 7-treated cells ($p = 0.002$) (Figure 10A). At the 72 h timepoint, we observed the most significant reduction in p53 expression (66%) after treating cells with siRNA 7 ($p < 0.001$) (Figure 10A).

Cyclin D1 expression was only found to be significantly altered at the 24 h timepoint, with a 38% reduction in its expression following siRNA 7 treatment compared to negative control (Figure 10B). No significant changes were observed at the remaining timepoints (Figure 10B).

p21 protein expression was reduced following siRNA 2 treatment. At 24 h, we observed a 27% decrease of expression compared to negative control ($p = 0.033$) and at the 48 h timepoint, a 58% reduction was found ($p = 0.0004$) (Figure 10C). However, at the 72 h timepoint with siRNA 2 treatment, cells seemed to recuperate p21 expression to the same level as in negative control cells (Figure 10C). Using siRNA 7, only at the 48 h timepoint a statistically significant association was found, interestingly with a marked increase of 140% (2.4-fold) of p21 expression compared to negative control ($p = 0.045$) (Figure 10C). When comparing p21 expression between the two treatments, a 2-fold difference was found ($p = 0.011$) (Figure 10C).

When treating cells with siRNA 2, we observed a reduction in p27 expression at the 24 h timepoint (33% reduction, $p = 0.003$) and at the 48 h timepoint (51% reduction, $p = 0.007$) (Figure 10D). As for siRNA 7 treatment, we found a statistically significant increase of 39% on p27 expression at the 72 h timepoint ($p = 0.014$) (Figure 10D).

3. Discussion

We report here the identification of a *USP42* germline mutation that segregated with the disease in a family compatible with the diagnosis of NSFNMT. The family was composed of a mother and two monozygotic twin daughters with an early-onset diagnosis of OSPTC. Despite filling the criteria for hereditary cancer, no alterations associated with syndromic forms of FNMT were found. Using WES, we identified a possible causative mutation for the disease in a Ubiquitin-Specific Protease gene-*[USP42 p.(Gly486Arg)]*.

USP42 is a thiol-dependent deubiquitinating enzyme (DUB) predicted to be active in cytosol and nucleus [15,16]. The ubiquitination pathway is a dynamic and complex post-transcriptional process, regulated by E3 enzymes and reversible through DUBs [11,17,18]. DUBs are enzymes capable of removing ubiquitin from ubiquitinated proteins, which may regulate their stability and activity, protecting them from proteasomal degradation [19–21].

USP42 gene alterations have been previously described as an underlying cause of cancer. Wang et al. (2022) suggested that *USP42* might be a target gene for breast cancer [22]. They have shown that USP42 was overexpressed in breast cancer tissues and further demonstrated, using in vitro studies, that USP42 overexpression promoted cell invasion and migration, as well as that *USP42* silencing led not only to a reduction in proliferation, but also to increased apoptosis, suggesting that this gene may act as an oncogene [22]. USP42 regulation of cellular metabolism through p53—histone H2B interaction has been demonstrated [13,14].

Other USPs have been put forward as regulators of tumor formation and proliferation in TC. Jianing et al. (2022) showed an interaction of USP26 protein with TAZ protein, leading to its stabilization and the consequent proliferation of Anaplastic Thyroid Carcinoma (ATC) [23]. Liang et al. (2021) reported that TC patients with low expression of USP18 had worse survival rates than those with high USP18 expression [24]. An et al. (2015) demonstrated that USP39 underexpression was associated with decreased cell proliferation due to G2/M cell cycle arrest in Medullary Thyroid Carcinoma (MTC) cells [25]. More recently, using WES, Maria et al. (2022) described a *USP13 p.(Val495Met)* mutation as an

underlying cause of PTC in a family with NSFNMTTC. In this family, the *USP13* mutation that was shown to result in *USP13* overexpression, which played a role in the tumorigenesis of PTC [26].

These results suggest that USPs may play a role in both sporadic and familial thyroid carcinoma. We examined the data available in the TCGA database and found that 17 PTC cases had somatic genetic alterations in USP genes. *USP9X* and *USP6* were the most frequently affected USP genes. Two cases presented variants in *USP42* (different from the one identified in the family examined in this study). *BRAF* hotspot mutations were present in 76% of these 17 PTCs, suggesting that USP alterations can cooperate with well-known TC genetic alterations during the transformation process. Recently, a polygenic mode of action in NSFNMTTC tumorigenesis has been proposed [8,9]. NSFNMTTC is described as presenting low- to moderate-penetrance susceptibility alleles with incomplete penetrance, which leads to TC susceptibility [5–7]. It is hypothesized that the combined influences of other modified genes along with these susceptibility alleles triggers the onset of cancer [6–9]. Of note, in the studied family we detected a somatic *HRAS* mutation in the proband tumor samples, but that alteration was not present in the other elements.

The *USP42 p.(Gly486Arg)* mutation was present in all of the tumor samples from the family members in heterozygosity. Using immunohistochemistry (IHC) we noted that, although *USP42* was expressed in the normal tissue, an increased expression was observed in the tumor specimens, indicating overexpression. The tumor samples also presented higher expression of p53 and Caspase-3 than adjacent tissues.

To our knowledge, no prior studies investigating the role of *USP42* gene alterations in NSFNMTTC have conducted, and we therefore performed several in vitro experiments to validate *USP42* mutation as an underlying cause of NSFNMTTC.

We undertook downregulation of *USP42* using two siRNAs, both of which efficiently silenced *USP42* at 24 h and 48 h, also showing some recuperation of the silencing effect at 72 h. A marked effect on apoptosis was shown, with a significant increase in apoptosis following silencing of the *USP42* gene. These results are consistent with those of Wang et al. (2022), who silenced *USP42* and observed increased apoptosis in breast cancer cells [22]. Other USPs, such as *USP15*, have been demonstrated to impact the stability and activity of Caspase-3 [27].

The effect of *USP42* siRNA silencing on proliferation was delayed and not so marked, although a significant reduction in the proliferation of *USP42*-silenced cells at 72 h post-treatment was noted.

The ubiquitination process is a dynamic process that has already been described to regulate actin filaments in the cells [28]. In our study, we observed that *USP42* silencing led to cytoskeletal alterations in the cells, as shown by phalloidin staining of actin filaments, and also a reduction in cytoskeletal proteins as shown by Western blot for actin, vinculin, and tubulin. Whether *USP42* silencing has a direct or indirect effect on cytoskeleton formation is still to be uncovered, but other USPs, such as *USP8* and *USP14*, have been shown to regulate cytoskeletal proteins [28,29].

It has been described that *USP42* stabilizes p53 by removing ubiquitin and preventing its proteasomal degradation [13,14,30]. Using IHC, increased expression of p53 was found in FFPE tissues for all family members. Usually, increased p53 expression results from mutations [31], but no p53 mutations were found in the samples from the proband (I). Therefore, we hypothesize that increased p53 expression in the tissues is related to *USP42* overexpression, which was also noted in the FFPE; supporting this hypothesis, after silencing *USP42* we observed a significant reduction in p53 expression at 48 h post-treatment for both siRNAs. However, at 72 h post-transfection, while siRNA 7 treatment maintained a significant reduction in p53, this effect was not verified for siRNA 2. This might be due to the transient effects of the siRNAs, which presented a recovery at 72 h, as referred to above. As the *USP42* gene can present different isoforms, we can also hypothesize that different siRNAs may target different isoforms, thus resulting in variable effects in the cell [13].

Hou et al. (2016) has shown that USP42 was overexpressed in gastric cancer patients and that overall survival of these patients was reduced compared to patients whose tumors presented lower expression of USP42 protein [32]. They further demonstrated, using siRNAs against the *USP42* gene, that cell cycle-related proteins, namely Cyclin D1, Cyclin E1, and the Proliferating Cell Nuclear Antigen (PCNA), were downregulated following *USP42* silencing [32]. Furthermore, Maria et al. (2022) showed that *USP13* silencing by siRNAs decreased expression of Cyclin D1, leading to cell cycle arrest [26]. In our study, in concordance with the aforementioned studies, we demonstrated that, following *USP42* silencing using siRNA 2, Cyclin D1 is significantly downregulated at 24 h post-treatment.

We also showed that p21 is downregulated at 24 h and 48 h after treating cells with siRNA 2, although the same effect is not evident when treating cells with siRNA 7 for 48 h. These discrepancies may be associated with the specific binding site of the siRNA that might lead to variable effects in the cells or they may be related to the cytotoxicity associated with siRNA 7. Hock et al. (2014) reported that *USP42* silencing resulted in lower expression of p21 mRNA and hypothesized that USP42 is recruited to the transcriptional site of p21, but further investigation on this interaction is still required [14]. A similar effect was obtained for p27 expression following *USP42*-silencing treatments. siRNA 2 treatment resulted in p27 downregulation between 24 h and 48 h of treatment, and siRNA 7 treatment resulted in the upregulation of p27 at the 72 h timepoint.

Our results, along with the available information in the literature, leads us to hypothesize that the *USP42* p.(Gly486Arg) missense mutation is the underlying cause of NSFNMTC in this family. The mutation seems to be activating, leading to overexpression of the mutated protein, thus possibly acting as an oncogene.

As for the limitations of this study, it is important to emphasize that our sample is small, being composed of only three affected elements. Furthermore, in the in vitro siRNA studies we cannot rule out the possibility that some of the observed changes might be due to the transient nature of siRNA and/or that some effects may be due to transfection cytotoxicity. Future assays using CRISPR-Cas9 or a similar methodology, in which the Nthy-Ori 3-1 cell line will be transformed to harbor the specific SNP that was found in this family by using WES [*USP42* p.(Gly486Arg)], will be necessary.

The genetics behind NSFNMTC is rather heterogeneous and poorly understood [2,4]. The causative genes in many affected families remain to be identified and the establishment of molecular markers would lead to better genetic counselling and to the development of new therapies. Disease prevention and active surveillance are key factors for reducing aggressive treatments, high comorbidities, and invasive surgeries, in addition to avoiding high burdens for the health care system.

4. Materials and Methods

4.1. Patients

The surgical procedures and sample collection were performed at Cluj-Napoca Municipal Clinical Hospital, Romania. Peripheral blood and FFPE tumor tissues were collected from the three family members. We obtained a total of 26 FFPE blocks from the three elements: 10 blocks from the mother (I), 12 from the first twin (II.1), and 4 from the second twin (II.2) (Figure 1A). DNA was extracted from all samples and preserved at -20°C until further processing was performed. All subjects gave their informed consent for inclusion before they participated in the study. This study was conducted in accordance with the Declaration of Helsinki.

4.2. Molecular Studies

4.2.1. DNA Extraction from Patient Samples

DNA was extracted from peripheral blood samples using the GRS Total DNA Kit—Blood & Cultured Cells (GRiSP Research Solutions, Porto, Portugal). DNA extraction from FFPE tissues was performed using the GRS Genomic DNA Kit BroadRange (GRiSP Research Solutions, Porto, Portugal), following the manufacturer's instructions. Quantitative and

qualitative sample analysis was performed using spectrophotometry with a Nanodrop N-1000 Spectrophotometer for microvolume UV–Visible measurements (Thermo Scientific, Waltham, MA, USA).

4.2.2. Immunohistochemistry

IHC was performed in FFPE samples of the three family members using the Ultravision Quanto Detection System HRP (Epredia®, Kalamazoo, MI, USA, TL-125-QHL) following the manufacturer's instructions. Dewaxing and antigen retrieval were performed using De-wax and HIER (Epredia, Kalamazoo, MI, USA) in a steamer for 45 min. Monoclonal USP42 (1:250, d-4 mouse, sc-390604, Santa Cruz Biotechnology, Dallas, Texas, USA), monoclonal Caspase-3 (1:100, E-8, sc-7272, Santa Cruz Biotechnology, Dallas, Texas, USA), and monoclonal p53 (1:250, mouse, NCL-L-p53-DO7, Leica, Chicago, Illinois) antibodies were used as primary antibodies. Detection was performed with a DAB chromogen for all antibodies (Epredia®, TA-125-QHDX, Kalamazoo, MI, USA). Slides were counterstained with Gill's hematoxylin. USP42 and Caspase-3 staining were evaluated by semi-quantitative analysis. Quantification of p53 expression was performed by counting at least 1000 cells per field ratio between positive cells and total cell number was calculated as a percentage.

4.3. In Silico Studies

4.3.1. Variant Calling and Annotation

Normal DNA was extracted from the blood of the three affected individuals, and the tumor DNA from the mother was microdissected from FFPE tissue blocks as previously described. All DNA samples were subjected to WES using the Illumina DNA Prep.

4.3.2. Whole-Exome Sequencing

The resulting sequencing files were aligned against the human GRCh37 reference genome using the Burrows-Wheeler Aligner (BWA, v0.7.10) [33]. PCR duplicates were removed using Picard (<http://broadinstitute.github.io/picard/> (accessed on 29 October 2023)). Single/nucleotide variants (SNVs) and insertions and deletions (indels) were annotated using GATK (version 3.1.1, Broad Institute, Cambridge, MA, USA) [34]. Reads around known and detected indels were realigned, and base quality was recalibrated using GATK (version 3.1.1, Broad Institute, Cambridge, MA, USA) [34].

All generated files were analyzed using The Cancer-Related Analysis of Variants Toolkit (CRAVAT (version 5.2.4, Johns Hopkins, Baltimore, MD, USA), Figure 1B) [35].

4.3.3. Variant Prioritization

A step-by-step approach was used to identify potential pathogenic germline variants, using the following criteria: (i) having an allele fraction $\leq 1\%$ in the overall population, as given by gnomAD (version 2.1.1) [36,37], (ii) being present in all three affected individuals, (iii) having a combined annotation dependent depletion (CADD (version 1.7, University of Washington, Hudson-Alpha Institute for Biotechnology, WA, USA and Berlin Institute of Health at Charité - Universitätsmedizin Berlin, Germany) [38] PHRED score 10), and (iv) being present on IGV (version 2.16.2 Broad Institute, Cambridge and Harvard, MA, USA) [39] when visualizing the sequenced reads. Shared candidate variants were subjected to confirmation by Sanger sequencing.

4.3.4. Analysis of Somatic Variants in the Tumor DNA

The presence of somatic SNVs and indels in the tumor DNA of the mother were obtained by filtering all germline variants present in the matched normal DNA and by visualizing the sequenced reads of each somatic variant on IGV (version 2.16.2, Broad Institute, Cambridge and Harvard, MA, USA) [39]. Mutational hotspots were annotated according to Chang et al. (2016) [40]. FACETS (version 0.5.6, Memorial Sloan-Kettering Cancer Center, New York, NY, USA) [41] was used to determine Copy Number Alterations (CNAs) and whether genes harboring somatic or germline mutations were targeted by

Loss Of Heterozygosity (LOH), as previously described [42]. The cancer cell fractions of all somatic mutations were computed using ABSOLUTE (version 1.0.6, Broad Institute, Cambridge and Harvard, MA, USA) [43]. A mutation was classified as clonal if its probability of being clonal was >50% or if the lower bound of the 95% confidence interval of its Cancer Cell Fraction (CCF) was >90%, as previously described [42].

4.3.5. Polymerase Chain Reaction and Sanger Sequencing Analysis

The candidate germline variant was validated by Polymerase Chain Reaction (PCR) using the QIAGEN multiplex PCR kit (QIAGEN, Hilden, Germany) and by Sanger sequencing analysis, as previously described [44]. Primer design was performed (Forward: 5'-CAGCCAAAATGGGTTCTGTT-3'; Reverse: 5'-TTTGGACAGAAGCTGAAGCA-3') (Integrated DNA Technologies, Coralville, IA, USA). The annealing temperature of 52 °C was established after protocol optimization for the *USP42* gene. All alterations were confirmed by performing a new and independent analysis.

4.3.6. USP Genetic Alterations in Papillary Thyroid Cancers

Bioinformatic analysis was conducted on The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/> accessed on 15 November 2023)). USP mutations, gene amplifications, gene deletions, frameshifts, truncations, and splice variants were searched. Other online databases describing *USP42* mutation status and effect prediction were searched, namely IntOGen (version 2.4.0) [45], gnomAD (version 2.1.1) [37] COSMIC (version 99) [46], Ensembl (version 111) [47] and ClinVar (version 1.0) [48].

4.4. Functional Characterization

4.4.1. Cell Culture

Nthy-Ori 3-1 cells were cultured in T75 flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) in RPMI-1640 medium (Capricorn scientific, Ebsdorfergrund, Germany), supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% Penicillin/Streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained at 37 °C with 5% CO₂ humidified environment in an incubator (HERACell 150i, Thermo Fisher Scientific, Waltham, MA, USA). Cells were routinely screened to confirm the absence of mycoplasma contamination.

4.4.2. siRNAs–*USP42* Silencing

Nthy-Ori 3-1 cells were plated in 6-well plates (1×10^5 cells/well). After 24 h, cells were transfected with pre-designed siRNAs (Hs_USP42_2-SI00758618 and Hs_USP42_7-SI03068583; QIAGEN, Aarhus, Denmark) or with a non-coding siRNA (Allstars Negative Control siRNA-SI03650318, QIAGEN, Aarhus, Denmark) using Lipofectamine 2000 transfection reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) diluted in Opti-MEM™ I Reduced Serum Medium (Gibco, Thermo Fisher, Waltham, MA, USA). siRNA complexes were formed for 20 min in the dark and were added to the cells at the concentration of 25 nM. Medium change was performed 4h post-transfection (t = 0 h). Each experiment was performed with two replicates and at least three independent experiments were conducted. Cells were collected at 24 h, 48 h, and 72 h timepoints and counted with Trypan Blue (T8154, Merck KGaA, Darmstadt, Germany) on a Neubauer chamber for proliferation assessment.

4.4.3. RNA Extraction and cDNA Conversion

RNA was extracted using the GRS Total RNA Kit–Blood & Cultured Cells (GrisP, Porto, Portugal), following the manufactures' instructions. RNA concentration and sample purity and quality were analyzed using spectrophotometry, as previously described. 1000 ng of RNA was converted to cDNA using Reverse Transcriptase following DNase I and EDTA treatments. Samples were amplified and converted using the SimplyAmp Thermal

Cycler (Applied Biosystems, Life Technologies, Thermo Fisher, Waltham, MA, USA) at 25 °C for 10 min, 42 °C for 60 min, and 70 °C for 10 min.

4.4.4. Real-Time Quantitative Polymerase Chain Reaction

mRNA evaluation of USP42 expression (probe: Hs.PT.58.670189; Integrated DNA Technologies—Integrated DNA Technologies IDT, Leuven, Belgium) was performed using TaqMan® Universal PCR Master Mix-No AmpErase® (ThermoFisher Scientific, Waltham, MA, USA) using TATA-binding protein (TBP) as an endogenous control (probe: Hs.PT.39a.22214825; Integrated DNA Technologies IDT, Leuven, Belgium). The reaction was performed in a MicroAmp™ Optical 96-Well Reaction Plate (ThermoFisher Scientific, Waltham, MA, USA) in a QuantStudio5 machine (Applied Biosystems, Life Technologies, Thermo Fisher, Waltham, MA, USA). Triplicates and non-template controls were performed for all samples. Quantification of mRNA expression was performed using the $2^{-\Delta\Delta C_t}$ method.

4.4.5. Protein Extraction

Cell lysis was performed by incubating the cell pellet for 15 min with RIPA buffer mixed with phosphatase and protease inhibitors. After centrifugation for 10 min at 14,000 rpm at 4 °C, the pellet was discarded. Protein quantification was performed in a 96-well plate using the Modified Protein Lowry assay according to the manufacturers' instructions. Sample absorbance was read at 650 nm on a SpectraMax® iD3 machine (Molecular Devices, San Jose, CA, USA).

4.4.6. Western Blot Analysis

Whole protein lysates (15 µg) were loaded on 10% polyacrylamide gels. The following antibodies were used: monoclonal USP42 (1:1000, d-4 mouse, sc-390604, Santa Cruz Biotechnology, Dallas, Texas, USA), monoclonal p53 (1:3000, mouse, NCL-L-p53-DO7, Leica Biosystems, Leica, Chicago, IL, USA), monoclonal Cyclin D1 (1:10000, ab134175, Abcam, Cambridge, UK), monoclonal p21 (1:500, rabbit, #2947s, Waf1/Cip1 (12D1), Cell Signaling Technology, Beverly, MA, USA), monoclonal p27 (1:500, rabbit, Kip1-D69C12, #36865, Cell Signaling Technology, Danvers, MA, USA), monoclonal β-actin (1:1000, mouse, Santa Cruz Biotechnology, Dallas, TX, USA), monoclonal α-Tubulin (1:5000, Mouse, T5168, Merck KGaA, Darmstadt, Germany), monoclonal Vinculin (1:3000, Mouse, V9131 Merck KGaA, Darmstadt, Germany), and monoclonal GAPDH (1:1000, Mouse, sc-32233 Santa Cruz Biotechnology, Dallas, TX, USA). Protein expression was analyzed in an Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, Cambridge, UK) and read at 800 nm absorbance using fluorescent IRDye® 800CW Donkey Anti-Rabbit antibody (LI-COR Biotech, USA) and IRDye® 800CW Goat Anti-Mouse antibody (LI-COR Biotech, Lincoln, NE, USA) as secondary antibodies. Target protein quantification was performed and compared against the total protein of the sample (Ponceau S staining) using Image Studio™ Lite software version 5.2.5 (LI-COR Biosciences, Cambridge, UK).

4.4.7. Phalloidin Staining

Cells were grown in sterilized round slides and transfected with siRNAs, as previously described, before being stained with “Flash Phalloidin™ Green 488” (Biolegend, San Diego, CA, USA) (1:200) and DAPI (1:1000) as previously described [49]. Samples were photographed using the fluorescence microscope Zeiss Axio Imager Z1 (Carl Zeiss, Oberkochen, Germany). Data was analyzed using Image J Version 1.53t software (National Institutes of Health, Bethesda, MD, USA).

4.4.8. Annexin V Assay

siRNA-transfected cells were collected, centrifuged at $300 \times g$ for 5 min, and resuspended in Binding Buffer 1× (ThermoFisher Scientific, Waltham, MA, USA). Cells were stained with 5 µL Annexin V FITC (ImmunoTools, Friesoythe, Germany) (30 min incubation in the dark) and 50 µg/mL propidium iodide (PI) (5 min incubation in the dark). Unstained

cells were used as autofluorescence controls. Cells were filtered (70 µm filter) and read in the BD Accuri C6 cytometer (BD Biosciences, Qume Drive San Jose, CA, USA). At least 20,000 events per sample were evaluated using FlowJo (FlowJo™ Software version 10.9, BD Biosciences, Qume Drive San Jose, CA, USA).

4.4.9. Statistical Analysis

Data analyses were performed using GraphPad Prism (version 8.0.2, La Jolla, CA, USA). Two-way ANOVA or mixed-effect model analysis (non-parametric two-way ANOVA) was performed when applicable. Results are presented as the respective mean and standard error. Samples were considered significantly different when $p < 0.05$ (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$) and confidence interval $\neq 1$.

5. Conclusions

We described a germline *USP42* p.(Gly486Arg) mutation as an underlying cause of FNMTc in a family presenting OSPTC in all the elements under study. Our in silico results support that USPs, other than *USP42*, are altered in sporadic thyroid cancer, and that USPs can be in line with other mutations associated with thyroid cancer development, such as *BRAF* p.(Val600Glu). Our in vitro results demonstrate that silencing of the *USP42* gene leads to changes in thyroid cell biology. This is shown by differences in cell proliferation, apoptotic rate, and cell cycle-related protein expression, which correlated with the altered protein expression observed in the patients' tumor samples. We hereby present evidence that *USP42* gene alterations could represent a new susceptibility factor for NSFNMTc.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of CHVNG/E (Project investigation 30/2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Dataset from The Cancer Genome Atlas (TCGA) analyses can be accessed/download in cBioPortal for Cancer Genomics at <https://www.cbioportal.org/> (accessed on 15 November 2023).

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References

- Wiltshire, J.J.; Drake, T.M.; Uttley, L.; Balasubramanian, S.P. Systematic review of trends in the incidence rates of thyroid cancer. *Thyroid* **2016**, *26*, 1541–1552. [\[CrossRef\]](#)
- Guilmette, J.; Nosé, V. Hereditary and familial thyroid tumours. *Histopathology* **2018**, *72*, 70–81. [\[CrossRef\]](#)
- Diquigiovanni, C.; Bonora, E. Genetics of Familial Non-Medullary Thyroid Carcinoma (FNMTc). *Cancers* **2021**, *13*, 2178. [\[CrossRef\]](#) [\[PubMed\]](#)
- Nosé, V.; Gill, A.; Teixeira, J.M.C.; Perren, A.; Erickson, L. Overview of the 2022 WHO Classification of Familial Endocrine Tumor Syndromes. *Endocr. Pathol.* **2022**, *33*, 197–227. [\[CrossRef\]](#) [\[PubMed\]](#)
- Capezzone, M.; Robenshtok, E.; Cantara, S.; Castagna, M.G. Familial non-medullary thyroid cancer: A critical review. *J. Endocrinol. Investig.* **2021**, *44*, 943–950. [\[CrossRef\]](#) [\[PubMed\]](#)
- Pereira, J.S.; da Silva, J.G.; Tomaz, R.A.; Pinto, A.E.; Bugalho, M.J.; Leite, V.; Cavaco, B.M. Identification of a novel germline FOXE1 variant in patients with familial non-medullary thyroid carcinoma (FNMTc). *Endocrine* **2015**, *49*, 204–214. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ngan, E.S.; Lang, B.H.; Liu, T.; Shum, C.K.; So, M.-T.; Lau, D.K.; Leon, T.Y.; Cherny, S.S.; Tsai, S.Y.; Lo, C.-Y. A germline mutation (A339V) in thyroid transcription factor-1 (TTF-1/NKX2. 1) in patients with multinodular goiter and papillary thyroid carcinoma. *J. Natl. Cancer Inst.* **2009**, *101*, 162–175. [\[CrossRef\]](#) [\[PubMed\]](#)
- Moses, W.; Weng, J.; Kebebew, E. Prevalence, clinicopathologic features, and somatic genetic mutation profile in familial versus sporadic nonmedullary thyroid cancer. *Thyroid* **2011**, *21*, 367–371. [\[CrossRef\]](#)
- Srivastava, A.; Giangioffe, S.; Skopelitou, D.; Miao, B.; Paramasivam, N.; Diquigiovanni, C.; Bonora, E.; Hemminki, K.; Försti, A.; Bandapalli, O.R. Whole Genome Sequencing Prioritizes CHEK2, EWSR1, and TIAM1 as Possible Predisposition Genes for Familial Non-Medullary Thyroid Cancer. *Front. Endocrinol.* **2021**, *12*, 600682. [\[CrossRef\]](#)
- Young, M.-J.; Hsu, K.-C.; Lin, T.E.; Chang, W.-C.; Hung, J.-J. The role of ubiquitin-specific peptidases in cancer progression. *J. Biomed. Sci.* **2019**, *26*, 42. [\[CrossRef\]](#)
- Cruz, L.; Soares, P.; Correia, M. Ubiquitin-specific proteases: Players in cancer cellular processes. *Pharmaceuticals* **2021**, *14*, 848. [\[CrossRef\]](#)
- He, M.; Zhou, Z.; Wu, G.; Chen, Q.; Wan, Y. Emerging role of DUBs in tumor metastasis and apoptosis: Therapeutic implication. *Pharmacol. Ther.* **2017**, *177*, 96–107. [\[CrossRef\]](#)
- Hock, A.K.; Vigneron, A.M.; Carter, S.; Ludwig, R.L.; Vousden, K.H. Regulation of p53 stability and function by the deubiquitinating enzyme USP42. *EMBO J.* **2011**, *30*, 4921–4930. [\[CrossRef\]](#)
- Hock, A.K.; Vigneron, A.M.; Vousden, K.H. Ubiquitin-specific peptidase 42 (USP42) functions to deubiquitylate histones and regulate transcriptional activity. *J. Biol. Chem.* **2014**, *289*, 34862–34870. [\[CrossRef\]](#) [\[PubMed\]](#)
- Harmonizing model organism data in the Alliance of Genome Resources. *Genetics* **2022**, *220*, iyac022. [\[CrossRef\]](#)
- Quesada, V.; Diaz-Perales, A.; Gutiérrez-Fernández, A.; Garabaya, C.; Cal, S.; López-Otín, C. Cloning and enzymatic analysis of 22 novel human ubiquitin-specific proteases. *Biochem. Biophys. Res. Commun.* **2004**, *314*, 54–62. [\[CrossRef\]](#)
- Komander, D.; Clague, M.J.; Urbé, S. Breaking the chains: Structure and function of the deubiquitinases. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 550–563. [\[CrossRef\]](#) [\[PubMed\]](#)
- Deng, L.; Meng, T.; Chen, L.; Wei, W.; Wang, P. The role of ubiquitination in tumorigenesis and targeted drug discovery. *Signal Transduct. Target. Ther.* **2020**, *5*, 11. [\[CrossRef\]](#) [\[PubMed\]](#)
- David, R. DUBs' key to selectivity. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 64–65. [\[CrossRef\]](#)
- Mevissen, T.E.; Komander, D. Mechanisms of deubiquitinase specificity and regulation. *Annu. Rev. Biochem.* **2017**, *86*, 159–192. [\[CrossRef\]](#) [\[PubMed\]](#)
- Clague, M.J.; Urbé, S.; Komander, D. Breaking the chains: Deubiquitylating enzyme specificity begets function. *Nat. Rev. Mol. Cell Biol.* **2019**, *20*, 338–352. [\[CrossRef\]](#)
- Wang, G.; He, C.; Chen, J.; He, W.; He, L.; Li, J. Deubiquitinating Enzyme USP42 Promotes Breast Cancer Progression by Inhibiting JNK/p38-Mediated Apoptosis. *Res. Sq.* **2022**. [\[CrossRef\]](#)
- Tang, J.; Luo, Y.; Xiao, L. USP26 promotes anaplastic thyroid cancer progression by stabilizing TAZ. *Cell Death Dis.* **2022**, *13*, 326. [\[CrossRef\]](#) [\[PubMed\]](#)

24. Liang, Q.; Zhong, W. Downregulated Expression of USP18 Is Associated with a Higher Recurrence Risk of Papillary Thyroid Carcinoma. *Tohoku J. Exp. Med.* **2021**, *255*, 203–212. [\[CrossRef\]](#)
25. An, Y.; Yang, S.; Guo, K.; Ma, B.; Wang, Y. Reduced USP39 expression inhibits malignant proliferation of medullary thyroid carcinoma in vitro. *World J. Surg. Oncol.* **2015**, *13*, 255. [\[CrossRef\]](#)
26. Maria, A.G.; Azevedo, B.; Settas, N.; Hannah-Shmouni, F.; Stratakis, C.A.; Faucz, F.R. USP13 genetics and expression in a family with thyroid cancer. *Endocrine* **2022**, *77*, 281–290. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Xu, M.; Takanashi, M.; Oikawa, K.; Tanaka, M.; Nishi, H.; Isaka, K.; Kudo, M.; Kuroda, M. USP15 plays an essential role for caspase-3 activation during Paclitaxel-induced apoptosis. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 366–371. [\[CrossRef\]](#)
28. Rodríguez-Pérez, F.; Manford, A.G.; Pogson, A.; Ingersoll, A.J.; Martínez-González, B.; Rape, M. Ubiquitin-dependent remodeling of the actin cytoskeleton drives cell fusion. *Dev. Cell* **2021**, *56*, 588–601. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Yan, G.; Liu, N.; Wang, J.; Tian, J.; Liu, H.; Li, S.; Liu, W.; Li, X.; Li, K.; Wang, H. Deubiquitylation and stabilization of Act7 by ubiquitin carboxyl-terminal hydrolase 14 (USP14) is critical for NSCLC migration. *J. Biosci.* **2021**, *46*, 19. [\[CrossRef\]](#)
30. Sun, X.X.; Dai, M.S. Deubiquitinating enzyme regulation of the p53 pathway: A lesson from Otub1. *World J. Biol. Chem.* **2014**, *5*, 75–84. [\[CrossRef\]](#)
31. Morita, N.; Ikeda, Y.; Takami, H. Clinical Significance of P53 Protein Expression in Papillary Thyroid Carcinoma. *World J. Surg.* **2008**, *32*, 2617–2622. [\[CrossRef\]](#)
32. Hou, K.; Zhu, Z.; Wang, Y.; Zhang, C.; Yu, S.; Zhu, Q.; Yan, B. Overexpression and biological function of ubiquitin-specific protease 42 in gastric cancer. *PLoS ONE* **2016**, *11*, e0152997. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Li, H.; Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **2009**, *25*, 1754–1760. [\[CrossRef\]](#) [\[PubMed\]](#)
34. McKenna, A.; Hanna, M.; Banks, E.; Sivachenko, A.; Cibulskis, K.; Kernysky, A.; Garimella, K.; Altshuler, D.; Gabriel, S.; Daly, M.; et al. The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* **2010**, *20*, 1297–1303. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Masica, D.L.; Douville, C.; Tokheim, C.; Bhattacharya, R.; Kim, R.; Moad, K.; Ryan, M.C.; Karchin, R. CRAVAT 4: Cancer-Related Analysis of Variants Toolkit. *Cancer Res.* **2017**, *77*, e35–e38. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Collins, R.L.; Brand, H.; Karczewski, K.J.; Zhao, X.; Alföldi, J.; Francioli, L.C.; Khera, A.V.; Lowther, C.; Gauthier, L.D.; Wang, H.; et al. A structural variation reference for medical and population genetics. *Nature* **2020**, *581*, 444–451. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Martínez-Jiménez, F.; Muiños, F.; Sentís, I.; Deu-Pons, J.; Reyes-Salazar, I.; Arnedo-Pac, C.; Mularoni, L.; Pich, O.; Bonet, J.; Kranas, H.; et al. A compendium of mutational cancer driver genes. *Nat. Rev. Cancer* **2020**, *20*, 555–572. [\[CrossRef\]](#)
38. Kircher, M.; Witten, D.M.; Jain, P.; O’Roak, B.J.; Cooper, G.M.; Shendure, J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat. Genet.* **2014**, *46*, 310–315. [\[CrossRef\]](#)
39. Robinson, J.T.; Thorvaldsdóttir, H.; Winckler, W.; Guttman, M.; Lander, E.S.; Getz, G.; Mesirov, J.P. Integrative genomics viewer. *Nat. Biotechnol.* **2011**, *29*, 24–26. [\[CrossRef\]](#)
40. Chang, M.T.; Asthana, S.; Gao, S.P.; Lee, B.H.; Chapman, J.S.; Kandath, C.; Gao, J.; Socci, N.D.; Solit, D.B.; Olshen, A.B.; et al. Identifying recurrent mutations in cancer reveals widespread lineage diversity and mutational specificity. *Nat. Biotechnol.* **2016**, *34*, 155–163. [\[CrossRef\]](#)
41. Shen, R.; Seshan, V.E. FACETS: Allele-specific copy number and clonal heterogeneity analysis tool for high-throughput DNA sequencing. *Nucleic Acids Res.* **2016**, *44*, e131. [\[CrossRef\]](#)
42. Kim, S.H.; Da Cruz Paula, A.; Basili, T.; Dopeso, H.; Bi, R.; Pareja, F.; da Silva, E.M.; Gualarte-Mérida, R.; Sun, Z.; Fujisawa, S.; et al. Identification of recurrent FHL2-GLI2 oncogenic fusion in sclerosing stromal tumors of the ovary. *Nat. Commun.* **2020**, *11*, 44. [\[CrossRef\]](#)
43. Carter, S.L.; Cibulskis, K.; Helman, E.; McKenna, A.; Shen, H.; Zack, T.; Laird, P.W.; Onofrio, R.C.; Winckler, W.; Weir, B.A.; et al. Absolute quantification of somatic DNA alterations in human cancer. *Nat. Biotechnol.* **2012**, *30*, 413–421. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Póvoa, A.A.; Teixeira, E.; Bella-Cueto, M.R.; Batista, R.; Pestana, A.; Melo, M.; Alves, T.; Pinto, M.; Sobrinho-Simões, M.; Maciel, J. Genetic determinants for prediction of outcome of patients with papillary thyroid carcinoma. *Cancers* **2021**, *13*, 2048. [\[CrossRef\]](#)
45. Siwei, C.; Laurent, C.F.; Julia, K.G.; Ryan, L.C.; Masahiro, K.; Qingbo, W.; Jessica, A.; Nicholas, A.W.; Christopher, V.; Laura, D.G.; et al. A genome-wide mutational constraint map quantified from variation in 76,156 human genomes. *bioRxiv* **2002**. [\[CrossRef\]](#)
46. Tate, J.G.; Bamford, S.; Jubb, H.C.; Sondka, Z.; Beare, D.M.; Bindal, N.; Boutselakis, H.; Cole, C.G.; Creatore, C.; Dawson, E.; et al. COSMIC: The Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Res.* **2018**, *47*, D941–D947. [\[CrossRef\]](#)
47. Martin, F.J.; Amode, M.R.; Aneja, A.; Austine-Orimoloye, O.; Azov, A.G.; Barnes, I.; Becker, A.; Bennett, R.; Berry, A.; Bhai, J.; et al. Ensembl 2023. *Nucleic Acids Res.* **2022**, *51*, D933–D941. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Landrum, M.J.; Chitipiralla, S.; Brown, G.R.; Chen, C.; Gu, B.; Hart, J.; Hoffman, D.; Jang, W.; Kaur, K.; Liu, C.; et al. ClinVar: Improvements to accessing data. *Nucleic Acids Res.* **2020**, *48*, D835–D844. [\[CrossRef\]](#)
49. Peixoto, J.; Príncipe, C.; Pestana, A.; Osório, H.; Pinto, M.T.; Prazeres, H.; Soares, P.; Lima, R.T. Using a Dual CRISPR/Cas9 Approach to Gain Insight into the Role of LRP1B in Glioblastoma. *Int. J. Mol. Sci.* **2023**, *24*, 1285. [\[CrossRef\]](#) [\[PubMed\]](#)

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Table S1. Somatic pathogenic genetic alterations affecting the UPS gene family in papillary thyroid carcinomas taken from TCGA.

Tumor Sample	Histology	Gene	Chr.	Position/Cytoband	Mutation type	Alternate Allele	Aminoacid change	PolyPhen	SIFT
TCGA-BJ-A3EZ-01	PTC NOS	<i>USP9Y</i>	Y	14924964	Missense	A > C	<i>p.Tyr1529Ser</i>	possibly_damaging	deleterious
TCGA-CE-A481-01	PTC NOS	<i>USP6</i>	17	5076261	Frameshift_Del	TA > -	<i>p.Met1404Valfs*23</i>	.	.
TCGA-DJ-A130-01	FVPTC	<i>USP40</i>	2	234468516	Missense	C > A	<i>p.Asp108Tyr</i>	probably_damaging	deleterious
TCGA-DJ-A1QE-01	PTC NOS	<i>USP51</i>	X	55514742	Missense	G > C	<i>p.Leu211Val</i>	possibly_damaging	deleterious
TCGA-DJ-A1QL-01	FVPTC	<i>USP28</i>	11	113705033	Nonsense	G > A	<i>p.Arg187*</i>	.	.
TCGA-DJ-A2Q9-01	PTC with oncocytic cells	<i>USP46</i>	4	53492228	Missense	T > C	<i>p.Gln173Arg</i>	benign	deleterious
TCGA-EB-A2EA-01	PTC NOS	<i>USP43</i>	17	9596525	Missense	C > A	<i>p.Leu479Ile</i>	probably_damaging	deleterious
TCGA-EL-A3GP-01	PTC NOS	<i>USP6</i>	17	5076150	Nonsense	T > A	<i>p.Lyr1366*</i>	.	.
TCGA-EL-A3H5-01	PTC NOS	<i>USP9X</i>	X	41075213	Missense	A > C	<i>p.Lys1798Thr</i>	probably_damaging	deleterious
TCGA-EL-A3MY-01	PTC with oncocytic cells	<i>USP9X</i>	X	41043681	Frameshift_Ins	- > A	<i>p.Pro1105Thrfs*4</i>	.	.
TCGA-EL-A3ZO-01	PTC NOS	<i>USP26</i>	X	132161783	Nonsense_Mutation	G > A	<i>p.Gln156*</i>	.	.
TCGA-EL-A4K7-01	PTC NOS	<i>USP49</i>	6	41767589	Missense	C > A	<i>p.Arg550Leu</i>	probably_damaging	deleterious
TCGA-EM-A22P-01	PTC NOS	<i>USP48</i>	1	22033083	Splice Site	C > G	<i>p.X697_splice</i>	.	.
TCGA-EM-A4FM-01	PTC NOS	<i>USP9X</i>	X	41058005	Splice Site	T > C	<i>p.X1535_splice</i>	.	.
TCGA-ET-A39P-01	PTC with oncocytic cells	<i>USP42</i>	7	6189833	Missense	C > A	<i>p.Pro669Gln</i>	benign	deleterious
TCGA-FE-A22Z-01	PTC with oncocytic cells	<i>USP9X</i>	X	40988337	Nonsense	G > T	<i>p.Glu61*</i>	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L10</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L11</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L12</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L13</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L15</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L17</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L18</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L19</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L20</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L24</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L25</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L26</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L27</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L28</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L29</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L30</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L6P</i>	4	p16.1	Amplification	.	.	.	.
TCGA-BJ-A0Z0-01	FVPTC	<i>USP17L9P</i>	4	p16.1	Amplification	.	.	.	.

*1 Chr. – Chromosome; PTC, papillary thyroid carcinoma; NOS, Not otherwise specified; FVPTC, follicular variant of papillary thyroid carcinoma.

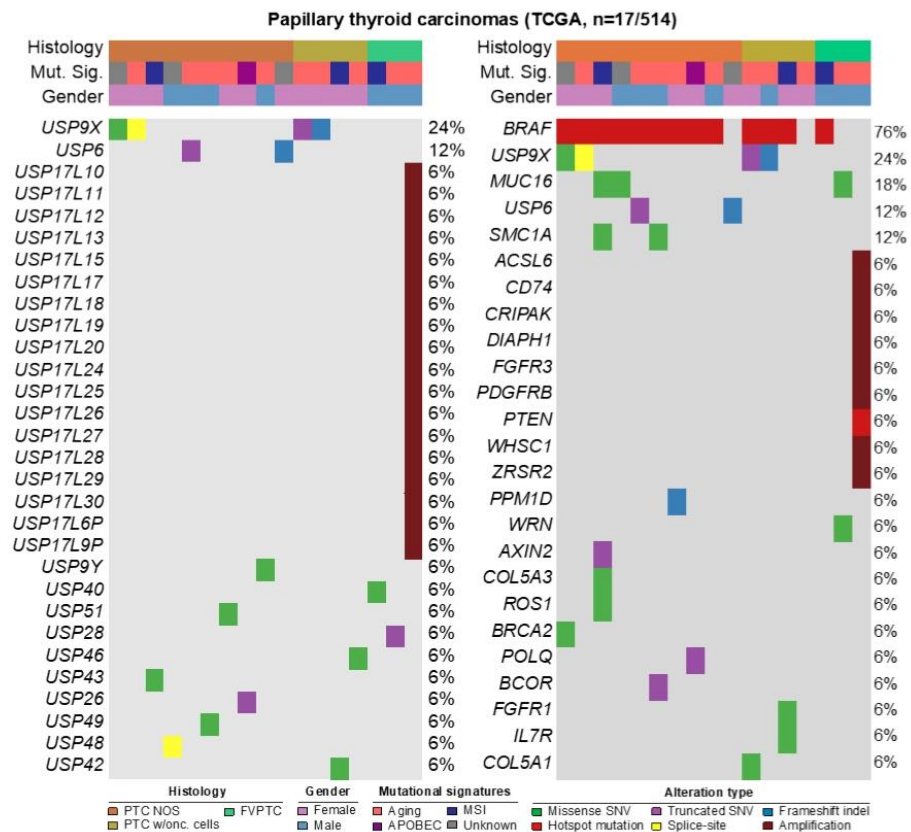


Figure S1 - *In silico* analysis of 514 papillary thyroid carcinomas subjected to whole-exome sequencing from TCGA. Heatmap depicting pathogenic somatic alterations affecting the USP gene family only in papillary thyroid carcinomas (PTC, left), and heatmap depicting all pathogenic somatic alterations affecting the USP-mutant PTCs (right). Cases are shown in columns and genes in rows. Histology, gender, mutational signatures and alteration types are color-coded according to the legend.

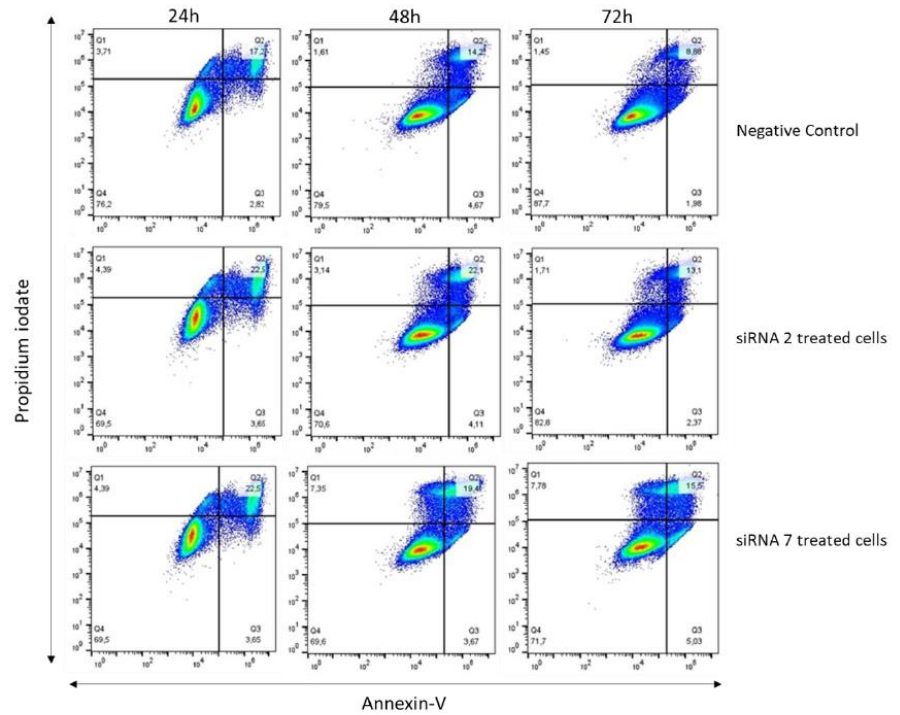


Figure S2 – Apoptosis assays: representative analysis of Annexin-V and Propidium iodide staining. Cell viability and cell mortality analysis at the studied timepoints. Q1 – Necrotic cells (AnnexinV⁺/PI⁺), Q2 - Late apoptotic (AnnexinV⁺/PI⁺), Q3 - Early apoptotic cells (AnnexinV⁺/PI⁻), Q4 – Viable cells (AnnexinV⁻/PI⁻). Each experiment was performed with duplicates and three independent experiments were conducted.

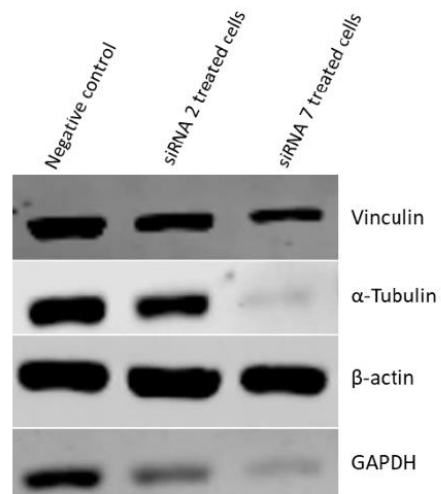


Figure S3 – Representative blots of housekeeping genes that were silenced by siRNA 2 and 7 treatments. This effect was more evident in siRNA 7 treated cells.

CHAPTER IV (continuation)

RESULTS

(in development)

4.6. *USP42* GENE KNOCKOUT SYNTHESIS USING CRISPR-RNP EDITING TECHNIQUE (in development)

Our **ARTICLE V** results showed promising results regarding the role of *USP42* gene silencing on thyroid cell biology. In continuation of this work, and with the aim of obtaining a permanent *USP42* knockout (KO) cell line, to understand the full impact of *USP42* loss, the Nthy-ori 3-1 were transfected using CRISPR-RNP gene editing methodology [200, 201].

The following data is related to unpublished results of work that is still ongoing.

4.6.1. MATERIALS AND METHODS

4.6.1.1. Knockout of *USP42* gene using CRISPR-RNP

With the aim of obtaining a permanent *USP42* knockout (KO) cell line, the Nthy-ori 3-1 thyroid cells were transfected using CRISPR-RNP gene editing methodology [200, 201]. Lipofectamine™ CRISPRMAX™ Cas9 transfection reagent (CMAX00015, Invitrogen™, Thermo Fisher Scientific, USA) was used [202]. *USP42* specific sgRNA, donor DNA and respective primers were designed using the Invitrogen TrueDesign Genome Editor online tool (<https://www.thermofisher.com/>) (Invitrogen™, Thermo Fisher Scientific, USA) (**Table 3**). CDK4 positive control (A35523, Invitrogen™, Thermo Fisher Scientific, USA) and negative control (A35526, Invitrogen™, Thermo Fisher Scientific, USA) sgRNAs were used as transfection controls (**Table 3**). Cell concentration was optimized to 2.5×10^3 cells/well in 96-well plates. Transfection was performed 24h after plating, following the indications of the manufacturer (Invitrogen™, Thermo Fisher Scientific, USA) and in duplicates for each condition. All reagents were prepared/diluted in Opti-MEM Reduced-Serum Medium (31985070, Gibco, USA) or TE buffer (Invitrogen, Thermo Fisher Scientific, USA), accordingly to manufacturer's instructions. Cells were maintained in RPMI 1640, with Stable Glutamine, (RPMI-STA, Capricorn Scientific, Germany) and 10% of heat-inactivated Fetal Bovine Serum (FBS) (10500064, Gibco, USA). Medium was changed 72h post-transfection.

Table 3 – Sequences of guides and primers used for the CRISPR-RNP assay to obtain a *USP42* knockout cell line

Material	Sequence
<i>USP42</i> Donor DNA	ATGTGTCAAATCACACACTTTCTTTAGGACCAGTATGATGATGATGACCTGG TGCTGTAGTTTATTCGAGTTCATCTGTACC
<i>sgRNA USP42</i>	5'- TAAACTACAGCACCAGGTAC -3'
<i>USP42</i> Forward primer	5'-ATCTTCAGACCCATCAGCCT -3'
<i>USP42</i> Reverse primer	5'- ATACAACTCTGCTGGCCACT - 3'
<i>CDK4</i> Forward primer	5'-GCACAGACGTCCATCAGCC-3'
<i>CDK4</i> Reverse primer	5'-GCCGGCCCAAGGAAGACTGGGAG-3'

<i>sgRNA CDK4</i>	5'- CACUCUUGAGGGCCACAAAG-3'
<i>sgRNA Negative Control</i>	5'- AAAUGUGAGAUCAAGUAAU -3'

Donor DNA synthesized to insert four STOP codons (TGA) at **Valine 57** in *USP42* **exon 2**, using the *USP42* normal sequence as template. Amplicon *USP42*: 343 bp; Amplicon *CDK4*: 557 bp; Materials designed and acquired at Invitrogen™, Thermo Fisher Scientific, USA.

4.6.1.2. Clonal selection of *USP42* and *CDK4* edited cells

CRISPR-RNP transfected cells that were cultured in a 96-well plate were allowed to grow and sequentially moved from a 96-well plate to 12-well plate, T25 flask until reaching a T75 flask. At this point, cells were harvested, and pellets were collected for DNA extraction of stock cells and for cell storage at -80°C. Cells were counted on a Neubauer chamber and subsequently diluted until reaching an estimated dilution of 8 cells/mL. Resuspended cells were then distributed through five 96-well plates, at a volume of 100 µL/well.

For *USP42* sgRNA plus donor DNA transfected cells, a total of 59 wells from replicate 1 (Flask 1) were selected as viable to proceed selection, for included wells in which only one cell was visibly present. Over time, only 30 *USP42* clones were grown and collected for Sanger Sequencing analysis. Reasons for the loss of 29 candidate clones were: 1 – due to the death of the single cell, 2 – more than one clone were visible in the well, thus was considered “not pure” and discarded.

For *CDK4* sgRNA transfected cells, from the two replicates (Flask 1 and 2) a total of 43 clones were selected as viable to proceed selection, but only 25 clones were grown and collected for Sanger Sequencing analysis.

4.6.1.3. *USP42* edited cells DNA extraction

Cell DNA was extracted using the GRS Total DNA Kit – Blood & Cultured Cells (GrisP, Porto, Portugal), following the manufactures' instructions. DNA concentration, sample purity and quality were analyzed by spectrophotometry, as previously described in **ARTICLE II**.

4.6.1.4. PCR and Sanger sequencing validation of *USP42*

DNA from candidate clones were analyzed by Polymerase Chain Reaction (PCR) using the QIAGEN multiplex PCR kit (QIAGEN, Hilden, Germany) and by Sanger sequencing analysis, as previously described in **ARTICLE II**. The annealing temperature of 62°C was established after protocol optimization for the *USP42* gene. All alterations were confirmed by performing two/three new and independent analysis. All PCR reactions were conducted in the SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA).

4.6.1.5. Selection of *USP42* Knockout/Knockdown clones

Electropherograms obtained by Sanger sequencing were independently analyzed by two different softwares to confirm if the clones were pure and if the four STOP codon sequences had been inserted after Valine 57 of exon 2 as intended. First, all electropherograms were ran on the SeqScreener Gene Edit Confirmation App (Invitrogen, Thermo Fisher Scientific, USA), followed by the Mutation Surveyor® DNA Variant Analysis Software (SoftGenetics, USA).

4.6.1.6. *USP42* CRISPR clones characterization (a preliminary analysis)

A preliminary analysis of the selected *USP42* CRISPR-RNP clones was performed by the evaluation of 1- *USP42* mRNA expression assessment by RT-PCR; 2- *USP42*, p53, cyclin D1, p21, p27, and β -actin protein expression assessment by Western blot; and 3 - Apoptosis analysis by the Annexin-V assay. Materials and Methods for this part of the work are described in **ARTICLE V**.

4.6.2. RESULTS

4.6.2.1. *USP42* CRISPR-RNP transfection evaluation

Contrary to siRNA assays performed in **ARTICLE V**, CRISPR-RNP transfection did not present with cytotoxicity to the Nthy-ori 3-1 cell line. 72h post-transfection, cells were thriving. 96h post-transfection cells presented with confluence over 80% in the 96-wells and were immediately harvested to 12-well plates. When reaching T75 flasks, cell images of the pool of cells for each condition were acquired (**Figure 6**). No evident differences between the clones were observed, however, control *CDK4* cells from replicate 2 (Flask 2) grew less and showed less confluence when comparing to Flask 1 and the other clones.

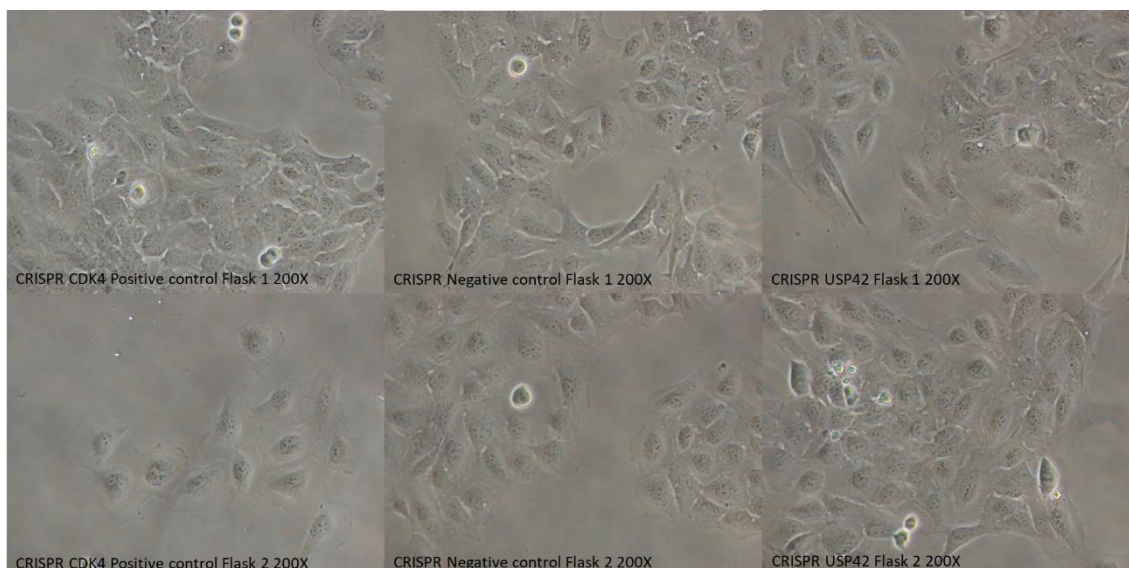


Figure 6 – Images of the pool of Nthy-ori 3-1 cells grew up to T75 flasks, post-transfection and before clonal selection. Magnification: 200X.

4.6.2.2. *USP42* CRISPR-RNP clone cells morphology

A total of 34 clones were obtained after single-cell clonal selection. Different clones presented with changes in cell morphology when comparing to Nthy-ori 3-1 parental cells (**Figure 7**). P1C12 clone presented a morphology similar to Nthy-ori 3-1 parental cells, while clones P2F12 and P5D2 presented an elongated morphology, as shown in **Figure 7**.

Cells were harvested and stabilized for four weeks before acquiring new images that confirmed cell morphology changes in CRISPR-RNP *USP42* edited cells (**Figure 8**).

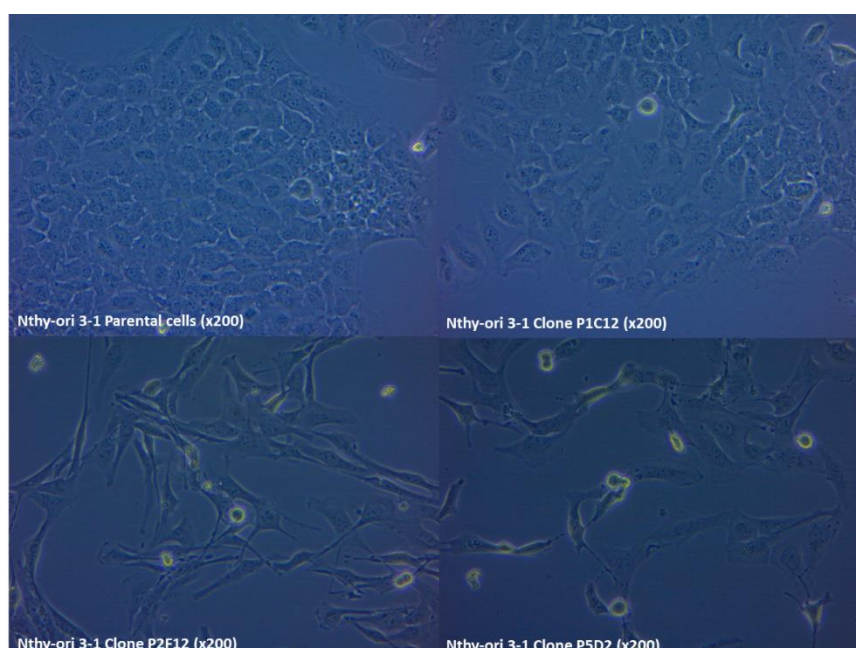


Figure 7 - Nthy-ori 3-1 parental cells morphology in comparison to CRISPR-RNP *USP42* edited cells. Magnification: 200X.

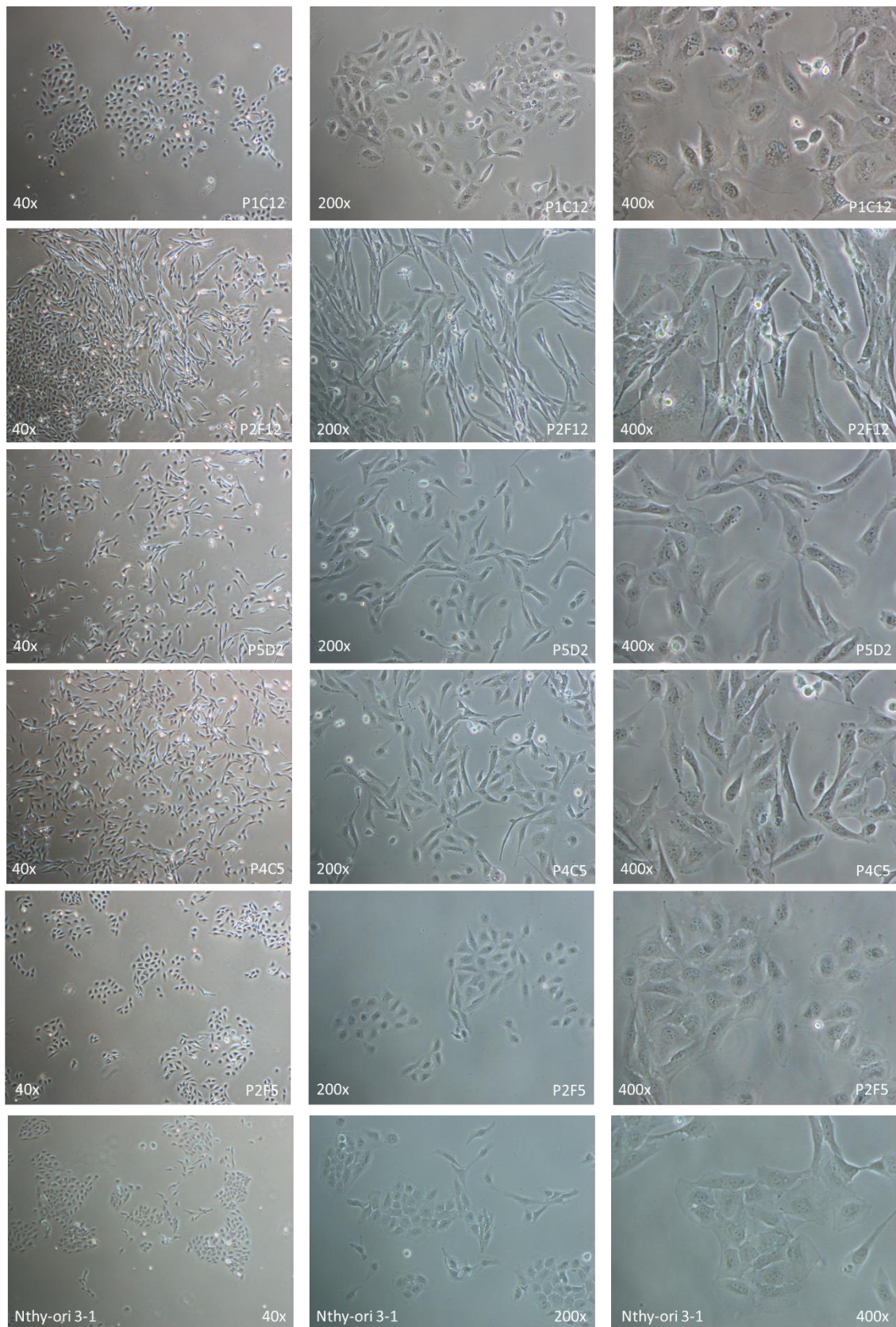


Figure 8 - Nthy-ori 3-1 parental cells morphology in comparison to CRISPR-RNP *USP42* edited cells four weeks after cell stabilization. Magnification: 40X, 200X and 400X.

4.6.2.3. *USP42* CRISPR-RNP candidate clones' selection

All 34 *USP42* viable clones were amplified and analyzed by electrophoresis before Sanger sequencing and purification, as previously described in **ARTICLE II**. All samples were amplified by PCR reaction (**Figure 9**). We observed that DNA samples from different clones presented with different molecular weight in several cases and that some samples presented more than one band (**Figure 9**).

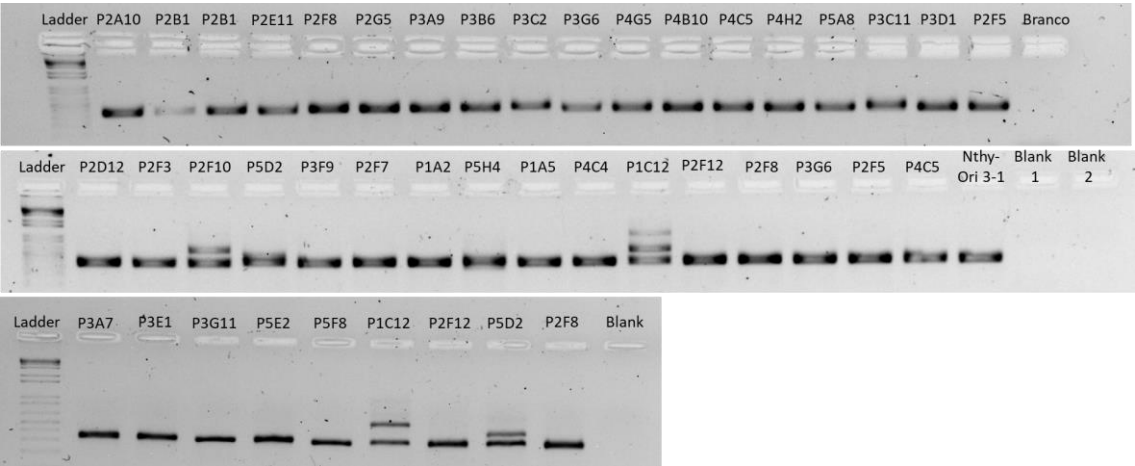


Figure 9 – Electrophoresis gels of the DNA of the *USP42* viable clones. DNA samples from different clones presented with different molecular weight in several cases and that some samples presented more than one band. All samples amplified by PCR reaction.

4.6.2.4. SeqScreener Gene Edit Confirmation

In a first analysis, the pool of cells collected after transfection and before clonal selection, was sequenced and analyzed in the SeqScreener Gene Edit Confirmation software (Invitrogen, ThermoFisher Scientific, USA) to access the success of transfection. Both pools of *CDK4* clones obtained through non-homologous end joining (NHEJ) were classified as good knockouts (KO), however, clone 1 (Flask 1) showed less percentage of DNA editing when comparing to the *wild-type* (WT) sequence (**Table 4**). The higher percentage of editing observed on *CDK4* clone 2 (Flask 2) may explain the slow growth and expansion observed in this pool of cells (**Table 4**, **Figure 6**). *USP42* clones, obtained by Homology-Directed Repair (HDR) by using a donor DNA during transfection, showed that clone 1 (Flask 1) was the most likely to harbor the targeted clone containing the TGATGATGATGA insertion (**Table 4**). As expected, Negative control pool of cells presented the WT *USP42* sequence (**Table 4**).

Table 4 - SeqScreener Gene Edit Confirmation software results of the eletropherograms of the pool of cells collected after CRIPR-RNP transfection and before clonal selection.

Grade	Experiment file	Target sequence	Model fit (R-square)	Editing (%)	HDR (%)	FrameShift (%)	Edits diversity
Good KO	CRISPR_Clone_CDK4_[2]	CACUCUUGAGGGCCACAA AG	0.91	78.4	0.0	76.4	5
Good KO	CRISPR_Clone_CDK4_[2]	CACUCUUGAGGGCCACAA AG	0.91	75.1	0.0	74.2	4
Good KO	CRISPR_Clone_CDK4_KO_[1]	CACUCUUGAGGGCCACAA AG	0.98	26.4	0.0	25.2	1
Good KO	CRISPR_Clone_CDK4_KO_[1]	CACUCUUGAGGGCCACAA AG	0.98	25.7	0.0	24.9	1
Good KI	CRISPR_Clone_USP42_[1]	TAAACTACAGCACCAGGTA C	0.99	25.1	13.1	9.2	1
KI needs review	CRISPR_Clone_USP42_[1]	TAAACTACAGCACCAGGTA C	0.98	19.9	6.2	6.3	1
KI needs review	CRISPR_Clone_USP42_[2]	TAAACTACAGCACCAGGTA C	0.99	18.0	6.5	3.8	1
WT	CRISPR_Clone_USP42_[2]	TAAACTACAGCACCAGGTA C	0.99	12.3	4.3	3.8	0
WT	CRISPR_Clone_Negative_control_[1]	TAAACTACAGCACCAGGTA C	1.00	0.5	0.0	0.3	0
WT	CRISPR_Clone_Negative_control_[2]	TAAACTACAGCACCAGGTA C	1.00	0.1	0.0	0.0	0
WT	CRISPR_Clone_Negative_control_[2]	TAAACTACAGCACCAGGTA C	1.00	0.1	0.0	0.0	0
WT	CRISPR_Clone_Negative_control_[1]	TAAACTACAGCACCAGGTA C	1.00	0.0	0.0	0.0	0

KO – Knockout; KI – Knockin; WT - *Wild-Type* HDR - Homology-Directed Repair.

After clonal selection of *USP42* pool of cells (Flask 1), a new SeqScreener Gene Edit Confirmation software analysis was conducted in the 34 obtained clones in order to find our target clone that contained the TGATGATGATGA insertion (**Table 5**). Out of the 34 clones, 12 presented the WT *USP42* sequence, being excluded from any further analysis. Three clones, P5D2, P1C12 and P2F12 were indicated by the software as interest clones, with insertion of the four STOP codons. However, edit diversity indicated that in two of the clones, (P1C12 and P2F12) more than one edit might be present, being dubious. Other 19 clones, presented with lower HDR %, 15 of which presented with 0% HDR, being excluded from further analysis. The remaining seven clones presented HDR% ranging from 14.2-24.5%, being selected for further analyses.

Table 5 - SeqScreener Gene Edit Confirmation software general results of the eletropherograms of the CRIPR-RNP *USP42* edited cells after clonal selection.

Grade	USP42 CRISPR-RNP Clone	Model fit (R-square)	Editing (%)	HDR (%)	FrameShift (%)	Edits diversity
Good KI	P5D2	0.93	99.4	83.2	7.8	1
Good KI	P1C12	0.92	100.0	70.9	13.1	2
Good KI	P2F12	0.86	100.0	42.9	56.7	2
KI needs review	P5H4	0.28	87.6	0.0	53.7	5
KI needs review	P5E2	0.48	86.0	16.6	57.3	5
KI needs review	P2E11	0.38	84.3	0.0	61.8	4
KI needs review	P2F8	0.87	61.2	21.6	38.5	2
KI needs review	P2F7	0.94	60.5	0.0	0.00	3
KI needs review	P3A9	0.58	56.8	0.0	46.5	2
KI needs review	P1A5	0.96	49.3	0.0	47.6	1
KI needs review	P3C11	0.77	31.5	0.6	15.8	0
KI needs review	P3F9	0.95	30.9	0.0	30.9	1
KI needs review	P3E1	0.94	29.8	0.0	29.3	2
KI needs review	P2B1	0.95	25.6	0.0	0.8	1
KI needs review	P3G6	0.95	25.3	23.0	0.0	1
KI needs review	P4C5	0.96	24.9	22.6	0.3	1
KI needs review	P2F5	0.96	23.1	21.2	0.1	1
KI needs review	P2D12	0.96	20.2	0.0	20.0	1
KI needs review	P5A8	0.95	17.9	0.0	15.9	1
KI needs review	P2F12	0.61	100.0	24.5	66.6	4
KI needs review	P4B5	0.60	100.0	14.2	80.5	5
KI needs review	P2F10	0.97	100.0	0.0	7.0	2
KI needs review	P1C12	0.00	100.0	0.0	66.5	9
KI needs review	P3B6	0.59	100.0	0.0	100.0	7
WT	P3D1	1.00	1.4	0.0	0.8	0
WT	P2G5	1.00	0.5	0.0	0.0	0
WT	P3G11	1.00	0.4	0.0	0.1	0
WT	P4B10	1.00	0.3	0.0	0.0	0
WT	P3A7	1.00	0.2	0.0	0.1	0
WT	P4H2	1.00	0.2	0.0	0.0	0
WT	P1A2	1.00	0.2	0.0	0.1	0
WT	P4C4	1.00	0.2	0.0	0.1	0
WT	P3C2	1.00	0.1	0.0	0.0	0
WT	P2F3	1.00	0.1	0.0	0.1	0
WT	P5F8	1.00	0.1	0.0	0.1	0
WT	P2A10	1.00	0.0	0.0	0.0	0

KO – Knockout; KI – Knockin; WT - *Wild-Type* HDR - Homology-Directed Repair.

4.6.2.5. Mutation Surveyor® DNA Variant Analysis

As seen in **Figure 10**, more than one sequence was present in all analyzed clones, except for WT specimens, dictating that *USP42* edition did not lead to homozygous alteration.

By the analysis of the clone's eletropherograms and given the results from the SeqScreener Gene Edit Confirmation software we could verify that the insertion of the four STOP codons was successful in several clones (**Figure 10 and Table 6**). However, whether the second allele was WT or if it presented other genetic alterations resulting from transfection, remained to be evaluated.

This way, Mutation Surveyor® DNA Variant Analysis software was applied to attempt separate both alleles to observe each sequence separately. Several analyzed clones were immediately discarded since it presented three sequences or many genetic alterations that could be unpredictable on following assays.

The three clones most likely to be *USP42* KO (P1C12 and P2F12, and P5D2) were shown to present one allele with the TGATGATGATGA insertion and one WT allele. However, other genetic alterations (substitutions, insertions and deletions) were present in these clones (**Table 6**). Upon this second eletropherogram analysis, we proceed to analyze two other clones, that presented the TGATGATCATAC insertion in Valine 57, P4C5 and P2F5 (**Table 6**).

Of note, given to the fact that in some eletropherograms allele separation was not possible, due to low signal, we performed an observational analysis in some of the sequenced clones.

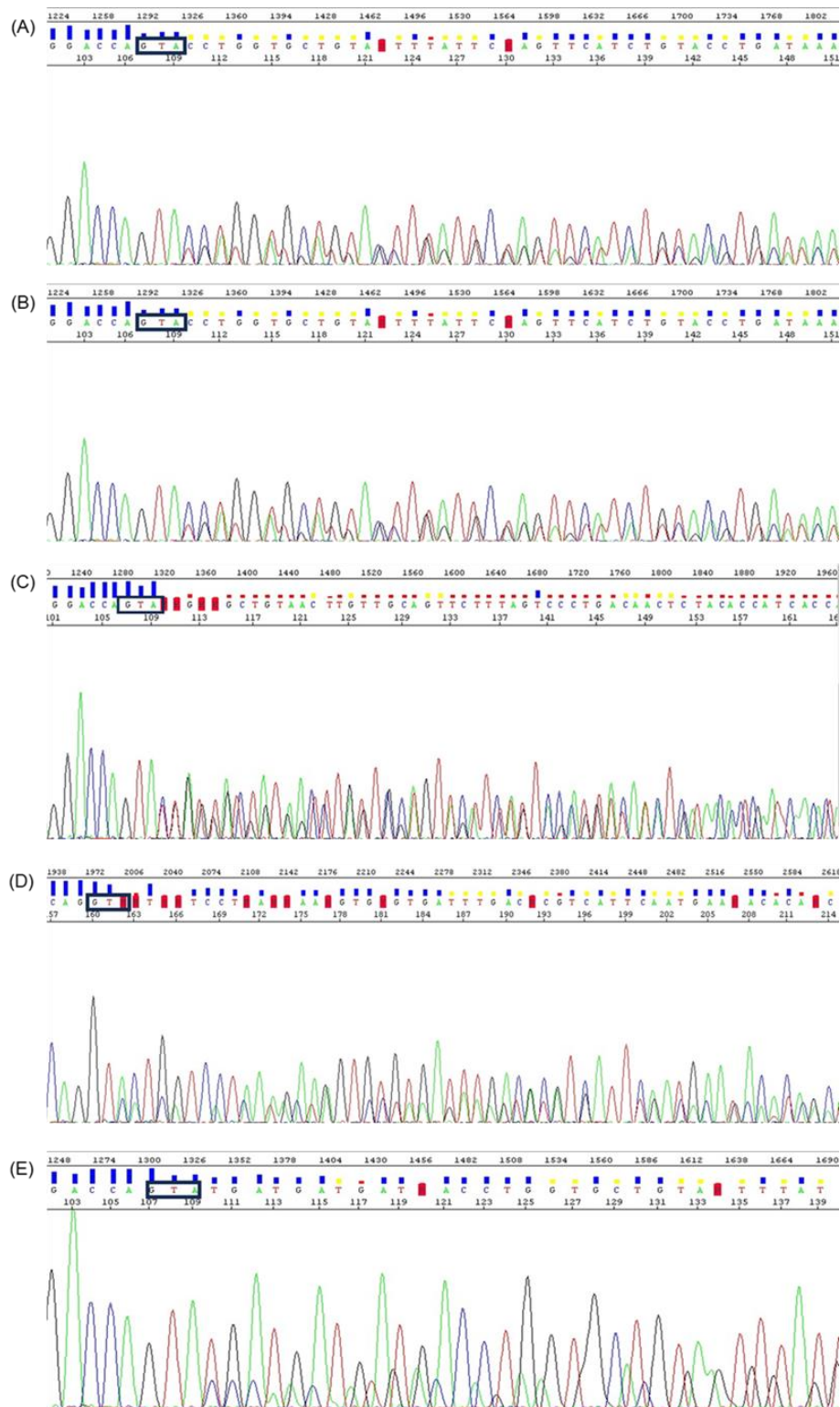


Figure 10 – CRISPR- RNP edited *USP42* clones eletropherograms - A) Clone P1C12; B) Clone P2F5; C) Clone P2F12; D) Clone P4C5; and E) Clone P5D2.

Table 6 - Mutation Surveyor DNA Variant Analysis software results of the eletropherograms of the CRIPR-RNP *USP42* clones most likely to be *USP42* knockouts

USP42 CRISPR-RNP Clone	Genetic alterations detected by the software
P5D2	30064_30065_ins: TGATGATGATGA 30165_30166_ins: CSAAACTCCKGTGTAATGCTGCTKGGGCTTGGTGGTTTCARAGAGGGGCTTATTTTG CAAGGCGTGAGGCCACCTGAGTGGCCAGCARAGTTGTATGCM
P1C12	30064_30065_ins: TGATGATGATGA Other genetic alterations
P2F12	30065_del_C 30065_30066het_ins: TGATGATGATGAC
P4C5	30064_30065het_ins: TGATGATCATAC 30069_30070het_ins: ATATGACCTGGT
P2F5	30064_30065het_ins: TGATGATCATAC Several other genetic alterations are present.

This analysis was performed in replicates. In some eletropherograms, allele separation was not possible due to low signal.

4.6.2.6. USP42 mRNA expression on *USP42* clones

A preliminary analysis of *USP42* mRNA expression was performed to assist clone selection for futures assays. Only slight variations on *USP42* mRNA expression were found in the five tested clones in comparison to the parental cell line. *USP42* mRNA was less expressed in P1C12 and P2F5 cell clones when comparing to the parental cell line, more expressed in P2F12 and P4C5 and maintained in the P5D2 clone (**Figure 11**). No studies with Negative control e CDK4 controls were performed at this point, only a preliminary assessment in comparison to the parental cell line was conducted.

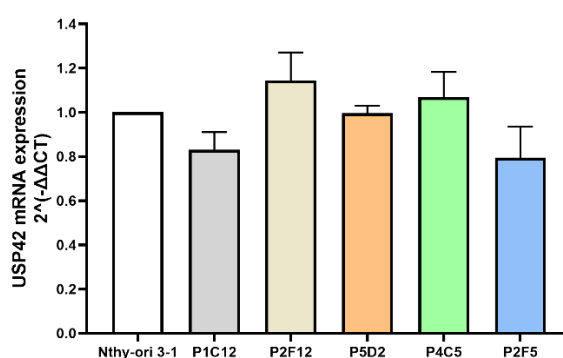


Figure 11 – *USP42* mRNA expression of CRISPR-RNP *USP42* edited clones in comparison to the parental cell-line. Only one experiment with two experimental replicates is presented in the graph. RT-PCR was performed in triplicates. No statistical analysis was conducted.

4.6.2.7. USP42 protein expression evaluation

USP42 protein expression was altered in the tested clones (**Figure 12, A**), despite mRNA expression being similar between clones and parental cells (**Figure 11**). Clones P5D2 and P4C5 showed the lowest USP42 protein expression, when comparing to parental cells and remaining clones (**Figure 12, A**). A decrease in USP42 protein expression was also observed in clone P2F12 (**Figure 12, A**). On the other hand clones P1C12 and P2F5 showed higher protein expression than parental cells. These results represent a preliminary analysis, since only one experiment using two replicates was performed. Expression estimates were calculated using total protein quantification on Ponceau (**Figure 12, B**).

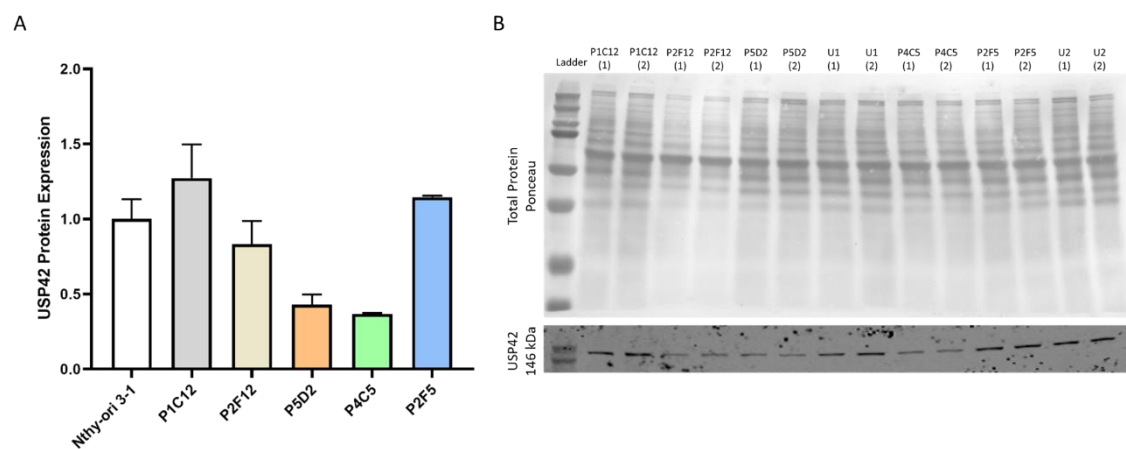


Figure 12 – A) USP42 protein expression of CRISPR-RNP *USP42* edited clones in comparison to the parental cell-line. Only one experiment with two experimental replicates is presented in the graph. No statistical analysis was conducted. B) Expression estimates were calculated using total protein quantification on Ponceau.

4.6.2.8. β -actin protein expression evaluation

In **ARTICLE V**, we demonstrate that structural proteins, like β -actin are altered upon siRNA silencing of *USP42* gene. To verify if the same would happen in CRISPR-RNP edited clones, we evaluated β -actin expression by western blot (**Figure 13**). The following results represent a preliminary analysis, since only one experiment using two replicates was performed. We show that P5D2 clone seems to overexpress β -actin, when comparing to parental cells (**Figure 13, A**). Clone P2F5 β -actin expression was similar to parental cells despite showing a high standard deviation, however, clones P1C12, P2F12 and P4C5 presented underexpression of β -actin when comparing to parental cells (**Figure 13, A**). Expression estimates were calculated using total protein quantification on Ponceau (**Figure 13, B**).

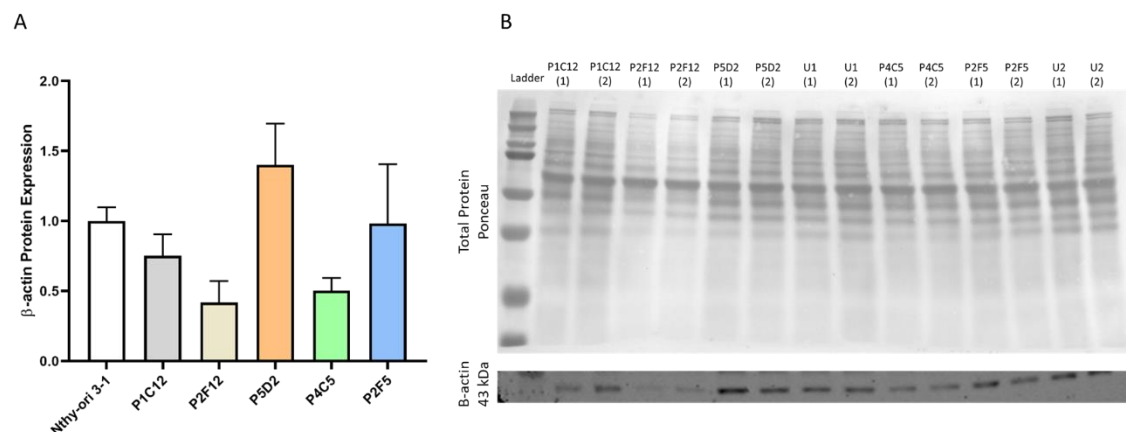


Figure 13 – A) β-actin protein expression of CRISPR-RNP *USP42* edited clones in comparison to the parental cell-line. Only one experiment with two experimental replicates is presented in the graph. No statistical analysis was conducted. B) Expression estimates were calculated using total protein quantification on Ponceau.

4.6.2.9. Cell cycle-related protein expression evaluation

Same as in **ARTICLE V**, we aimed to assess if *USP42* clones presented altered expression of cell cycle-related proteins. The following results represent a preliminary analysis, since only one experiment using two replicates was performed. We verified that clones P2F12, P4C5 and P2F5 presented lower expression of p53, when comparing to parental cells (**Figure 14, A**). Regarding Cyclin D1 analysis, clone P5D1 presented overexpression of Cyclin D1 when comparing to parental cells or any other clone (**Figure 14, B**). A slight increase of Cyclin D1 expression was seen in clone P2F12 and a decrease of protein expression was verified in clone P2F5 (**Figure 14, B**). p21 and p27 protein expression was markedly increased in clones P1C12, P2F12 and P5D2 (**Figure 14, C and D**). Expression estimates were calculated using total protein quantification on Ponceau (**Figure 14, E**).

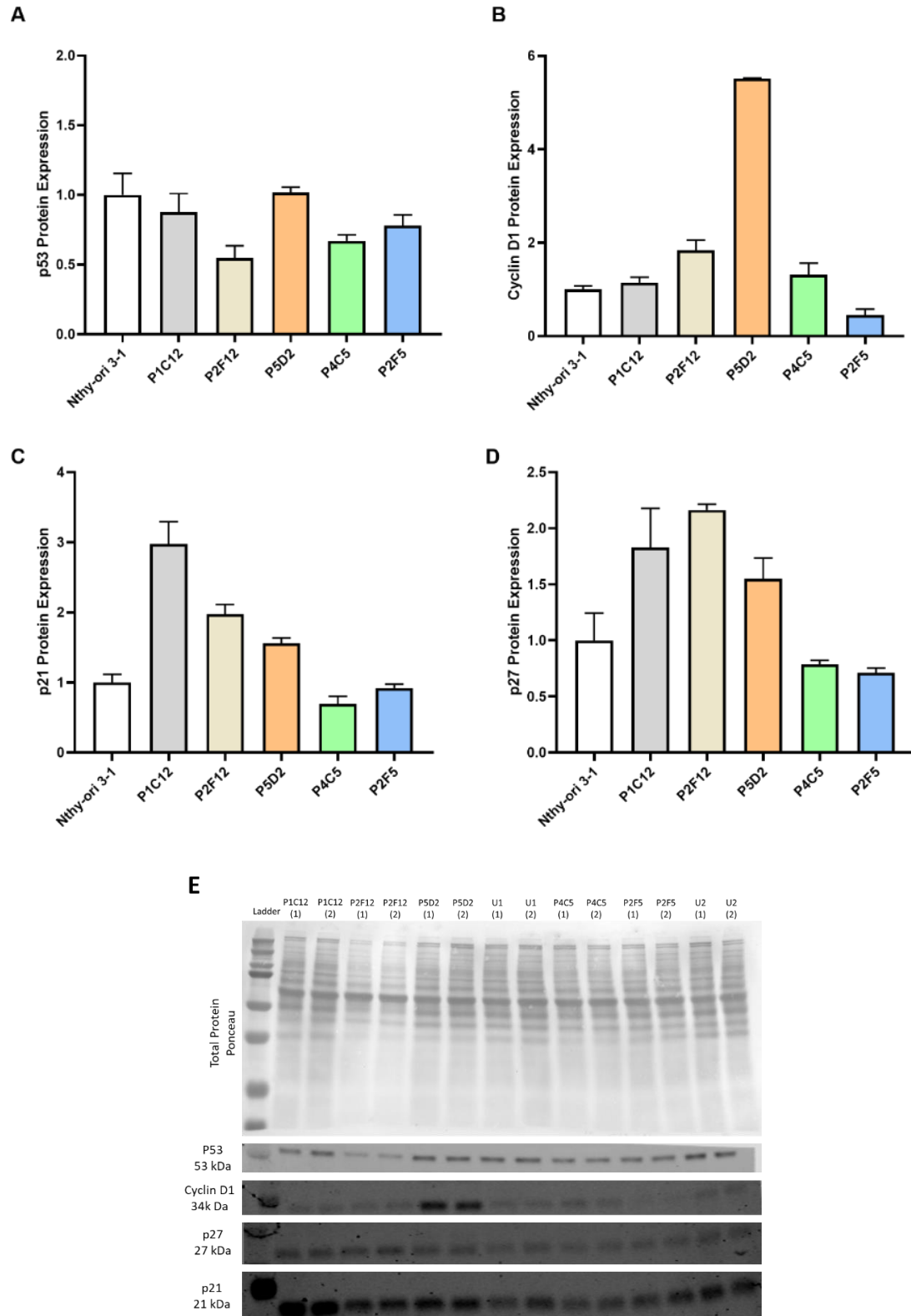


Figure 14 – Cell cycle-related protein expression of CRISPR-RNP *USP42* edited clones in comparison to the parental cell-line. A) p53 protein expression; B) Cyclin D1 protein expression; C) p21 protein expression; D) p27 protein expression. Only one experiment with two experimental replicates is presented in the graph. No statistical analysis was conducted. E) Expression estimates were calculated using total protein quantification on Ponceau.

4.6.3. DISCUSSION

USP42 variants had not been described as cancer drivers but rather as benign variants or variants of uncertain significance, according to IntOGen and gnomAD databases [203, 204].

Although the specific *USP42* *p.(Gly486Arg)* variant is described in IntOGen, gnomAD, Essembl and Clinvar databases as a variant of *uncertain significance*, no other data regarding this variant or its pathogenesis were found [203-206]. In thyroid tumors, only ten missense *USP42* variants are described on COSMIC, none of which is the *p.(Gly486Arg)* variant found in family R [207].

In the next paragraph, we will deepen the results obtained in the TCGA database. Our literature search, together with bioinformatic analysis, showed that USPs, other than *USP42* may be implicated in thyroid cancer. When searching the TCGA database, *USP9X* and *USP6* were the most frequently encountered altered USPs in Papillary thyroid carcinoma. *USP9X* is well-described in several forms of cancer, including TC [208, 209]. However, due to the complex nature of *USP9X* as both a proto-oncogene and a tumor suppressor gene, many questions remain to be answered [208]. In TC, in the context of a RET rearrangement, *USP9X* expression was higher in the primary tumors and in metastatic lesions when comparing to respective normal tissues, indicating this protein as an attractive strategy to combat RET-mediated oncogenesis [209]. In the context of prostate cancer, *USP9X* has been identified as a promoter of cell invasion and migration, through the regulation of MMP9 and activation of DRP1 [210, 211]. In breast cancer, *USP9X* was described to regulate cell cycle through CDC123 protein [208]. In hepatocarcinoma, *USP9X* targeting using miRNA-26, lead to inhibition of epithelial-to-mesenchymal transition [212]. In melanoma, this gene was proposed to have therapeutic implications [213]. *USP9X* was shown to prevent Ets-1 proteasomal degradation, indirectly resulting in increased NRAS expression, having an impact in the regulation of the MAPK and PI3K/AKT pathways [213].

Despite being present in TCGA database, no findings of *USP6* role in TC were found in the literature. *USP6* rearrangements were described in aneurysmal bone cyst, nodular fasciitis, among other diseases well-described by Teramura *et al.* (2019) [214, 215]. *USP6* presents many fusion partners all of them promoting transcriptional activation [215]. Despite presenting rapid growth, these neoplasms usually follow a benign clinical course and spontaneous regression has been described [215]. Several other punctual USPs alterations were found in this database search, but due to their low frequency, these won't be further explored in this thesis. However, we would like to emphasize that the majority of USP mutated cases also presented *BRAF* mutations (76%) and several other sporadic alterations are also present. This further supports

the notion that NSFNMTTC may be a polygenic disorder, in which key genes confer susceptibility to develop TC but sporadic mutations may be the “*second hit*” that promotes tumor formation.

In family R we detected a sporadic hotspot *HRAS* mutation in the proband, however, this alteration was not present in the other family members. No other sporadic alterations were indicated as possible causes of disease in our evaluation.

In familial cases, such as in Family R, tumors with oncocytic component are frequently described (OA, OCA, Oncocytic PTC) [179, 181, 184, 198]. This led us to wonder if *USP42* could be associated with mitochondrial changes, but no information regarding this interaction was found in the literature. However, other USPs were demonstrated to regulate mitochondria. *USP30*, a mitochondrial DUB located within the mitochondrial outer membrane, plays a role in enhancing mitochondrial fusion while suppressing fission and arrests mitochondrial clearance via mitophagy [216, 217]. Knockdown of *USP9X*, in prostate cancer cells was shown to induced mitochondrial fission [210, 211]. *DRP1*, a mitochondrial fission protein, was shown to be regulated by *USP9X* [210]. In thyroid cancer, our group has previously shown that the active (phosphorylated) form of *DRP1* (S616-p-*DRP1*) may be a molecular marker of malignancy in TC and in patients' risk assessment [218]. *USP15* was also described to antagonize mitophagy in the liver [219, 220].

To obtain a better knowledge of *USP42* protein role on thyroid cancer, after silencing assays (**ARTICLE V**) we proceed to perform CRISPR-RNP technique in which we inserted four STOP codons in Valine 57 of *USP42* exon 2 to arrest protein translation. *USP42* CRISPR-RNP edited clones showed different morphologies when comparing to Nthy-ori 3-1 parental cells. Some cells presented an elongated morphology not typical of parental cells. Despite STOP codon insertion, all clones presented with *USP42* mRNA expression, however, when looking at *USP42* protein expression, the latter was reduced in some clones while being higher in other clones when comparing to parental cells. Selected clones also demonstrated variable β -actin protein expression, thus supporting the observation of altered morphology. Cell cycle-related proteins were also differently expressed in all clones.

As limitations of the phenotypic evaluation of the clones resulting from the CRISPR-RNP assay, we recognize that only two experimental replicates are presented for protein assessment and therefore, we present only a preliminary analysis of the top five clones selected for the study. Each one of the five clones was validated at least three times by Sanger sequencing analysis, followed by electropherogram evaluation. Due to low signal in some electropherograms we were not able to analyze both alleles separately, leaving some doubts relating to other molecular alterations resulting from CRISPR-RNP editing.

Our software analysis leads us to hypothesize that 1 - *USP42* mutation may be present in heterozygosity, hence the clones present *USP42* mRNA and protein expression, given that one allele may be sufficient for gene expression; 2 - our selected sgRNA may have lack of specificity, resulting in several off-targets; 3 - we may have to consider that we might have impure clones suggesting that more than one clone might have been selected in the well during clonal selection assays. This could be solved by a new clonal selection on already selected clones or by performing a new transfection with fluorescent sgRNAs to ameliorate and accelerate the clonal selection process. For the already selected clones, new Sanger sequencing analysis with higher concentration of ABI Prism Big Dye Terminator kit v3.1 Cycle Sequencing may lead to better electropherogram signal and software evaluation to help determine if both DNA sequences present in electropherograms are in fact due to heterozygosity or due to clone impurity.

Overall, we can conclude that no *USP42* Knockout clone was obtained, however, more than one Knockdown clone was achieved through CRISPR-RNP methodology. Morphologic and cell-cycle alterations were induced in CRISPR clones that might be interesting to explore. Further analysis conducted in *USP42* CRISPR-RNP clones will be compared to Negative Control transfected cells. Of note, *CDK4* pool of cells 2 (Flask 2) presented with 75% prediction of frameshift being classified as a good KO for this gene. This demonstrated that cell transfection was effective and further provided us with an interesting cell line to evaluate the consequences of *CDK4* depletion on thyroid cells. *CDK4* inhibitors have been indicated as putative therapy in cancer [221, 222].

If possible, in future assays and to complete our work, CRISPR-RNP will be used to transform Nthy-Ori 3-1 cells to harbor the specific SNP that was found in Family R by WES. This will allow us to understand the true impact of the *USP42* *p.(Gly486Arg)* missense mutation in thyroid cell biology.

4.7. EVALUATION OF THE PATHOGENIC ROLE OF *KCNB2* AS A PUTATIVE GENE IN NSFNMTC (in development)

4.7.1. INTRODUCTION

Our team employed WES in a family (Family C) diagnosed with FNMTC and detected a new potentially pathogenic germline mutation in the *KCNB2* gene [*p.(Gly106Arg)*], which codes for the ion channel kv2.2, a voltage-gated potassium channel (vgKCN).

Ion channels activity is vital for development and homeostasis of almost all living organisms and ion channels are virtually present in all cell types, establishing a membrane potential [223, 224]. Ion channel's implication in cellular morphology, differentiation and migration has been recognized [224].

Particularly, vgKCNs channels are tetrameric proteins of α subunits that form aqueous pores on plasmatic membranes, mediating K^+ ions flow upon an action potential generation [225] (**Figure 15**). The active site is composed of conserved TVGYG motifs highly specific for K^+ ligation. These channels are divided into different classes according to their structure and function (**Figure 15**). Specifically, vgKCNs comprise a family of 40 genes further subdivided into 12 subfamilies [226]. These are composed of 6 subunits of α -helices from which S1-S4 helices form the voltage sensor domain and S5-S6 helices form the pore-forming domain (**Figure 15**). Positively charged aminoacids at the S4 subunit make the vgKCN sensitive to electric charge [224, 225, 227] (**Figure 15**). *KCNB2 p.(Gly106Arg)* missense mutation is predicted to be located in the protein's tetramerization domain (T1) [228]. T1 promotes channel assembly and hinders the assembly of homo- and heterotetramers by obstructing tetramerization between subunits that are incompatible [228]. Other than affecting channel assembly, alterations in T1 domain could result in impaired ion gating, alterations of cytoskeletal dynamics, altered protein targeting for ubiquitination and alterations in transcription [229-231].

In **Figure 16**, we show a map of the location of the 40 vgKCN genes. Of note, some of this tetrameric channels are functional by the assembly of four α subunits of themselves (e.g. kv2.2) however, other channels are gating modulators/modifiers, that must be associated with other subunits to be functional [232]. However, the kv2.2 channel can form heterodimers and be modulated by its family channel kv2.1 (*KCNB1* gene) and with the silent subunits kv9.1 (*KCNS1* gene) and kv9.2 (*KCNS2* gene) [232].

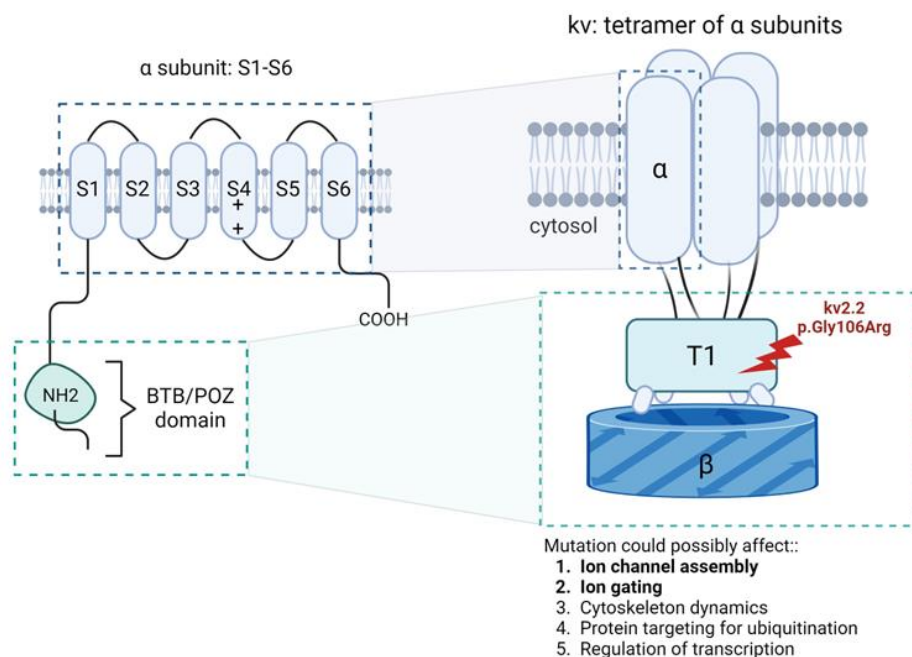


Figure 15 – kv2.2 channel and predicted mutation location. *KCNB2* p.(Gly106Arg) missense mutation is predicted to be located in the protein's tetramerization domain (T1) [228]. T1 promotes channel assembly and hinders the assembly of homo- and heterotetramers by obstructing tetramerization between subunits that are incompatible [228]. Other than affecting channel assembly, alterations in T1 domain could result in impaired ion gating, alterations of cytoskeletal dynamics, altered protein targeting for ubiquitination and alterations in transcription [229-231]. Created with Biorender.com

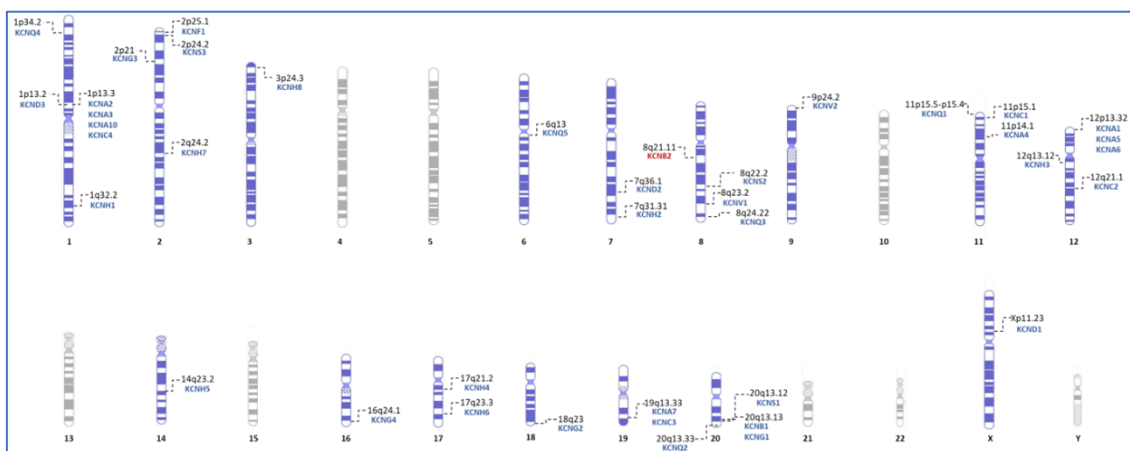


Figure 16 – vgKCns location in the human genome. Chromosome images were collected from NCBI Genes and Disease (<https://www.ncbi.nlm.nih.gov/books/NBK22266/>) . Gene localization was obtained in NCBI Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/browser/genome/?id=GCF_000001405.39) and HUGO (<https://www.genenames.org/tools/search/#!/all?query=kcn>) databases.

Several lines of data are supportive of vgKCns alterations to be involved in cancer [233-235]. Interestingly, mice studies have shown that the disruption of the *KCNE2* subunit impairs I^- uptake by the cell, therefore leading to hypothyroidism or FND [236-238].

In the thyroid parenchyma, NIS function is fundamental for the entering of ^{131}I in the tumor cell which constitutes the standard treatment for TC, as previously discussed in chapter 1.6.5. [34] (**Figure 17**). We hypothesize that vgKCns alterations may indirectly impair I^- uptake by

the follicular cells, leading to higher levels of TSH on the blood and resulting in cellular proliferation [231, 239, 240]. Secondly, vgKCN alterations may explain why some patients with sporadic TC lose the capacity to intake I^- and to respond to RAI therapy. This way, by indirect effect on NIS function, vgKCN mutations may increase predisposition to TC and maybe explain why some patients do not respond to RAI therapy. If our hypothesis is verified, it can have therapeutic repercussions and introduce vgKCNs as new pharmacological targets in TC therapy.

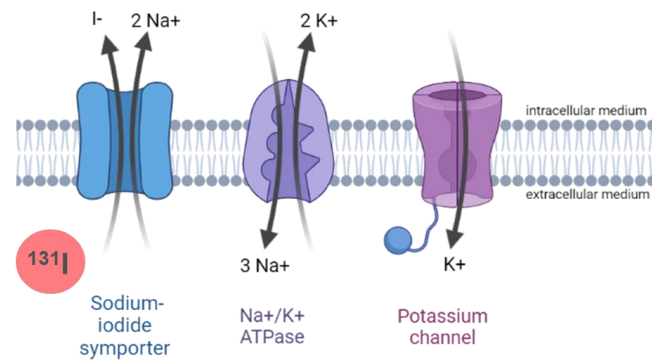


Figure 17 – Normal ionic conductivity in thyroid follicular cells. Cell homeostasis is tightly regulated by the ionic conductivity and membrane potential. The disruption of a vgKCN channel may be sufficient to impact the other channels nearby, namely, the Sodium-iodide symporter (NIS), which is fundamental for thyroid hormone metabolism. This mechanism was previously studied by Purtell et al. (2012) [238] that showed that the disruption of the potassium channel KCNE2, resulted in defaulted iodide uptake by the follicular thyroid cell. Since NIS function is fundamental for the entering of radioiodine (^{131}I) in the tumor cell, its function must be maintained to assure the efficacy of thyroid cancer standard treatment.

The innovative nature of our proposal comes from genetically and functionally addressing vgKCNs as novel targets for TC susceptibility. To our knowledge, how *KCNB2* (8q21.11) or other vgKCN mutations can affect thyroid cell proliferation/differentiation and cell malignant potential was never studied in TC.

4.7.2. MATERIALS AND METHODS

4.7.2.1. Family presentation

Family C is comprised of 15 relatives, in which, five were diagnosed with FNMTc between the age of 26 and 38 years, and two were submitted to thyroid surgery of unknown diagnosis. A germline missense mutation that segregated within the family was found in the *KCNB2* gene [*p.(Gly106Arg)*]. *KCNB2* encodes a voltage-gated potassium channel (vgKCN) and was found in all affected family members, while being absent in the tested healthy individuals and random population controls (**Figure 18**).

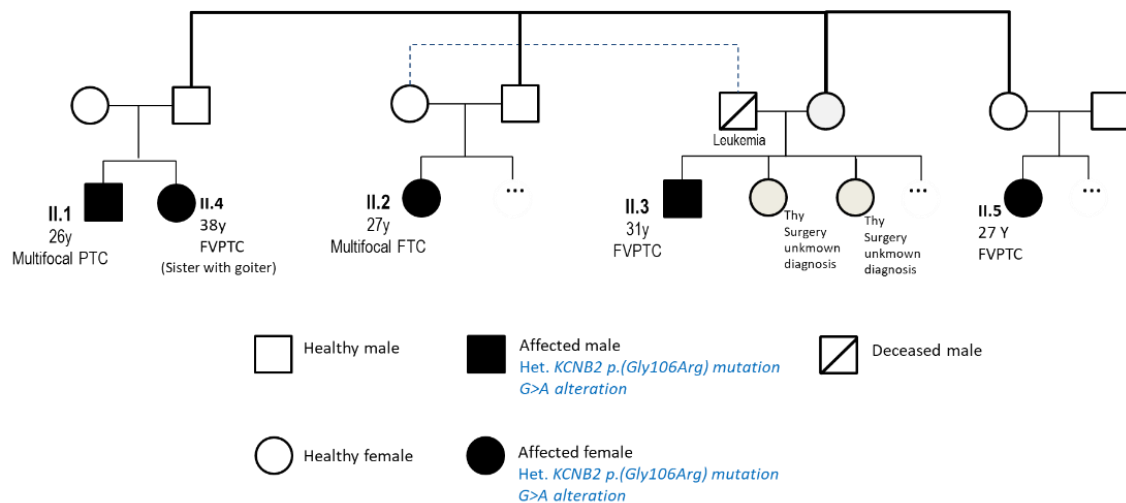


Figure 18 - Heredogram of family C. Our team had access to information of two generations reaching a total of 15 relatives, in which, five were diagnosed with FNMTCT between the age of 26 and 38 years, and two were submitted to thyroid surgery of unknown diagnosis. Benign (FND) and malignant lesions (PTC- Papillary Thyroid Carcinoma; FVPTC – Follicular Variant of Papillary Thyroid Carcinoma, non-otherwise specified; FCT – Follicular Thyroid Carcinoma) were found in the family. The heterozygous missense *KCNB2* p.(Gly106Arg) segregated within the family. Het. – Heterozygous. Of note: Tumor classification was performed before the 2022 WHO update, according to the 4th edition of the WHO Classification of Thyroid Tumors [241].

4.7.2.2. *In silico* studies

The same *in silico* methodology, as the one described in **ARTICLE V** was applied to investigate vgKCN mutations in thyroid cancer. Bioinformatic analysis was conducted on The Cancer Genome Atlas (TCGA) base (<https://portal.gdc.cancer.gov/>) and in a *in house* database. When possible, clinicopathological variables were analyzed according to vgKCN mutation status on TCGA database. Statistical analysis was conducted using parametric or non-parametric tests, when appropriate. For some categories, cases presented missing information (e.g. age at diagnosis – 2 cases). Other online databases namely IntOGen [204], gnomAD [203], COSMIC [207], Ensembl [205] and ClinVar [206] were searched in order to verify mutation status and effect prediction.

4.7.2.3. *In vitro* studies

4.7.2.3.1. FRTL-5 cell line

FRTL-5 cells and respective clones were cultured at 37°C with 5% CO₂ in Nutrient Mixture F-12 Coon's modification (F6636, Sigma) and supplemented with 10µg/ml Insulin (I9278, Sigma-Aldrich, USA), 10nM Hydrocortisone (H0888-1G, Sigma-Aldrich, USA), 5µg/ml apo-Transferrin (T2036, Sigma-Aldrich, USA), 10ng/ml Gly-His-Lys acetate (G1887, Sigma-Aldrich, USA), 10ng/ml Somatostatin (S9124, Sigma-Aldrich, USA), 5% of heat-inactivated Foetal Bovine Serum (FBS) (Gibco), 1mU/ml TSH. Penicillin-Streptomycin (L0022-100, Biowest) and Fungizone

(30-003-CF, Corning™ Amphotericin B, Fisher Scientific, UK) were added at prophylactic concentration.

FRTL-5 cells do not naturally express the *KCNB2* gene, therefore these were previously transfected with human WT and Mutated *KCNB2* gene, using the overexpression pcDNA3.1+/C-(K)-DYK Vector (GenScript, USA). Two clones were obtained: *KCNB2* wild-type (WT) (OHu25595, GenScript, USA), and *KCNB2* Mutated (Mut) (OHu25595M, GenScript, USA), in which the specific *KCNB2* *p.(Gly106Arg)* mutation detected in Family C was inserted. Empty vector (negative control) was achieved by the insertion of the pCMV6-AC-Myc vector (PS100003, OriGene) available in our lab, since it presents the same backbone as the pcDNA3.1+/C-(K)-DYK Vector.

4.7.2.3.2. FRTL-5 transfected cells clonal selection.

Transfected cells were treated with the selection antibiotic G418 (Geneticin) at the concentration of 0.4 mg/mL (Invitrogen, France) and then maintained at 0.2 mg/mL to prevent non-transfected clones from growing. Cells were counted on a Neubauer chamber and subsequently diluted until reaching an estimated dilution of 8 cells/mL. Resuspended cells were then distributed through five 96-well plates, at a volume of 100 µL/well. Surviving clones were maintained and grown up to T75 flasks where at this point cells were harvested, and cell pellets were collected for DNA extraction and storage at -80°C.

4.7.2.3.3. *KCNB2* edited cells DNA extraction

Cell DNA was extracted using the GRS Total DNA Kit – Blood & Cultured Cells (GrisP, Porto, Portugal), following the manufacturer's instructions. DNA concentration, sample purity and quality were analyzed by spectrophotometry, as previously described in **ARTICLE II**.

4.7.2.3.4. PCR and Sanger Sequencing validation of *KCNB2* transfected cells

DNA from candidate clones was analyzed by Polymerase Chain Reaction (PCR) using the QIAGEN multiplex PCR kit (QIAGEN, Hilden, Germany) and by Sanger sequencing analysis, as previously described [215]. Utilized primers for clonal selection are depicted in **Table 7**. All alterations were confirmed by performing two new and independent analyses. All PCR reactions were conducted in the SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA).

Table 7 – Characteristics of *KCNB2* and *pCMV6* primers used for clonal selection – primer sequence, amplicon size and annealing temperature.

Primer	Primer sequence	Amplicon size (bp)	Annealing temperature
<i>KCNB2_hotspot Forward</i>	5' - AACCACGAAGTCCTGTGGAG - 3'	364	54°C
<i>KCNB2_hotspot Reverse</i>	5' - TCTTCTCCTTCTCGCTCTCG - 3'		
<i>KCNB2_middle_ORF Forward</i>	5' - CATTGTGCTTTCCACCATTG - 3'	194	60°C
<i>KCNB2_middle_ORF Reverse</i>	5' - TCAGTGGGCCTTTGAAGAAC - 3'		
<i>KCNB2_end_ORF Forward</i>	5' - GCCACCGACAGTCCTAAGAG - 3'	150	60°C
<i>KCNB2_end_ORF Reverse</i>	5' - CGAAAAGTCAGCTGTGGTGA - 3'		
<i>pCMV6 Forward</i>	5' - CTCAGTGACAACGTCGAGC - 3'	254	52°C
<i>pCMV6 Reverse</i>	5' - TGAACAAGATGGATTGCACG - 3'		

4.7.2.3.5. FRTL-5 proliferation assessment

Proliferation assessment was performed by plating the Empty vector (negative control) cells at 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 7.5×10^4 , and 1×10^5 cells/wells. Only negative control cells were used at this point due to similar growth in all clones. Cells were observed at 24h, 48h and 72h post-plating, harvested and counted on the Z2 Coulter - Particle Count and Size Analyzer (Beckman Coulter Diagnostics, USA).

4.7.2.3.6. RNA extraction and cDNA conversion

RNA was extracted using the GRS Total RNA Kit – Blood & Cultured Cells (GrisP, Porto, Portugal), following the manufactures' instructions. RNA concentration, sample purity and quality were analyzed by spectrophotometry, as previously described in **ARTICLE V**. 1000 ng of RNA were converted to cDNA by Reverse Transcriptase reaction upon DNase I and EDTA treatments. Samples were amplified and converted, using the SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA) at 25°C for 10 minutes, 42°C for 60 minutes and 70°C for 10 minutes.

4.7.2.3.7. Real-Time quantitative Polymerase Chain Reaction

Human *KCNB2* (Hs.PT.58.39309562, Integrated DNA Technologies - IDT, USA), rat *slc5a5* (nis) (Rn.PT.58.10552131, Integrated DNA Technologies - IDT, USA), and rat *tshR* (Rn.PT.58.44590530, Integrated DNA Technologies - IDT, USA) RNA expression was accessed, using rat *tbp* (Rn.PT.39a.22214837, Integrated DNA Technologies - IDT, USA) as our endogenous control gene. cDNA was amplified using the following cycle: 50X (95° for 10 min, 95 for 15 sec and 60° for 1 min, for TSH and 58°C for 1 min for *slc5a5* and *Tpo*). Quantification of mRNA expression was performed using the $2^{-\Delta\Delta Ct}$ method.

4.7.2.3.8. *Mycoplasma spp.* contamination elimination

Routine mycoplasma *spp.* assessment revealed *Mycoplasma spp.* contamination of transfected cells. Cells were treated with Plasmocin® - Mycoplasma Elimination Reagent (Invivogen, France) at the concentration of 25 µg/mL in complete medium for 15 days. Treatment medium was changed all 2-to-3 days and cells were trypsinized always below a confluence of 40%. After treatment, cells were left in T25 flasks for 7 days with Plasmocin, penicillin-streptomycin and fungizone free-medium until full confluence. Medium was collected for mycoplasma *spp.* PCR testing. Upon positivity, treatment was repeated and Plasmocin concentration was raised for 37.5 µg/mL, but resistance to treatment was still verified. Plasmocure® - Mycoplasma Elimination Reagent (Invivogen, France) used to treat mycoplasma *spp.* resistant strains, was applied to at 50 µg/ml for 3 weeks, as indicated by the manufacturer.

4.7.3. RESULTS

4.7.3.1. *In silico* results

In IntOGen database, *KCNB2* mutations are not described as “cancer drivers” [204] In gnomAD, *KCNB2* alterations are classified as variants of uncertain significance (VUS), benign variants or probably benign variants [203].

Alterations of vgKCN were searched on the TCGA database from Cell, 2014 [242] and in an *in-house* database composed of oncogenic tumors (OA and OCAs) (**Figure 19**). In TCGA, 19/488 (4%) of the cases presented pathogenic alterations in vgKCNs, with *KCNH2* (7q36.1), *KCNH1* (1q32.2) and *KCNIP4* (4p15.31-p15.2) being the most frequently altered channels (**Figure 19**). In our oncogenic tumors database 3/18 (17%) of the cases presented vgKCN mutations (**Figure 19**). One patient presented with homozygous deletion on *KCND3*, *KCNC4*, *KCNA2* and *KCNA10* channels, all located in cytogenetic band 1p13.2-1p13.3. The other two patients showed amplification of *KCNIP2* (10q24.32) and deletion of *KCNE3* (11q13.4) channels (**Figure 19**). No *KCNB2* pathogenic mutations were found in any of the databases.

The specific *KCNB2* p.(Gly106Arg) mutation described in Family C was previously described as a somatic mutation in prostate cancer, as result of an WES analysis, but no prediction of the mutation's impact or its clinical significance is described [243]. In thyroid, only the *KCNB2* p.(T666=) (c.1998G>T) silent mutation is described [207].

Altered vgKCNs cases also presented *BRAF* hotspot mutation in 11/19 (58%) of the cases and *NRAS* alterations in 3/19 (16%) of the cases (data not shown).

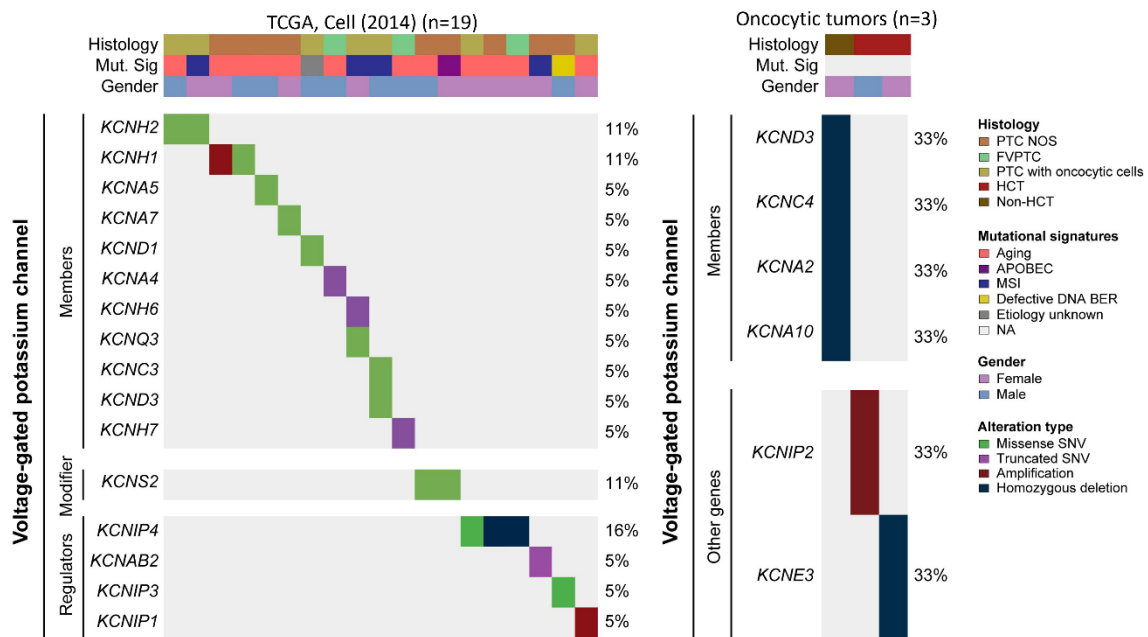


Figure 19 – vgKCN genetic alterations found in TCGA database from Cell, 2014 [221] and in an in-house database composed of oncocytic tumors (OA and OCAs).

In a broader analysis, our team searched cBioportal (<https://www.cbioportal.org/>) and to the TCGA Cell (2014) database, added the TCGA Firehose Legacy (2016) and PanCancer Atlas (2018) databases. Genes coding for 40 vgKCNs were searched. Since several cases of these databases were overlapping, we performed a detailed merge of the three databases. Duplicate/triplicate cases were excluded, collecting the most informative data for each case. Cases without clinical information were excluded from the analysis. A total of 513 cases were considered valid. Statistical analysis for patient stage, clinicopathological characteristics, and molecular status was conducted for the global TCGA database.

The series was comprised of 372 females and 139 males (two cases had missing information on sex), 410 (79.9%) tumors were histologically classified as PTC, including 37 (7.2%) Tall cell PTC, and 103 were classified as FTC (20.1%). Mean age at diagnosis was 46.1 ± 15.22 years for women and 49.9 ± 17.07 years for men ($p=0.013$).

For the 513 cases, 41 (8%) presented alterations in vgKCNs (missense mutations, amplifications, or deletions). Correlation between vgKCN-altered tumors and prognostic factors was analyzed. No statistically significant association between vgKCN-altered tumors and any of the analyzed prognostic factors depicted in **Figure 20** was found, namely: A) Patient sex, B) Clinical stage, C) Distant metastases, D) Extrathyroidal extension, E) Tumor location, F) RAI treatment, G) Disease recurrence, H) Disease-free survival, and I) Overall survival. Although no significant association was found between the presence of vgKCN alterations and patient prognosis, we observed that patients with vgKCN-altered tumors were more frequently at

disease stage IV (**Figure 20, B**) and presented more often with DM at diagnosis (**Figure 20, C**). These tumors seemed to be more frequently present in the isthmus when comparing to thyroid lobes (**Figure 20**).

At a molecular level, in vgKCN-altered cases, *BRAF* point mutations were concomitantly present in 22/41 (54%) of the cases, *RAS* point mutations in 4/41 (10%), *TERT* mutations in 1/41 (2%), and 2/41 (5%) of the cases presented both *BRAF* point mutation and *USP42* point mutations.

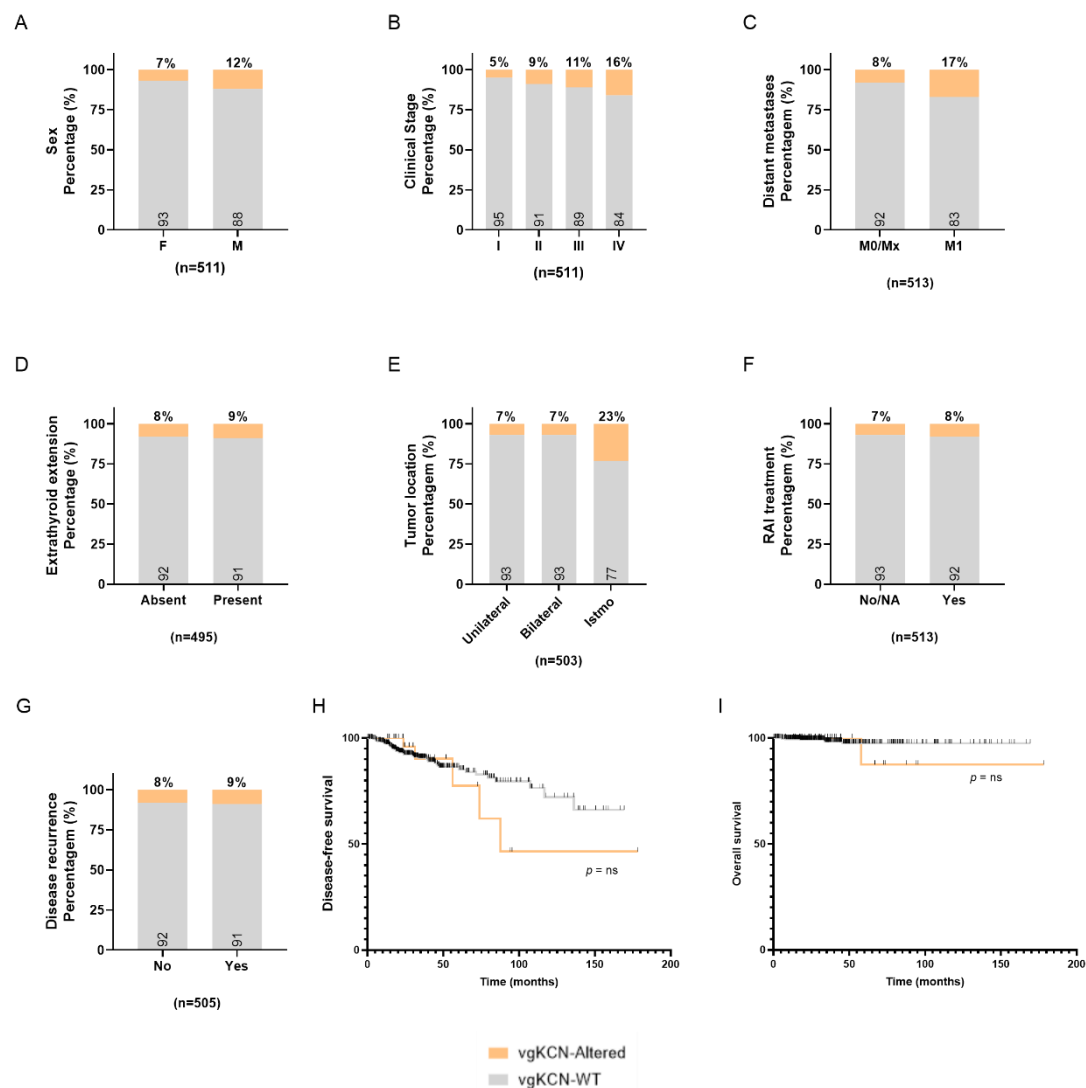


Figure 20 – Analysis of clinicopathological factors in vgKCN-altered (orange) and in vgKCN-WT cases (grey) – A) Patient sex, B) Clinical stage, C) Distant metastases, D) Extrathyroidal extension, E) Tumor location, F) RAI treatment, G) Disease recurrence, H) Disease-free survival, and I) Overall survival. No statistically significant association was found in any of the analysed prognostic factors.

4.7.3.2. *In vitro* results

4.7.3.2.1. *KCNB2* transfected cell clonal selection results

FRTL-5 cells were successfully transfected with the pcDNA3.1+/C-(K)-DYK Vector (GenScript, USA) or pCMV6-AC-Myc vector (OriGene), as shown by the electrophoresis and Sanger sequencing results in **Figure 21**. Antibiotic selection with G418 at the concentration of 0.4 mg/mL, led to very clean electropherograms, indicating almost pure clones (**Figure 21**).

To assure clone purity, after G418 selection cells were subjected to further clonal selection by the plating of one cell per well in 96-well plates. A G418 prophylactic concentration of 0.2 mg/mL was maintained to prevent non-transfected cells from growing. Surviving clones were grown up to T75 flasks, and DNA was collected for testing.

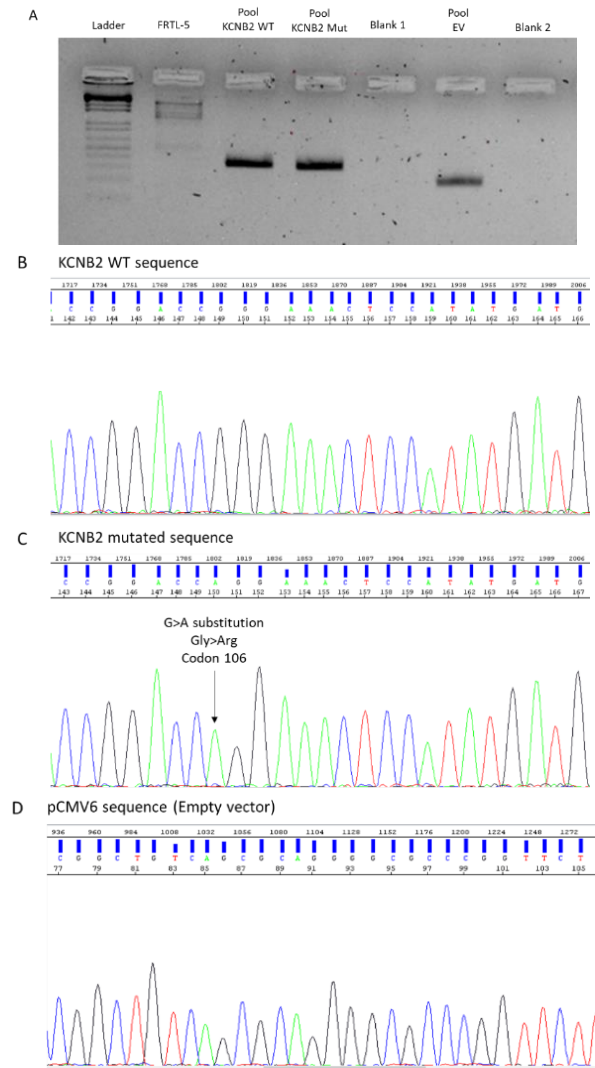


Figure 21 – Electropherograms from the analysis of transfection in the pool of cells. A) Electrophoresis results of *KCNB2* WT and Mutated sequences and of pCMV6 vector, B) *KCNB2* WT hotspot region, C) *KCNB2* Mutated hotspot region, D) pCMV6 vector representative region.

From single cell clonal selection, we obtained one Empty vector clone (Clone G5) (**Figure 22, A**), five *KCNB2* WT clones (Clones B1, B5, D5, F9 and G5) and two *KCNB2* mutated clones (Clones D3 and D8) (**Figure 22, B**). As shown in **Figure 22 (B)** the selected clones did not amplify for the hotspot region of *KCNB2*. New PCR annealing temperature's optimization was performed but unsuccessful. The middle and end regions of the inserted ORF were amplified for the pools of cells and for two *KCNB2* clones (one WT, G5, and one mutated, D8) as to test if these cells contained the vector. When comparing to the pool of cells, only the end segment of the ORF was inserted in clone *KCNB2* WT Clone G5, while in *KCNB2* Mutated Clone D8, most of the ORF was successfully inserted (**Figure 22, C**), however, our *KCNB2* target region is not present in any of the surviving clones (**Figure 22, B and C**).

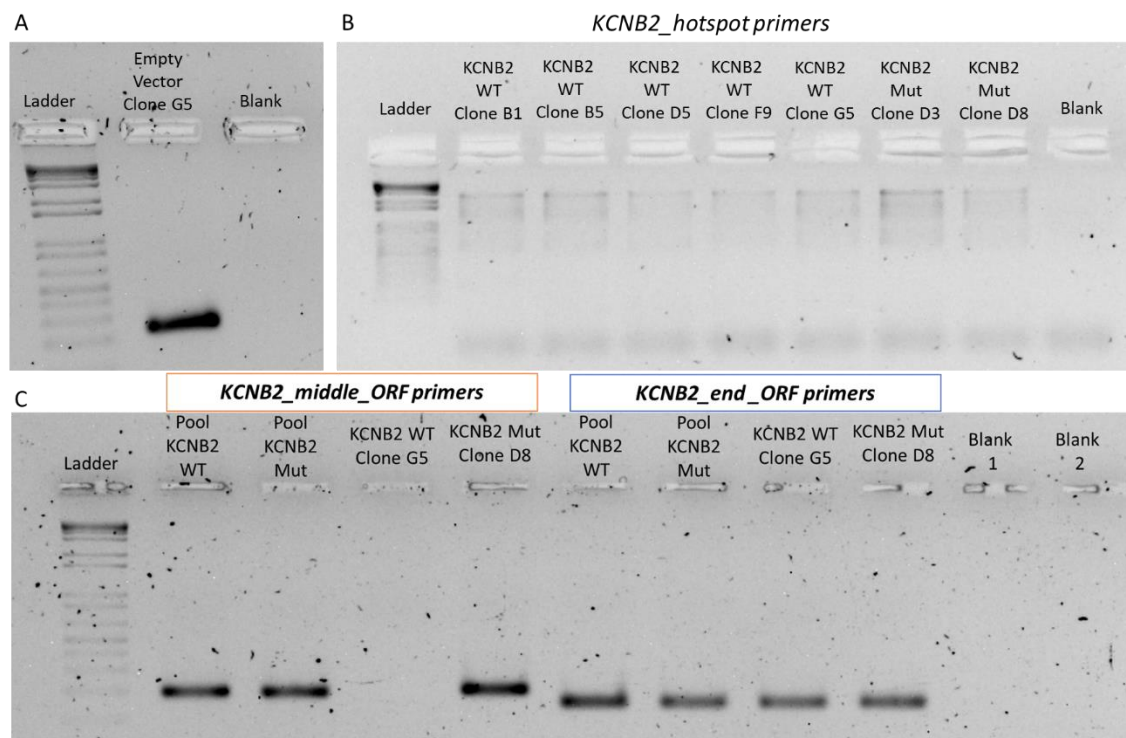


Figure 22 – Electrophoresis results of antibiotic and single cell clonal selection in 1% agarose gel. A) Empty vector clone G5 amplification, B) *KCNB2* WT and Mutated clones' amplification of *KCNB2* hotspot region, C) Amplification of the middle and end section of the ORF of *KCNB2* WT and Mutated pool of cells and of tested *KCNB2* WT G5 and *KCNB2* Mutated D8 clones.

4.7.3.2.2. Cell proliferation analysis

Cell proliferation assays showed that plating concentrations $\geq 7.5 \times 10^4$ cells/well lead to excessive cell confluence and growth arrest (**Figure 23**). The concentration of 3×10^4 cells/well was established as the optimal concentration for *in vitro* assays after cell observation and fold-

change assessment (**Figure 24**). Since cell growth was similar in all clones, the same concentration was used for the following assays.

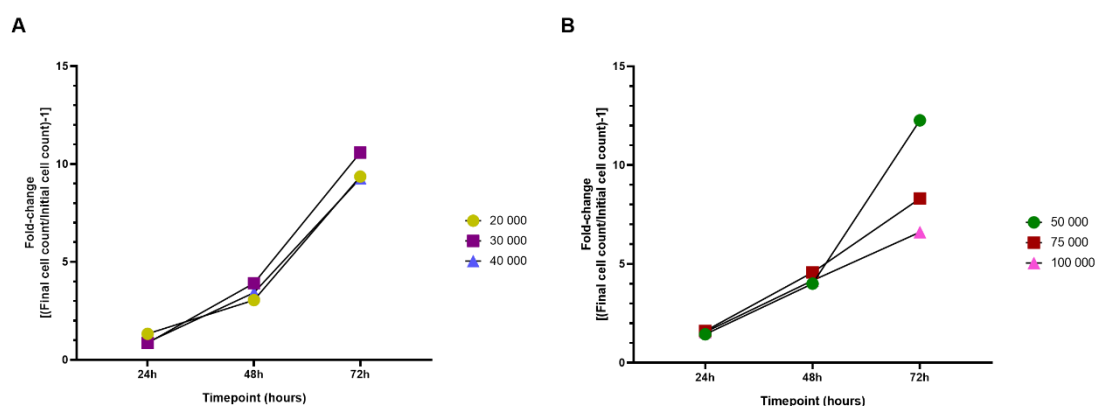


Figure 23 – Cell proliferation assay using Empty vector transfected cells. Fold change $[(\text{Final cell count}/\text{Initial cell count}) - 1]$ was calculated at 24h, 48h and 72h after plating.

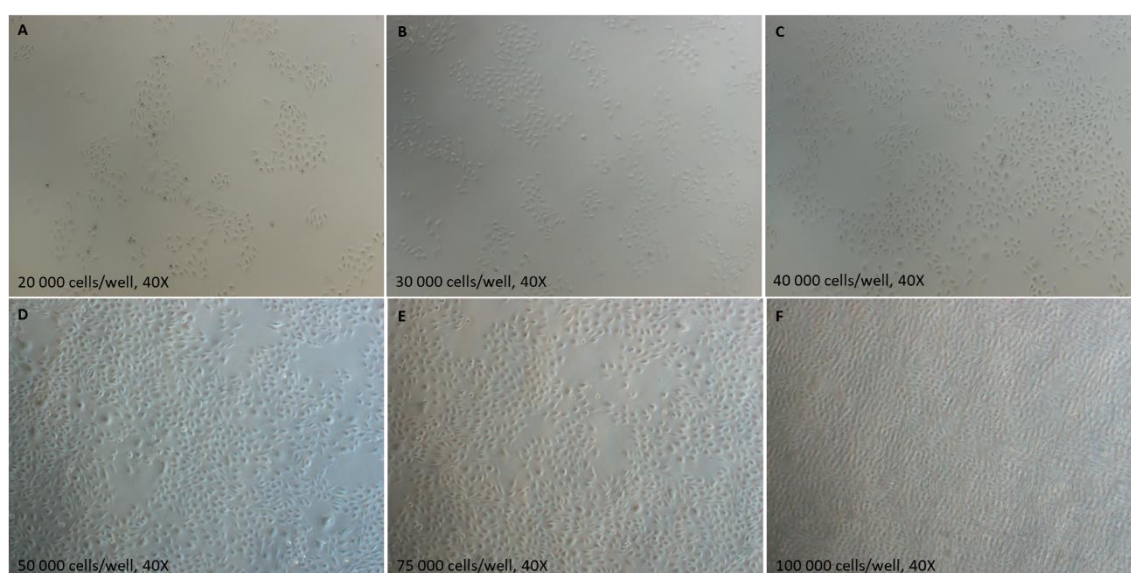


Figure 24 – Representative images of the six tested cell concentrations of Empty vector transfected cells at 48h timepoint. A) 2×10^4 , B) 3×10^4 , C) 4×10^4 , D) 5×10^4 , E) 7.5×10^4 , and F) 1×10^5 cells/well.

4.7.3.2.3. KCNB2 mRNA expression evaluation

KCNB2 mRNA expression was evaluated by RT-PCR in FRTL-5, Nthy-ori 3-1, TPC-1, 8505 and T241 parental cells lines, using HEK293 embryonic kidney cells as control, and none of the thyroid cell lines presented KCNB2 mRNA amplification (data not shown).

KCNB2 mRNA expression was evaluated by RT-PCR. *KCNB2* mutated cells (pool) showed higher mRNA expression of KCNB2 channel (8.8 times higher) when comparing to cells transfected with *KCNB2* WT segment (**Figure 25**). Given the low number of samples, no statistical significance was found between both clones.

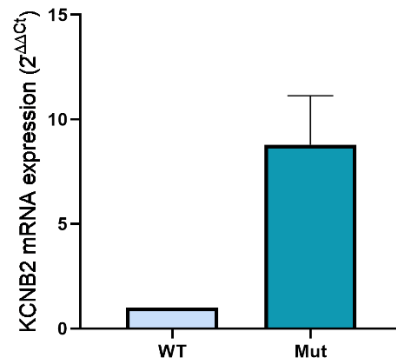


Figure 25 – KCNB2 mRNA expression evaluation. *KCNB2* mutated cells (pool) showed higher expression of *KCNB2* channel when comparing to cells transfected with *KCNB2* WT segment. The experiment was conducted using two experimental replicates. mRNA expression was assessed using triplicates. WT – wild-type; Mut – Mutated.

4.7.3.2.4. Thyroid cell markers mRNA expression evaluation

Expression of *slc5a5* was 50% higher in *KCNB2* transfected cells (pools), when comparing to negative control (Empty Vector – EV) and expression was similar between *KCNB2* WT and *KCNB2* mutated cells (**Figure 26, A**). Regarding *tsh* receptor, expression was higher in *KCNB2* transfected cells (pools), when comparing to negative control, however, *KCNB2* WT cells seem to present 74% increase in *KCNB2* expression, while mutated cells present 54% increase. Given high standard deviations, and low number of samples results must be analyzed with caution.

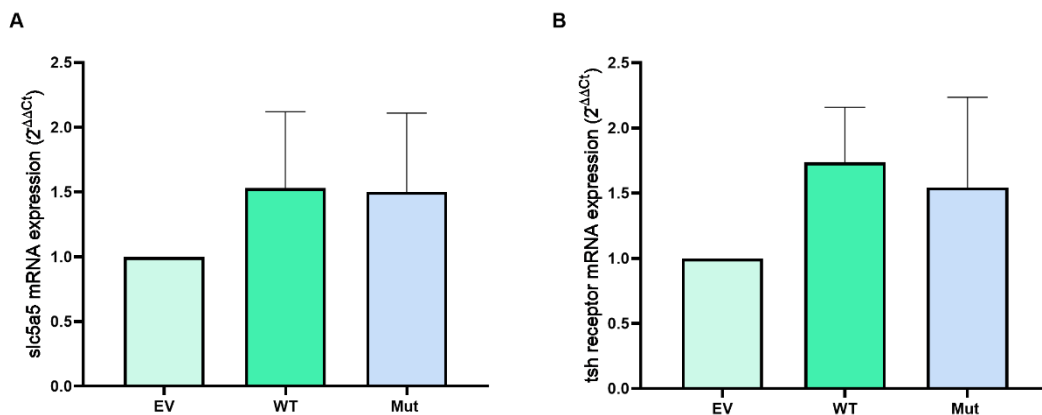


Figure 26 - Thyroid markers mRNA expression. A) Rat *slc5a5* expression on FRTL-5 transfected pool of cells, B) Rat *tsh* receptor expression on FRTL-5 transfected pool of cells. The experiment was conducted using two experimental replicates. mRNA expression was assessed using triplicates. EV – Empty vector; WT – wild-type; Mut – Mutated.

4.7.4. DISCUSSION

Most *vgKCN* alterations are characteristic of illnesses such as neurological disorders (epilepsy, ataxia, encephalopathy, hearing loss, and intellectual disability) [244-246] and cardiovascular disorders (cardiac arrhythmia syndromes) [247, 248]. In cancer, *vgKCNs* have been gaining attention over the years. High expression of *KCNH2* channel was shown to be

associated with reduced survival in glioblastoma multiforme and hepatocellular carcinoma [249]. *In vitro* studies in breast cancer, appointed KCNQ4 as a cancer marker, since channel overexpression resulted in tumor cell apoptosis and inhibited cell migration and invasion [250]. Other vgKCNs were appointed as potential therapeutic targets for breast cancer metastasis [251]. The *KCNB1* gene, that translates the kv2.1 channel was already demonstrated in prostate cancer cells to play an important role, since inhibition of this channel using stromatoxin-1 or siRNAs lead to inhibition of cell migration [252]. *KCNB1 SNP rs3331* found in a subset of Tunisian patients with colorectal cancer was shown to be associated with treatment response [253]. The study shows that patients with homozygous mutation were 4 times more likely of treatment failure than patients with the heterozygous mutation. However, another study reporting *KCNB1* in colorectal and gastric cancers showed that this channel (along with *KCNA5*) were similarly expressed in tumoral and adjacent tissues [254]. In gliomas, *KCNB1* was previously associated with malignant progression and outcome [255].

Since *KCNB1* and *KCNB2* genes (kv2.1 and kv2.2 channels, respectively) belong to the same family and may form heterotetramers, this relation can have physiological implications when one of the channels is altered [256]. As shown in our *in silico* results, *KCNB2* mutations are rare alteration in thyroid cancer. The role of *KCNB2* is not well-established or studied. To our knowledge, almost no information relating *KCNB2* alterations and cancer are found in the literature. A study in which NGS was applied in 875 breast tumors, *KCNB2* mutations were associated with luminal B breast cancer subtype [257]. *KCNB2* is highly expressed in human medulloblastoma (pediatric tumor), influencing tumor growth [258]. Knocking out *Kcnb2* in mice showed reduced replicative potential in medulloblastoma cells, hindering tumor growth [258]. Mechanistically, *Kcnb2* controls potassium homeostasis, modulating EGFR signaling and cell proliferation in medulloblastoma, being hypothesized as a therapeutic marker for this cancer [258]. In MalaCards database, *KCNB2* alterations are appointed in colorectal and extragonadal germ cell cancer, but no other reports in cancer were found [259].

Despite not finding *KCNB2* alteration in our *in silico* analysis of TCGA and in our *in-house* database, other vgKCNs possibly pathogenic alterations were found. Furthermore, these alterations seem to be concomitant with somatic mutations such as *BRAF* and *NRAS*, which could be implicated in the tumorigenic process working as a “second hit”. However, no association between vgKCNs and patient prognosis was found in our analysis.

Since other vgKCNs have been demonstrated to be implicated in carcinogenesis, and since a large gap of information about *KCNB2* gene exists, we proceed to perform *in vitro* studies.

We successfully transfected FRTL-5 cells with both WT and *KCNB2* mutated genes. Our pools of cells were almost pure after antibiotic selection. We still proceeded to perform single cell

clonal selection which was only successful for the selection of Empty vector Clone G5. *KCNB2* WT and Mutated cells did not show upon PCR amplification of the hotspot region of the mutation. Our first hypothesis was that during selection, we may have selected parental cells present in the pool. Our second hypothesis was that during proliferation, selected clones may have lost the vector. To test our hypotheses, we tested one WT and one Mutated *KCNB2* clone, amplifying more downstream regions of the inserted ORF (after the hotspot region) and compared with the pool of cells. These results showed that these clones were antibiotic resistant so probably harbored the vector, but the hotspot region was not present in the two single cell selected clones. This indicated that vector insertion of the clones that were tested was not properly performed by the cell machinery and a part of the *KCNB2* gene was probably missing. A new single-cell clonal selection may be more successful in separating the targeting clones.

Since the pool of cells was almost pure and the majority of the clones presented the intended insertions, we proceed to perform functional assays in the pool of cells. Our *in vitro* studies showed that *KCNB2* mRNA expression was notably higher in *KCNB2* mutated cells when comparing to *KCNB2* WT cells, although not statistically significant.

We performed an exploratory analysis of the expression of thyroid markers in *KCNB2* WT and Mutated transfected cells and verified that when comparing to negative control *KCNB2* cells (WT and Mutated) presented twice the expression of *slc5a5*. This is a possible indication that *nis* may be regulated by *KCNB2* expression. Furthermore, expression of this receptor was also affected by *KCNB2* expression. When comparing to negative control cells, both *KCNB2* transformed pools showed increased expression of *tsh* receptor, with *KCNB2* WT cells showing the highest increase.

We are currently continuing mRNA evaluation assays in order to validate the results from the exploratory analysis. Cell cycle, apoptosis, cell morphology and migrations assays will also be performed in short time. Additionally, our group recently acquired the Guangxitoxin-1E (GTx), a *kv2.1/kv2.2* selective inhibitor (toxin) extracted from the *Plesiophrictus guangxiensis* tarantula [260]. GTx was shown to be a very potent and specific inhibitor of the *kv2* family in a study regarding diabetes treatment [261]. In our upcoming experiments, transfected *KCNB2* WT and Mutated cells will be subjected to *KCNB2* inhibition and functional assays will be performed to assess the impact of this inhibition in *nis* function. It would also be interesting to evaluate *KCNB2* function in a human thyroid cell line, such as the Nthy-ori 3-1.

However, given that the tested thyroid cell lines do not naturally express the *KCNB2* gene, we hypothesize that the mutation found in family C may be an activating missense mutation. In the TT cell line, derived from Familial MTC (Multiple endocrine neoplasia type 2), *KCNB2* is described to be highly expressed [262].

CHAPTER V

GENERAL DISCUSSION

CHAPTER V – GENERAL DISCUSSION

Comparing to other cancers (e.g. breast, lung, colorectal), TC remains understudied, with less attention from the National Institutes of Health [72, 263]. In recent years, several improvements in TC research and translational knowledge and management have been made regarding clinicopathological factors, genetic landscape and the issue of overdiagnosis [112, 263]. However, a large gap in TC knowledge is still present when it comes to diagnosis criteria and patient management [264]. Difficulty persists in separating low-risk disease from patients at risk of recurrence, progression and PTC-related mortality. This is true for both sporadic and familial forms of the disease, with the latter posing additional challenges [263]. The complexity of TC familial forms and their genetic landscape are still puzzling researchers, highlighting the importance of specialized care, tailored treatment strategies, and ongoing research efforts to unravel the clinicopathological and molecular factors influencing the outcomes of these patients [183].

Our team explored the clinicopathological and genetic characteristics of sporadic and familial forms of TC.

As previously stated, PTC is the most frequently diagnosed TC and despite generally portraying a favorable prognosis, some cases present highly aggressive and metastatic disease [112, 241]. To contribute to better knowledge regarding patient management, our first aim (**AIM 1**) was to identify clinicopathological and molecular factors that can contribute to better predict the outcome of PTC patient's prognosis (recurrent/persistent disease, structural disease and overall survival).

Since we aimed to have a trustworthy representation of the everyday clinical practice, patient selection was performed in an unbiased way. Patients that presented with sporadic PTC diagnosis between January 2002 and December 2015 were selected from one central hospital. History of previous irradiation and familial history of TC were exclusive criteria. Our cohort was mostly composed of middle-aged women (80%), thus representing the population most affected by PTC and the vast majority of the cases were at stage I/II according to the AJCC/TNM guidelines, with only four cases showing DM at diagnosis [155]. After a 10-year follow-up, the overall survival rate among patients with PTC exceeded 96%, indicating the success of patient management strategies in the central hospital.

We have demonstrated that necrosis was the strongest prognostic factor associated with recurrent/persistent disease, structural disease and overall survival, thus becoming overpowering in all multivariate analysis, which is in line with 5th edition of the WHO Classification of Thyroid Tumors, in which necrosis is itself a prognostic factor associated with

tumor aggressiveness and poor prognosis [112, 113]. Differentiated tumors (e.g. PTC, FTC or OCA) that present necrosis are immediately classified in the new DHGTC histotype [112]. We further performed a comprehensive analysis, shedding light on the pivotal role of calcifications in PTC diagnosis, the impact of surgical margins, and the importance of the level of ETE (minimal or gross) on PTC prognosis. Contrary to previous studies [265, 266], that analyzed the impact of lymphovascular invasion, we performed a separate evaluation of the presence of lymph vessel invasion and venous invasion, demonstrating that while lymph vessel invasion was associated with recurrent/persistent disease, venous invasion was associated with PTC-specific mortality.

Regarding our exploration of thyroid cancer molecular markers associated with (recurrent/persistent disease, structural disease and overall survival), our results challenge the previous notion that *BRAF* mutations alone are the primary indicators of PTC aggressiveness. Several previous studies have indicated *BRAF* to be associated with poor outcome, RAI resistance and disease-specific mortality [267, 268]. However, this effect of *BRAF* mutation may be overestimated in these studies since no other molecular markers known to be associated with poor outcome were evaluated [269, 270]. Additionally, the frequency of *BRAF* mutations in PTC is high (40%-80%), so if *BRAF* by itself was a PTC poor prognosis factor, a lot more cases with dismal prognosis would be expected [271]. *TERTp* mutations on the other hand, are described to be present in 10% of PTC cases, which is about the percentage of PTC cases that present poor prognosis, thus supporting this mutation as a prognostic factor in PTC [241, 242].

Our study shows that *BRAF* mutation is associated with locoregional tumor aggressiveness, while the presence of *TERTp* mutations associates with structural disease and PTC-related mortality. We further tested the possible relationship of molecular status combinations and different outcomes to obtain a more trustworthy effect of each mutation separately. *RAS* mutations were not found in more aggressive subtypes of PTC nor found to be associated with worse prognosis in our series, which is in line with previous studies stating that thyroid cancers harboring *RAS* mutations alone have an excellent prognosis [272]. One patient that presented a *KRAS* mutation died of PTC-related death, however, a *TERTp* mutation was also present in the same lesion (**AIM 1.2**).

PTC tumor's size has also been a target of debate over the years regarding thyroid cancer prognosis, with larger tumors being associated with worse prognosis [273]. Some authors argue that the impact of tumor size in disease recurrence and LNM is limited to older patients [274, 275]. "Papillary microcarcinomas (mPTCs)" usually present mild biological behavior and good prognosis, being recognized as low-risk malignancies. However, some mPTCs, though infrequent, can display aggressive behavior and metastasize to distant sites [276, 277]. Brain metastases are uncommon in TC, and even rarer in mPTC (0.8-1.3%), but are indicative of highly

aggressive disease [278]. Over the past 13 years, only 24 instances of “mPTCs” with DM have been documented in the literature [279]. For this reason, the “mPTC” category disappeared from the new 2022 WHO Classification of Thyroid Tumors and tumors ≤ 1 cm are now evaluated by their histopathologic characteristics, invasiveness and aggressiveness [112]. We discuss a case report detailing the demise of a patient who succumbed to PTC a decade after being diagnosed with a 2 mm FVPTC, previously classified as a microcarcinoma (mPTC) due to its size (according to the 4th edition of the WHO Classification of Thyroid Tumors) [241]. Being a male patient over the age of 55 years and presenting at diagnosis invasion of LNM, already indicated a higher risk for aggressive disease, despite having a primary subcentimetric well-differentiated malignant tumor [153-155, 274, 275]. Upon recurrence in the retrotracheal region, new lesions presented the Tall cell subtype of PTC, indicating a more aggressive tumor [112, 121].

Our study further supports the 2022 alterations of the WHO Classification of Thyroid Tumors, demonstrating PTC prognosis should not be evaluated by size alone, but rather by the patient characteristics, histological component, and molecular landscape of the tumor, as well by its risk of infiltration and/or invasion to surrounding tissues and distant sites. Even in subcentimetric tumors, a patient risk stratification must be carefully considered, to differentiate low-risk cases that may undergo active surveillance from patients whose subcentimetric tumors are aggressive and indicated for TC surgical treatment.

To better understand the impact of subcentimetric PTCs, we presented a study evaluating subcentimetric PTC prognosis between cases that were clinically diagnosed and those that were incidental findings [280]. Our cohort showed that medium- to long-term survival prognosis was excellent in both groups. In 97 patients, only two died of PTC-related mortality during the time of follow-up, both elderly and presenting LNM at diagnosis, characteristics previously discussed as associated with guarded prognosis [274, 275]. We further showed that subcentimetric tumors above 5.5 mm discriminated clinically detected PTC and that tumor size was a marker of higher aggressiveness in this subset of tumors. No statistical association between tumor histology and worse prognosis were encountered, however we are aware that this study presents some limitations: incomplete data in several studied cases, a relatively small sample size, and a restricted follow-up. An extended follow-up of these patients could possibly provide with more concise data on patient outcome. Thus, we emphasize the need for continued exploration and refinement of our understanding of subcentimetric PTCs.

Our case report molecular analysis revealed that while *BRAF V600E* mutation was initially present at diagnosis, the subsequent recurrence featured both *BRAF V600E* and *TERTp* mutations. Ki-67 index was also higher in the recurrent lesions compared to the LNM detected at diagnosis. Subsequently, the patient developed DM in the lung, dorsal lumbar spine, and

brain. Analyses of the resected brain lesions showed Hobnail variant PTC subtype and clear cells, also demonstrating a more aggressive subtype of TC [112, 123]. Additionally, these lesions showed concomitant *BRAF V600E* and *TERTp* promoter mutations and even a higher ki-67 index, when comparing to previous lesions (**AIM 1.2**).

Our investigation challenges some treatment guidelines by showing that patients treated without RAI had excellent outcomes, supporting the idea that RAI may not be necessary in all cases [264, 281]. To this assessment we divided the patients regarding type of surgery and RAI treatment, since hormone levels (e.g. Tg, TSH) are affected by the selected treatment. Our analysis of the literature supports that low-risk malignancy may be handled by less invasive surgery (hemithyroidectomy) and post-operative surveillance. In some cases, no RAI treatment is necessary. This allows to reduce the health care system burden and avoids overtreatment of indolent tumors. Furthermore, by categorizing tumors accordingly to their risk groups, we not only enhance clinical approach but also alleviate patient anxiety associated with a diagnosis labeled as "cancer." Categorization of low-risk neoplasms permits for a more personalized communication strategy, providing patients with a clearer understanding of their specific situation and promoting a more informed and confident decision-making process.

Overall, our investigation states that male gender, presence of psammoma bodies, gross extrathyroidal extension, lateral compartment LNM, lymph vessel invasion, venous invasion, necrosis, incomplete surgical resection, and tumor molecular status should be taken into consideration regarding treatment and follow-up of PTC patients (**AIM 1.1**).

As limitations of our studies, we recognize that at the time of publication, tumor histology assessment was performed using the 4th edition of the WHO Classification of Thyroid Tumors [241]. Accordingly, the Follicular Variant PTCs (FVPTCs) were evaluated as a group. In a new case revision, these would be separated into IEFV-PCT and IFV-PCT. Oncocytic tumors (either Oncocytic PTC, Whartin-like PTC or FTC) were evaluated together due to the small number of cases. (n=18). We further acknowledge that we had a small number of *BRAF*^{wt}/*TERTp*^{mut} cases, which leads us to interpret these results with caution.

Despite these limitations, our findings allow us to answer our first aim (**AIM 1**) and provide valuable insight to the distinction of low-risk patients and patients at risk of poorer prognosis, urging a personalized approach in treatment decisions based on the evaluation of patient-specific characteristics, tumor histologic characteristics, and molecular profile.

Our second aim consisted of performing an in-depth exploration of familial thyroid cancer (**AIM 2**). The genetics behind NSFNMTC is rather heterogeneous and poorly understood. In numerous families, the genes behind FNMTC remain to be identified. The establishment of

molecular markers holds the potential to enhance genetic counseling and facilitate the development of novel therapies.

It is important that physicians can properly identify risk factors that might indicate familial disease, namely: 1 - follicular cell derived thyroid carcinoma in at least three first-degree relatives; 2- papillary thyroid carcinoma in two or more first-degree relatives; 3 – young male presenting papillary thyroid carcinoma; 3 – three family members diagnosed with FND [181, 182, 184, 185]. The identification of these families and the understanding of the genetic profile of these tumors could result in many benefits. Also, disease prevention or active surveillance would prevent invasive surgeries and more aggressive treatments in those who are affected by the illness. Besides reducing patient morbidity, preventive measures would be more economical for the health care system. Additionally, detecting familial disease and identifying the responsible gene(s), we can, through assisted reproduction, eradicate the segregation of the disease in the family tree. This could also lead to the development of new therapies targeting these specific genes.

While several families have been studied, there's limited progress in identifying a shared genetic event that could pinpoint the hereditary changes responsible for disease [194]. NSFNMTTC patients have more frequently early onset disease, when comparing to sporadic cases and this is usually more aggressive, showing large tumors and LNM involvement. Furthermore, in each following generation patients presents at an earlier age with more severe symptoms, suggesting clinical anticipation of the disease in the family tree [282, 283]. Hence the need to identify consistent patterns of disease. Our research team detected two families (denoted as Family R and Family C) with familial TC diagnosis. Both families met the criteria for hereditary thyroid cancer: 1- disease manifesting in more than three first-degree relatives; and 2- an early-age diagnosis. However, no syndromic forms of the disease were recognized. Employing WES, we unveiled two novel germline mutations, potentially pathogenic in nature. In Family R, the mutation was identified in the *USP42* gene, a ubiquitin-specific protease, while in Family C, it manifested in the *KCNB2* gene, a voltage-gated potassium channel.

We were able to demonstrate the pathogenic role of *USP42* as a putative gene in NSFNMTTC through *in silico* and *in vitro* studies (**AIM 2.1**). Our *in silico* analysis showed that although USP mutations are a rare event in thyroid cancer, this assessment identifies the existence of pathogenic USP alterations within the analyzed dataset. *USP42* variants had not been described as cancer drivers but rather as benign variants or variants of uncertain significance, according to IntOGen and gnomAD databases [203, 204]. However, our assessment showed that *USP42* mutation is associated with pathogenicity in the thyroid cell biology.

Interestingly, another USP similar to USP42, USP13, was already described as an underlying cause of NSFNMTTC in a Brazilian family [192].

Family R tumors presented a strong oncocytic component. Oncocytic tumors have been shown to present a higher incidence in familial cases than in sporadic TC [194]. However, the genetic and factors that contribute to the onset of familial oncocytic tumors are yet to be understood [194]. Genetic alterations on the *TCO* locus seem to be associated with oncocytic change [194]. Histologically, familial tumors can be very heterogenous lesions, as seen in both families studied by our group. Family R showed histological patterns that included follicular, solid, trabecular and papillary patterns of growth, all with a strong oncocytic component. Family C presented with FTC and PTC with papillary and follicular patterns of growth. Histological and genetic heterogeneity present in familial tumors increases the challenges of understanding NSFNMTTC, adding to the fact that histologically, familial tumors are similar to the sporadic counterpart [182, 185]. Also, the fact that some genetic alterations seem to be “private” and therefore unique in each family, complicates researchers’ work when attempting to establish a consistent pattern of disease [194]. Given the high heterogeneity of Family R’s tumors, our ongoing efforts involve a rigorous third review of the cases by an experienced pathologist (MSS) taking into account the new update on the WHO Classification of Thyroid Tumors. Simultaneously, we are in the process of initiating a new manuscript based on NSFNMTTC tumor pathology. The forthcoming manuscript will adhere to the WHO 2022 guidelines, with all lesions being meticulously reviewed and reclassified. A comprehensive analysis will be conducted to identify prognostic factors associated with disease progression: e.g. invasion, infiltration, necrosis, mitotic rate, surgical margins. Benign lesions and adjacent tumor tissues will also be thoroughly analyzed. Selection of additional immunohistochemical markers is currently underway for a comprehensive tumor analysis (in development).

Literature findings on the association between vgKCNs and cancer are strong [249-255], although almost no information regarding *KCNB2* alterations and cancer are described. A huge gap exists on the *KCNB2* role in carcinogenesis, while alterations in the similar gene *KCNB1*, which forms complexes with kv2.2 are already well described [253-255]. One could speculate that alterations in *KCNB2* could affect the function of kv2.1. We further hypothesized that *KCNB2* mutations would lead to impaired Nis function (**AIM 2.2**). We showed that *KCNB2* mutated cells presented higher expression of channel mRNA when comparing to *KCNB2* WT cells. Preliminary analysis points out to a possible influence of kv2.2 expression on slc5a5 and tsh receptor expression.

Since thyroid cells do not naturally express the *KCNB2* gene, we hypothesize that the mutation detected in Family C may be activating. Ongoing studies on this correlation are being

performed. FRTL-5 *KCNB2* WT and Mutated cells will be challenged with GTx selective kv2.2 inhibitor and I⁻ assays will be performed to establish a correlation between vgKCN mutations, I⁻ uptake and patient response to treatment (in development).

NSFNMTTC has been hypothesized to be a polygenic disorder usually coupled with various low- to moderate-susceptibility genes and incomplete penetrance, indicating that germline mutations may lead to TC susceptibility but a “second hit” may be necessary for carcinogenesis initiation [182, 183, 186-189]. *BRAF* mutations seem to be a highly prevalent event in USPs and vgKCNs mutated tumors, which could contribute to disease initiation. However, other than histology and genetics, environmental disruptors may also be key players in thyroid carcinogenesis, or even be a non-genetic “second hit”. Our group performed a systematic review in which explores the role of Endocrine-Disrupting Chemicals (EDCs) in the carcinogenesis of six endocrine-related tumors. Thyroid was the endocrine organ that showed the highest cancer risk after EDC exposure which may help explain the increasing thyroid cancer incidence over the last decades [104]. We found that exposure to phthalates showed higher risk for TC development [104-106]. 75% of the studies showed an increased risk for thyroid cancer upon pesticide exposure, with exposure to atrazine being the most significant, representing almost five times increased risk of TC [104, 284]. Heavy metals, e.g. cadmium and lead, and phenols like bisphenol A (BPA), bisphenol AF (BPAF), bisphenol E (BPE) were also associated with increased risk for thyroid tumors, as shown by our systematic review [104, 105, 285, 286]. Two recent studies demonstrated an environmental-genetic interaction, one demonstrating that BPA results in decreased expression of *KCNB2* mRNA, and other showing that BPA leads to decreased methylation of *KCNB2* gene [287, 288].

Much yet is to be discovered on NSFNMTTC and we are still far from establishing a consistent pattern of disease. Since *in vitro* models may fail to represent the full spectrum of disease, studies in animal models, like zebrafish could be very informative on the overall implications of *USP42* and *KCNB2* germline mutations in TC. Most components of zebrafish thyroid axis have been described and shown to be structurally and functionally similar to humans, given that 70% of human genes have at least one zebrafish orthologue, including *USP42* and *KCNB2*.

More studies in this field are needed. As a long-term aims, we wish to promote research in other families affected by non-syndromic FNMTTC and conduct to the recognition of disease manifestation and molecular patterns, as well as to contribute to the development of a molecular panel of genes for improved patient risk stratification in PTC.

CHAPTER VI

GENERAL CONCLUSIONS

CHAPTER VI – GENERAL CONCLUSIONS

This thesis aimed to enhance early diagnosis, precise risk stratification, and personalized medicine for thyroid cancer patients. We conducted a comprehensive investigation into both sporadic and familial thyroid cancers, searching for clinicopathological and molecular aspects of these tumors.

Our findings highlight that male gender, presence of psammoma bodies, gross extrathyroidal extension, and lateral compartment LNM, together with pathological factors, namely lymph vessel invasion, venous invasion, necrosis, and incomplete surgical resection, should be taken into consideration regarding treatment and follow-up of PTC patients. Additionally, we showed how tumor molecular status may help predict patient outcome. *BRAF* mutation is associated with locoregional tumor aggressiveness, while the presence of *TERTp* mutations associates with structural disease and PTC-related mortality. In addition, concomitant *BRAF* and *TERTp* mutations were associated with disease progression and poorer prognosis.

Our analysis supports that low-risk malignancy may be handled by less invasive surgery (hemithyroidectomy) and post-operative surveillance. However, PTC prognosis should not be evaluated by size alone, but rather by the patient characteristics, histological component, and molecular landscape of the tumor, as well by its risk of infiltration and/or invasion to surrounding tissues and distant sites.

This characterization, allows for an improved communication strategy, providing patients with a clearer understanding of their specific situation and promoting a more informed and confident decision-making process.

To our knowledge we describe for the first time mutations on *USP42* and *KCNB2* genes in the context of NSFNMTc. Considering our *in silico* and *in vitro* assays, along with the information available in the literature, we hereby propose *USP42* mutation as an underlying cause of NSFNMTc. We are currently continuing our promising investigation into the potential pathogenic role of *KCNB2* mutations and hope to contribute to bridging the information void on *KCNB2* gene in NSFNMTc.

REFERENCES

REFERENCES

1. Khan, Y.S. and A. Farhana, 2019, *Histology, thyroid gland*.
2. Arrangoiz, R., et al., 2018, *Comprehensive review of thyroid embryology, anatomy, histology, and physiology for surgeons*. International Journal of Otolaryngology and Head & Neck Surgery. **7**(4): p. 160-188.
3. Maenhaut, C., et al., 2015, *Ontogeny, anatomy, metabolism and physiology of the thyroid*. Endotext [Internet].
4. Kamrani, P., G. Marston, and A. Jan, 2019, *Anatomy, connective tissue*.
5. Kumar, V., et al., *Robbins & Cotran Pathologic Basis of Disease*. 7 ed. 2009: Philadelphia, Pa. : Elsevier Saunders.
6. Rocha, T., et al., 2017, *A Study on maturation of thyroid gland in human fetuses*. Journal of Morphological Sciences. **27**(2): p. 0-0.
7. Dom, G., et al., 2021, *Transcriptomic Signature of Human Embryonic Thyroid Reveals Transition From Differentiation to Functional Maturation*. Frontiers in Cell and Developmental Biology. **9**.
8. Patel, J., et al., 2011, *Thyroid hormones and fetal neurological development*. Journal of Endocrinology. **209**(1): p. 1.
9. Bernal, J., 2000, *Thyroid Hormones in Brain Development and Function*. [Updated 2022 Jan 14]. Endotext [Internet]. South Dartmouth (MA): MDText. com, Inc.
10. Portulano, C., M. Paroder-Belenitsky, and N. Carrasco, 2014, *The Na⁺/I⁻ Symporter (NIS): Mechanism and Medical Impact*. Endocrine Reviews. **35**(1): p. 106-149.
11. de Escobar, G.M., et al. *The changing role of maternal thyroid hormone in fetal brain development*. in *Seminars in perinatology*. 2008. Elsevier.
12. Ravera, S., et al., 2017, *The sodium/iodide symporter (NIS): molecular physiology and preclinical and clinical applications*. Annual review of physiology. **79**: p. 261-289.
13. Shrestha, S., et al., 2016, *Thyroid function and neuropsychological status in older adults*. Physiology & Behavior. **164**: p. 34-39.
14. Siegmann, E.-M., et al., 2018, *Association of depression and anxiety disorders with autoimmune thyroiditis: a systematic review and meta-analysis*. JAMA psychiatry. **75**(6): p. 577-584.
15. Zivaljevic, V.R., et al., 2015, *Quality of life improvement in patients with Hashimoto thyroiditis and other goiters after surgery: A prospective cohort study*. International Journal of Surgery. **21**: p. 150-155.
16. Mullur, R., Y.-Y. Liu, and G.A. Brent, 2014, *Thyroid Hormone Regulation of Metabolism*. Physiological Reviews. **94**(2): p. 355-382.
17. Silva, J.E., 2011, *Physiological importance and control of non-shivering facultative thermogenesis*. Front Biosci (Schol Ed). **3**(1): p. 352-71.
18. Sengupta, P. and S. Dutta, 2018, *Thyroid disorders and semen quality*. Biomedical and Pharmacology Journal. **11**(1): p. 01-10.
19. Crawford, N.M. and A.Z. Steiner. *Thyroid autoimmunity and reproductive function*. in *Seminars in Reproductive Medicine*. 2016. Thieme Medical Publishers.
20. Messenger, A.G., 2000, *Thyroid hormone and hair growth*. British Journal of Dermatology. **142**(4): p. 633-634.
21. Kasumagic-Halilovic, E., 2014, *Thyroid disease and the skin*. Annals Thyroid Res. **1**(2): p. 27-31.
22. Begić, A., A. Begić, and A. Arnautović-Halimić, 2020, *Influence of thyroid hormones on bone density*. Medicinski Glasnik. **17**(2).
23. Tuncel, M., 2017, *Thyroid Stimulating Hormone Receptor*. Mol Imaging Radionucl Ther. **26**(Suppl 1): p. 87-91.

24. Feldt-Rasmussen, U., G. Effraimidis, and M. Klose, 2021, *The hypothalamus-pituitary-thyroid (HPT)-axis and its role in physiology and pathophysiology of other hypothalamus-pituitary functions*. Molecular and Cellular Endocrinology. **525**: p. 111173.
25. Mariotti, S. and P. Beck-Peccoz, 2021, *Physiology of the hypothalamic-pituitary-thyroid axis*. Endotext [Internet].
26. Pesce, L. and P. Kopp, 2014, *Iodide transport: implications for health and disease*. International journal of pediatric endocrinology. **2014**(1): p. 1-12.
27. Riesco-Eizaguirre, G. and P. Santisteban, 2006, *A perspective view of sodium iodide symporter research and its clinical implications*. **155**(4): p. 495.
28. Baumann, E., 1986, *Über den Jodgehalt der Schilddrüsen von Menschen und Tieren*. Hoppe Seyler's Z Physiol Chem. **22**: p. 1-17.
29. Dai, G., O. Levy, and N. Carrasco, 1996, *Cloning and characterization of the thyroid iodide transporter*. Nature. **379**(6564): p. 458-460.
30. Liu, Q.-R. and K.-Y. Ryu, 1996, *Cloning of the human sodium iodide symporter*. Biochemical and biophysical research communications. **226**: p. 339-345.
31. Dohan, O., et al., 2003, *The sodium/iodide symporter (NIS): characterization, regulation, and medical significance*. Endocrine reviews. **24**(1): p. 48-77.
32. Laura, A.S., et al., 2002, *PKA Modulation of Kv4.2-Encoded A-Type Potassium Channels Requires Formation of a Supramolecular Complex*. The Journal of Neuroscience. **22**(23): p. 10123.
33. Purtell, K., et al., 2012, *The KCNQ1-KCNE2 K⁺ channel is required for adequate thyroid I⁻ uptake*. FASEB J. **26**(8): p. 3252-9.
34. Spitzweg, C. and J.C. Morris, 2002, *The sodium iodide symporter: its pathophysiological and therapeutic implications*. Clinical Endocrinology. **57**(5): p. 559-574.
35. Targovnik, H.M., C.E. Citterio, and C.M. Rivolta, 2017, *Iodide handling disorders (NIS, TPO, TG, IYD)*. Best Practice & Research Clinical Endocrinology & Metabolism. **31**(2): p. 195-212.
36. Nicola, J.P., N. Carrasco, and L.M. Amzel, 2014, *Physiological sodium concentrations enhance the iodide affinity of the Na⁺/I⁻ symporter*. Nature communications. **5**(1): p. 1-10.
37. Kopp, P., 2014, *Mutations in the Pendred Syndrome (PDS/SLC26A) gene: an increasingly complex phenotypic spectrum from goiter to thyroid hypoplasia*, Oxford University Press.
38. Kopp, P.A., 2008, *Reduce, recycle, reuse—Iodotyrosine deiodinase in thyroid iodide metabolism*, Mass Medical Soc.
39. Pesce, L. and P. Kopp, 2014, *Iodide transport: implications for health and disease*. International journal of pediatric endocrinology. **2014**(1): p. 8.
40. Kaminsky, S.M., et al., 1994, *Na⁺/I⁻ symport activity is present in membrane vesicles from thyrotropin-deprived non-I⁻-transporting cultured thyroid cells*. Proceedings of the National Academy of Sciences. **91**(9): p. 3789-3793.
41. Paire, A., et al., 1997, *Characterization of the rat thyroid iodide transporter using anti-peptide antibodies relationship between its expression and activity*. Journal of Biological Chemistry. **272**(29): p. 18245-18249.
42. Kogai, T., et al., 1997, *Regulation by thyroid-stimulating hormone of sodium/iodide symporter gene expression and protein levels in FRTL-5 cells*. Endocrinology. **138**(6): p. 2227-2232.
43. Levy, O., et al., 1998, *N-linked Glycosylation of the Thyroid Na⁺/I⁻ Symporter (NIS) IMPLICATIONS FOR ITS SECONDARY STRUCTURE MODEL*. Journal of Biological Chemistry. **273**(35): p. 22657-22663.
44. Riedel, C., O. Levy, and N. Carrasco, 2001, *Post-transcriptional regulation of the sodium/iodide symporter by thyrotropin*. Journal of Biological Chemistry. **276**(24): p. 21458-21463.

45. Postiglione, M., et al., 2002, *Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland*. Proceedings of the National Academy of Sciences. **99**(24): p. 15462-15467.
46. Wolff, J. and I. Chaikoff, 1948, *The inhibitory action of excessive iodide upon the synthesis of diiodotyrosine and of thyroxine in the thyroid gland of the normal rat*. Endocrinology. **43**(3): p. 174-179.
47. Yoshihara, A., et al., 2012, *Regulation of dual oxidase expression and H₂O₂ production by thyroglobulin*. Thyroid. **22**(10): p. 1054-1062.
48. Suzuki, K., et al., 2011, *Role of thyroglobulin on negative feedback autoregulation of thyroid follicular function and growth*. Journal of endocrinology. **209**: p. 169-174.
49. Pisarev, M.A., L. Thomasz, and G.J. Juvenal, 2009, *Role of transforming growth factor beta in the regulation of thyroid function and growth*. Thyroid. **19**(8): p. 881-892.
50. Kawaguchi, A., et al., 1997, *Transforming Growth Factor- β 1 Suppresses Thyrotropin-Induced Na⁺/I⁻ Symporter Messenger RNA and Protein Levels in FRTL-5 Rat Thyroid Cells*. Thyroid. **7**(5): p. 789-794.
51. García, B. and P. Santisteban, 2002, *PI3K is involved in the IGF-I inhibition of TSH-induced sodium/iodide symporter gene expression*. Molecular Endocrinology. **16**(2): p. 342-352.
52. Spitzweg, C., et al., 1999, *Regulation of Sodium Iodide Symporter Gene Expression in FRTL-5 Rat Thyroid Cells*. Thyroid. **9**(8): p. 821-830.
53. Furlanetto, T.W., et al., 2001, *Estradiol decreases iodide uptake by rat thyroid follicular FRTL-5 cells*. Brazilian Journal of Medical and Biological Research. **34**: p. 259-263.
54. Santin, A.P. and T.W. Furlanetto, 2011, *Role of estrogen in thyroid function and growth regulation*. Journal of Thyroid Research. **2011**.
55. Yuk, J.S. and J.M. Kim, 2022, *Uterine fibroids increase the risk of thyroid goiter and thyroid nodules*. Sci Rep. **12**(1): p. 6620.
56. Xu, W., et al., 2023, *Clinical observation of menopause hormone therapy in postmenopausal women with euthyroid and mild subclinical hypothyroidism*. BMC Endocrine Disorders. **23**(1): p. 21.
57. Chung, T., et al., 2015, *Glycosylation of sodium/iodide symporter (NIS) regulates its membrane translocation and radioiodine uptake*. PloS one. **10**(11).
58. National Center for Biotechnology Information (NCBI) - SLC5A5 solute carrier family 5 member 5 [Homo sapiens (human)]. 6528 2020, June 9th [cited 2020 June 17th]; <https://www.ncbi.nlm.nih.gov/gene/6528>].
59. Antonelli, A., et al., 2015, *Autoimmune thyroid disorders*. Autoimmunity Reviews. **14**(2): p. 174-180.
60. Ehlers, M. and M. Schott, 2014, *Hashimoto's thyroiditis and papillary thyroid cancer: are they immunologically linked?* Trends in Endocrinology & Metabolism. **25**(12): p. 656-664.
61. Pearce, E.N., A.P. Farwell, and L.E. Braverman, 2003, *Thyroiditis*. New England Journal of Medicine. **348**(26): p. 2646-2655.
62. Bocchetta, A., et al., 2007, *Affective psychosis, Hashimoto's thyroiditis, and brain perfusion abnormalities: case report*. Clinical Practice and Epidemiology in Mental Health. **3**(1): p. 31.
63. Pyzik, A., et al., 2015, *Immune disorders in Hashimoto's thyroiditis: what do we know so far?* J Immunol Res. **2015**: p. 979167.
64. Ewa Machała, M.R., Iulia Iavorska, Piotr Machała, 2019, *Hashimoto's thyroiditis*. World Scientific News. p. 302-314.
65. Carta, M.G., et al., 2005, *A case control study on psychiatric disorders in Hashimoto disease and Euthyroid Goitre: not only depressive but also anxiety disorders are associated with thyroid autoimmunity*. Clinical Practice and Epidemiology in Mental Health. **1**(1): p. 23.

66. Pop, V.J., et al., 1998, *Are Autoimmune Thyroid Dysfunction and Depression Related?* The Journal of Clinical Endocrinology & Metabolism. **83**(9): p. 3194-3197.
67. Carta, M., et al., 2004, *The link between thyroid autoimmunity with anxiety and mood disorders in the community: a field of interest for public health in the future.* BMC Psychiatry. **4**: p. 25.
68. Kumar, V., et al., *Robbins and Cotran pathologic basis of disease, professional edition e-book.* 2014: elsevier health sciences.
69. Laura, C.L., W. Claire Louise, and C. Tim, 2023, *Graves' disease: moving forwards.* Archives of Disease in Childhood. **108**(4): p. 276.
70. Wu, Y.-J., et al., 2023, *Combination of treatments with transoral endoscopic thyroidectomy vestibular approach (TOETVA) for Graves' disease.* Scientific Reports. **13**(1): p. 2764.
71. Wiltshire, J.J., et al., 2016, *Systematic review of trends in the incidence rates of thyroid cancer.* Thyroid. **26**(11): p. 1541-1552.
72. Sung, H., et al., 2021, *Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries.* CA Cancer J Clin. **71**(3): p. 209-249.
73. Kitahara, C.M. and A.B. Schneider, 2022, *Epidemiology of Thyroid Cancer.* Cancer Epidemiology, Biomarkers & Prevention. **31**(7): p. 1284-1297.
74. Li, Y., et al., 2022, *The global burden of thyroid cancer in high-income Asia-Pacific: a systematic analysis of the Global Burden of Disease study.* Ther Adv Endocrinol Metab. **13**: p. 20420188221090012.
75. Raposo, L., et al., 2017, *Trends in thyroid cancer incidence and mortality in Portugal.* European Journal of Cancer Prevention. **26**(2): p. 135-143.
76. Shah, J.P., 2015, *Thyroid carcinoma: epidemiology, histology, and diagnosis.* Clin Adv Hematol Oncol. **13**(4 Suppl 4): p. 3-6.
77. Haymart, M.R., et al., 2008, *Higher serum thyroid stimulating hormone level in thyroid nodule patients is associated with greater risks of differentiated thyroid cancer and advanced tumor stage.* J Clin Endocrinol Metab. **93**(3): p. 809-14.
78. Kwong, N., et al., 2015, *The Influence of Patient Age on Thyroid Nodule Formation, Multinodularity, and Thyroid Cancer Risk.* J Clin Endocrinol Metab. **100**(12): p. 4434-40.
79. Lin, J.D., et al., 2005, *Thyroid cancer in the thyroid nodules evaluated by ultrasonography and fine-needle aspiration cytology.* Thyroid. **15**(7): p. 708-17.
80. Furukawa, K., et al., 2013, *Long-term trend of thyroid cancer risk among Japanese atomic-bomb survivors: 60 years after exposure.* Int J Cancer. **132**(5): p. 1222-6.
81. Drozdovitch, V., 2021, *Radiation exposure to the thyroid after the Chernobyl accident.* Frontiers in endocrinology. **11**: p. 569041.
82. Yamashita, S., et al., 2018, *Lessons from Fukushima: latest findings of thyroid cancer after the Fukushima nuclear power plant accident.* Thyroid. **28**(1): p. 11-22.
83. Jegerlehner, S., et al., 2017, *Overdiagnosis and overtreatment of thyroid cancer: A population-based temporal trend study.* PLoS One. **12**(6): p. e0179387.
84. Sobrinho-Simões, M.A., M.C. Sambade, and V. Gonçalves, 1979, *Latent thyroid carcinoma at autopsy: A study from Oporto, Portugal.* Cancer. **43**(5): p. 1702-1706.
85. Valle, L.A. and R.T. Kloos, 2011, *The prevalence of occult medullary thyroid carcinoma at autopsy.* The Journal of Clinical Endocrinology & Metabolism. **96**(1): p. E109-E113.
86. Vigneri, R., P. Malandrino, and P. Vigneri, 2015, *The changing epidemiology of thyroid cancer: why is incidence increasing?* Current opinion in oncology. **27**(1): p. 1-7.
87. Kitahara, C.M. and J.A. Sosa, 2016, *The changing incidence of thyroid cancer.* Nature Reviews Endocrinology. **12**(11): p. 646-653.
88. Andersson, M., et al., *Iodine deficiency in Europe: a continuing public health problem.* 2007: World Health Organization Geneva.

89. De Benoist, B., et al., 2004, *Iodine status worldwide*. WHO Global Database on Iodine Deficiency. Geneva: World Health Organization. **83**: p. 518-525.
90. Zhang, Y.-L., et al., 2019, *Does relatively low iodine intake contribute to thyroid cancer? An ecological comparison of epidemiology*. Medicine. **98**(41).
91. Carrasco, N., 1993, *Iodide transport in the thyroid gland*. Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes. **1154**(1): p. 65-82.
92. Organization, W.H. and UNICEF, 1994, *World summit for children mid-decade goal: iodine deficiency disorders*. Geneva: WHO, January.
93. Farebrother, J., M.B. Zimmermann, and M. Andersson, 2019, *Excess iodine intake: sources, assessment, and effects on thyroid function*. Annals of the New York Academy of Sciences. **1446**(1): p. 44-65.
94. Blomberg, M., et al., 2012, *Thyroid cancer in Denmark 1943–2008, before and after iodine supplementation*. International Journal of Cancer. **131**(10): p. 2360-2366.
95. Rose, N.R., R. Bonita, and C.L. Burek, 2002, *Iodine: an environmental trigger of thyroiditis*. Autoimmunity reviews. **1**(1-2): p. 97-103.
96. Carlé, A., A. Krejbjerg, and P. Laurberg, 2014, *Epidemiology of nodular goitre. Influence of iodine intake*. Best practice & research Clinical endocrinology & metabolism. **28**(4): p. 465-479.
97. Santos, J.E.C., et al., 2015, *Iodine deficiency and thyroid nodular pathology-epidemiological and cancer characteristics in different populations: Portugal and South Africa*. BMC research notes. **8**(1): p. 1-7.
98. Huszno, B., et al., 2003, *Influence of iodine deficiency and iodine prophylaxis on thyroid cancer histotypes and incidence in endemic goiter area*. Journal of endocrinological investigation. **26**(2 Suppl): p. 71-76.
99. Yamashita, H., et al., 1990, *Effects of dietary iodine on chemical induction of thyroid carcinoma*. Pathology International. **40**(10): p. 705-712.
100. Boltze, C., et al., 2002, *Radiation-Induced Thyroid Carcinogenesis as a Function of Time and Dietary Iodine Supply: An in Vivo Model of Tumorigenesis in the Rat*. Endocrinology. **143**(7): p. 2584-2592.
101. Guan, H., et al., 2009, *Association of high iodine intake with the T1799A BRAF mutation in papillary thyroid cancer*. The Journal of Clinical Endocrinology & Metabolism. **94**(5): p. 1612-1617.
102. Vuong, H.G., et al., 2016, *Genetic alterations of differentiated thyroid carcinoma in iodine-rich and iodine-deficient countries*. Cancer medicine. **5**(8): p. 1883-1889.
103. Yoon, K., et al., 2014, *Estrogenic endocrine-disrupting chemicals: molecular mechanisms of actions on putative human diseases*. Journal of Toxicology and Environmental Health, Part B. **17**(3): p. 127-174.
104. Macedo, S., et al., 2022, *Endocrine-disrupting chemicals and endocrine neoplasia: A forty-year systematic review*. Environmental Research. p. 114869.
105. Marotta, V., et al., 2019, *Human exposure to bisphenol AF and diethylhexylphthalate increases susceptibility to develop differentiated thyroid cancer in patients with thyroid nodules*. Chemosphere. **218**: p. 885-894.
106. Miao, H., et al., 2020, *Associations of urinary phthalate metabolites with risk of papillary thyroid cancer*. Chemosphere. **241**: p. 125093.
107. Dohán, O., et al., 2007, *The Na⁺/I⁻ symporter (NIS) mediates electroneutral active transport of the environmental pollutant perchlorate*. Proceedings of the National Academy of Sciences. **104**(51): p. 20250-20255.
108. Bogović Crnčić, T., et al., 2020, *Risk Factors for Thyroid Cancer: What Do We Know So Far?* Acta Clin Croat. **59**(Suppl 1): p. 66-72.
109. Pasqual, E., et al., 2023, *Obesity, obesity-related metabolic conditions, and risk of thyroid cancer in women: results from a prospective cohort study (Sister Study)*. The Lancet Regional Health–Americas. **23**.

110. Kitahara, C.M., et al., 2016, *Anthropometric factors and thyroid cancer risk by histological subtype: pooled analysis of 22 prospective studies*. *Thyroid*. **26**(2): p. 306-318.
111. Derwahl, M. and D. Nicula, 2014, *Estrogen and its role in thyroid cancer*. *Endocrine-Related Cancer*. **21**(5): p. T273-T283.
112. Baloch, Z.W., et al., 2022, *Overview of the 2022 WHO classification of thyroid neoplasms*. *Endocrine pathology*. **33**(1): p. 27-63.
113. Juhlin, C.C., O. Mete, and Z.W. Baloch, 2023, *The 2022 WHO classification of thyroid tumors: novel concepts in nomenclature and grading*. *Endocrine-Related Cancer*. **30**(2).
114. Jung, C.K., A. Bychkov, and K. Kakudo, 2022, *Update from the 2022 World Health Organization Classification of Thyroid Tumors: A Standardized Diagnostic Approach*. *enm*. **37**(5): p. 703-718.
115. Mete, O. and S.L. Asa, 2012, *Pitfalls in the Diagnosis of Follicular Epithelial Proliferations of the Thyroid*. *Advances in Anatomic Pathology*. **19**(6).
116. Lebrun, L. and I. Salmon, 2024, *Pathology and new insights in thyroid neoplasms in the 2022 WHO classification*. *Current Opinion in Oncology*. **36**(1).
117. Basolo, F., et al., 2023, *The 5th edition of WHO classification of tumors of endocrine organs: changes in the diagnosis of follicular-derived thyroid carcinoma*. *Endocrine*. **80**(3): p. 470-476.
118. Hong, S., et al., 2022, *Distinct molecular subtypes of papillary thyroid carcinoma and gene signature with diagnostic capability*. *Oncogene*. **41**(47): p. 5121-5132.
119. Cavaco, D., et al., 2022, *Diffuse sclerosing variant of papillary thyroid carcinoma: outcomes of 33 cases*. *Eur Thyroid J*. **11**(1).
120. Kilcarslan, A., et al., 2023, *What is the contribution of tumor capsule thickness and tumor nucleus diameter to differential diagnosis in encapsulated variant thyroid papillary carcinomas?*
121. Villar-Taibo, R., et al., 2017, *Aggressiveness of the tall cell variant of papillary thyroid carcinoma is independent of the tumor size and patient age*. *Oncol Lett*. **13**(5): p. 3501-3507.
122. Wang, S., et al., 2019, *Columnar cell papillary thyroid carcinoma prognosis: findings from the SEER database using propensity score matching analysis*. *Am J Transl Res*. **11**(9): p. 6262-6270.
123. Donaldson, L.B., et al., 2021, *Hobnail variant of papillary thyroid carcinoma: a systematic review and meta-analysis*. *Endocrine*. **72**(1): p. 27-39.
124. Ohashi, R., 2020, *Solid variant of papillary thyroid carcinoma: an under-recognized entity*. *Endocrine Journal*. **67**(3): p. 241-248.
125. Xu, B., et al., 2022, *The solid variant of papillary thyroid carcinoma: a multi-institutional retrospective study*. *Histopathology*. **81**(2): p. 171-182.
126. Olmos, R., et al., 2020, *Warthin-like and classic papillary thyroid cancer have similar clinical presentation and prognosis*. *Archives of endocrinology and metabolism*. **64**: p. 542-547.
127. Lukovic, J., et al., 2021, *Oncocytic Papillary Thyroid Carcinoma and Oncocytic Poorly Differentiated Thyroid Carcinoma: Clinical Features, Uptake, and Response to Radioactive Iodine Therapy, and Outcome*. *Front Endocrinol (Lausanne)*. **12**: p. 795184.
128. Zhang, T., et al., 2023, *Risk factors for death of follicular thyroid carcinoma: a systematic review and meta-analysis*. *Endocrine*. **82**(3): p. 457-466.
129. Sobrinho-Simões, M., et al., 2011, *Follicular thyroid carcinoma*. *Modern Pathology*. **24**: p. S10-S18.
130. Nojima, S., et al., 2023, *Deep Learning-Based Differential Diagnosis of Follicular Thyroid Tumors Using Histopathological Images*. *Modern Pathology*. **36**(11): p. 100296.

131. Wang, Z., et al., 2022, *Application of competing risk model in the prognostic prediction study of patients with follicular thyroid carcinoma*. Updates in Surgery. **74**(2): p. 735-746.
132. Melo, M., et al., 2023, *Non-invasive follicular thyroid neoplasm with papillary-like nuclear feature: clinical, pathological, and molecular update 5 years after the nomenclature revision*. European Journal of Endocrinology. **188**(2): p. lvad004.
133. Syed, A., et al., 2022, *Oncocytic Cell Carcinoma of the Thyroid: A Case Report and an Overview of the Diagnosis, Treatment Modalities, and Prognosis*. Cureus. **14**(10): p. e30298.
134. Grawe, F., et al., 2021, *Course of Disease and Clinical Management of Patients with Poorly Differentiated Thyroid Carcinoma*. Cancers (Basel). **13**(21).
135. Tong, J., et al., 2022, *Poorly differentiated thyroid carcinoma: a clinician's perspective*. Eur Thyroid J. **11**(2).
136. Limaïem, F., et al., 2019, *Anaplastic thyroid cancer*.
137. Jannin, A., et al., 2022, *Anaplastic Thyroid Carcinoma: An Update*. Cancers. **14**(4): p. 1061.
138. Brierley, J.D., M.K. Gospodarowicz, and C. Wittekind, *TNM classification of malignant tumours*. 2017: John Wiley & Sons.
139. R. Michael Tuttle, B.H., and Nancy D. Perrier, 2017, *Updated American Joint Committee on Cancer/Tumor-Node-Metastasis Staging System for Differentiated and Anaplastic Thyroid Cancer (Eighth Edition): What Changed and Why?* Thyroid®. **27**(6): p. 751-756.
140. Haugen, B.R., et al., 2016, *2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer*. Thyroid. **26**(1): p. 1-133.
141. Schmidbauer, B., et al. *Differentiated Thyroid Cancer—Treatment: State of the Art*. International Journal of Molecular Sciences, 2017. **18**, DOI: 10.3390/ijms18061292.
142. Lohia, S., et al., 2020, *Active surveillance for patients with very low-risk thyroid cancer*. Laryngoscope Investig Otolaryngol. **5**(1): p. 175-182.
143. Luster, M., et al., 2008, *Guidelines for radioiodine therapy of differentiated thyroid cancer*. European journal of nuclear medicine and molecular imaging. **35**(10): p. 1941.
144. Paschke, R., et al., 2015, *The treatment of well-differentiated thyroid carcinoma*. Deutsches Ärzteblatt International. **112**(26): p. 452.
145. Carvalho, D.P. and A.C. Ferreira, 2007, *The importance of sodium/iodide symporter (NIS) for thyroid cancer management*. Arquivos Brasileiros de Endocrinologia & Metabologia. **51**(5): p. 672-682.
146. Dadachova, E. and N. Carrasco. *The Na⁺/I⁻ symporter (NIS): imaging and therapeutic applications*. in *Seminars in nuclear medicine*. 2004. Elsevier.
147. Paroder-Belenitsky, M., et al., 2011, *Mechanism of anion selectivity and stoichiometry of the Na⁺/I⁻ symporter (NIS)*. Proceedings of the National Academy of Sciences. **108**(44): p. 17933-17938.
148. Connor, C.S., et al., 1988, *Radioiodine therapy for differentiated thyroid carcinoma*. The American Journal of Surgery. **156**(6): p. 519-521.
149. Su, J., et al., 2022, *Efficacy and safety of multi-kinase inhibitors in patients with radioiodine-refractory differentiated thyroid cancer: a systematic review and meta-analysis of clinical trials*. Expert Review of Anticancer Therapy. **22**(9): p. 999-1008.
150. Vaisman, F., D.P. Carvalho, and M. Vaisman, 2015, *A new appraisal of iodine refractory thyroid cancer*. Endocrine-related cancer. **22**: p. R301-R310.
151. De Leo, S., M. Trevisan, and L. Fugazzola, 2020, *Recent advances in the management of anaplastic thyroid cancer*. Thyroid Research. **13**(1): p. 17.
152. Eghtedari, B. and R. Correa, 2019, *Levothyroxine*.

153. Rahbari, R., L. Zhang, and E. Kebebew, 2010, *Thyroid cancer gender disparity*. *Future Oncology*. **6**(11): p. 1771-1779.
154. Jonklaas, J., et al., 2012, *The impact of age and gender on papillary thyroid cancer survival*. *The Journal of Clinical Endocrinology & Metabolism*. **97**(6): p. E878-E887.
155. Kim, K., et al., 2018, *The Updated AJCC/TNM Staging System for Papillary Thyroid Cancer (8th Edition): From the Perspective of Genomic Analysis*. *World Journal of Surgery*. **42**(11): p. 3624-3631.
156. Shi, W., et al., 2023, *Extrathyroidal extension or tumor size of primary lesion influences thyroid cancer outcomes*. *Nucl Med Commun*. **44**(10): p. 854-859.
157. Durante, C., et al., 2006, *Long-term outcome of 444 patients with distant metastases from papillary and follicular thyroid carcinoma: benefits and limits of radioiodine therapy*. *The Journal of Clinical Endocrinology & Metabolism*. **91**(8): p. 2892-2899.
158. Ho, A.L., et al., 2012, *Reacquisition of RAI uptake in RAI-refractory metastatic thyroid cancers by pretreatment with the MEK inhibitor selumetinib*. *Journal of Clinical Oncology*. **30**(15_suppl): p. 5509-5509.
159. Tavares, C., et al., 2018, *mTOR pathway in papillary thyroid carcinoma: Different contributions of mTORC1 and mTORC2 complexes for tumor behavior and SLC5A5 mRNA expression*. *International journal of molecular sciences*. **19**(5): p. 1448.
160. Brose, M.S., et al., 2014, *Sorafenib in radioactive iodine-refractory, locally advanced or metastatic differentiated thyroid cancer: a randomised, double-blind, phase 3 trial*. *The Lancet*. **384**(9940): p. 319-328.
161. Anderson, R.T., et al., 2013, *Clinical, safety, and economic evidence in radioactive iodine-refractory differentiated thyroid cancer: a systematic literature review*. *Thyroid*. **23**(4): p. 392-407.
162. Vaisman, F., et al., 2011, *In differentiated thyroid cancer, an incomplete structural response to therapy is associated with significantly worse clinical outcomes than only an incomplete thyroglobulin response*. *Thyroid*. **21**(12): p. 1317-1322.
163. Tavares, C., et al., 2018, *NIS expression in thyroid tumors, relation with prognosis clinicopathological and molecular features*. *Endocrine connections*. **7**(1): p. 78-90.
164. Mian, C., et al., 2008, *Molecular characteristics in papillary thyroid cancers (PTCs) with no 131I uptake*. *Clinical endocrinology*. **68**(1): p. 108-116.
165. Elia, G., et al., 2022, *Molecular features of aggressive thyroid cancer*. *Frontiers in Oncology*. **12**.
166. Nylén, C., et al., 2020, *Molecular Markers Guiding Thyroid Cancer Management*. *Cancers (Basel)*. **12**(8).
167. Gozu, H.I., et al., 2010, *Genetics and phenomics of inherited and sporadic non-autoimmune hyperthyroidism*. *Molecular and Cellular Endocrinology*. **322**(1): p. 125-134.
168. Jeong, S.H., et al., 2018, *Analysis of RAS mutation in thyroid nodular hyperplasia and follicular neoplasm in a Korean population*. *Endocrinology, Diabetes & Metabolism*. **1**(4): p. e00040.
169. Sauer, M. and J.A. Barletta, 2022, *Proceedings of the North American Society of Head and Neck Pathology, Los Angeles, CA, March 20, 2022: DICER1-Related Thyroid Tumors*. *Head Neck Pathol*. **16**(1): p. 190-199.
170. Macerola, E., A. Proietti, and F. Basolo, 2020, *Noninvasive follicular neoplasm with papillary-like nuclear features (NIFTP): a new entity*. *Gland Surg*. **9**(Suppl 1): p. S47-s53.
171. Gilani, S.M., et al., 2022, *RAS mutation and associated risk of malignancy in the thyroid gland: An FNA study with cytology-histology correlation*. *Cancer Cytopathology*. **130**(4): p. 284-293.
172. Marotta, V., M. Bifulco, and M. Vitale, 2021, *Significance of RAS Mutations in Thyroid Benign Nodules and Non-Medullary Thyroid Cancer*. *Cancers*. **13**(15): p. 3785.

173. Prescott, J.D. and M.A. Zeiger, 2015, *The RET oncogene in papillary thyroid carcinoma*. *Cancer*. **121**(13): p. 2137-2146.
174. Pillai, S., et al., 2015, *Diffuse sclerosing variant of papillary thyroid carcinoma—an update of its clinicopathological features and molecular biology*. *Critical Reviews in Oncology/Hematology*. **94**(1): p. 64-73.
175. Cavadas, B., et al., 2019, *Genomic and transcriptomic characterization of the mitochondrial-rich oncocytic phenotype on a thyroid carcinoma background*. *Mitochondrion*. **46**: p. 123-133.
176. Gasparre, G., et al., 2008, *Clonal expansion of mutated mitochondrial DNA is associated with tumor formation and complex I deficiency in the benign renal oncocytoma*. *Human molecular genetics*. **17**(7): p. 986-995.
177. Schubert, L., et al., 2023, *MAPK Pathway Inhibitors in Thyroid Cancer: Preclinical and Clinical Data*. *Cancers (Basel)*. **15**(3).
178. Nozhat, Z. and M. Hedayati, 2016, *PI3K/AKT Pathway and Its Mediators in Thyroid Carcinomas*. *Molecular Diagnosis & Therapy*. **20**(1): p. 13-26.
179. Guilmette, J. and V. Nosé, 2018, *Hereditary and familial thyroid tumours*. *Histopathology*. **72**(1): p. 70-81.
180. Yang, S.P. and J. Ngeow, 2016, *Familial non-medullary thyroid cancer: unraveling the genetic maze*. *Endocrine-related cancer*. **23**(12): p. R577-R595.
181. Cameselle-Teijeiro, J.M., et al., 2021, *Inherited Follicular Epithelial-Derived Thyroid Carcinomas: From Molecular Biology to Histological Correlates*. *Endocrine Pathology*. **32**(1): p. 77-101.
182. Nosé, V., et al., 2022, *Overview of the 2022 WHO Classification of Familial Endocrine Tumor Syndromes*. *Endocrine Pathology*. **33**(1): p. 197-227.
183. Capezzone, M., et al., 2021, *Familial non-medullary thyroid cancer: a critical review*. *J Endocrinol Invest*. **44**(5): p. 943-950.
184. Orois, A., et al., 2021, *Familial non medullary thyroid carcinoma: Beyond the syndromic forms*. *Endocrinología, Diabetes y Nutrición (English ed.)*. **68**(4): p. 260-269.
185. Alsanea, O. and O.H. Clark, 2001, *Familial thyroid cancer*. *Current opinion in oncology*. **13**(1): p. 44-51.
186. Diquigiovanni, C. and E. Bonora, 2021, *Genetics of Familial Non-Medullary Thyroid Carcinoma (FNMTTC)*. *Cancers (Basel)*. **13**(9).
187. Pereira, J.S., et al., 2015, *Identification of a novel germline FOXE1 variant in patients with familial non-medullary thyroid carcinoma (FNMTTC)*. *Endocrine*. **49**: p. 204-214.
188. Ngan, E.S., et al., 2009, *A germline mutation (A339V) in thyroid transcription factor-1 (TTF-1/NKX2. 1) in patients with multinodular goiter and papillary thyroid carcinoma*. *Journal of the National Cancer Institute*. **101**(3): p. 162-175.
189. Gara, S.K., et al., 2015, *Germline HABP2 mutation causing familial nonmedullary thyroid cancer*. *New England Journal of Medicine*. **373**(5): p. 448-455.
190. Capezzone, M., et al., 2021, *Familial non-medullary thyroid cancer: a critical review*. *Journal of Endocrinological Investigation*. **44**: p. 943-950.
191. Ye, F., et al., 2019, *Whole exome and target sequencing identifies MAP2K5 as novel susceptibility gene for familial non-medullary thyroid carcinoma*. *International journal of cancer*. **144**(6): p. 1321-1330.
192. Maria, A.G., et al., 2022, *USP13 genetics and expression in a family with thyroid cancer*. *Endocrine*. **77**(2): p. 281-290.
193. Taylor, P.N., et al., 2015, *Whole-genome sequence-based analysis of thyroid function*. *Nat Commun*. **6**: p. 5681.
194. Correia, M., et al., 2021, *Inherited Thyroid Tumors With Oncocytic Change*. *Front Endocrinol (Lausanne)*. **12**: p. 691979.
195. He, H., et al., 2013, *SRGAP1 is a candidate gene for papillary thyroid carcinoma susceptibility*. *The Journal of Clinical Endocrinology & Metabolism*. **98**(5): p. E973-E980.

196. Zhao, Y., et al., 2020, *A germline CHEK2 mutation in a family with papillary thyroid cancer*. *Thyroid*. **30**(6): p. 924-930.
197. Bonora, E., et al., 2006, *Novel germline variants identified in the inner mitochondrial membrane transporter TIMM44 and their role in predisposition to oncocyctic thyroid carcinomas*. *British journal of cancer*. **95**(11): p. 1529-1536.
198. Sarquis, M., et al., 2020, *Germline mutations in familial papillary thyroid cancer*. *Endocrine pathology*. **31**: p. 14-20.
199. Orois, A., et al., 2019, *NOP53 as a candidate modifier locus for familial non-medullary thyroid cancer*. *Genes*. **10**(11): p. 899.
200. Liang, X., et al., 2017, *Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA*. *Journal of biotechnology*. **241**: p. 136-146.
201. Liang, X., et al., 2015, *Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection*. *Journal of biotechnology*. **208**: p. 44-53.
202. Yu, X., et al., 2016, *Improved delivery of Cas9 protein/gRNA complexes using lipofectamine CRISPRMAX*. *Biotechnology letters*. **38**: p. 919-929.
203. Siwei, C., et al., 2022, *A genome-wide mutational constraint map quantified from variation in 76,156 human genomes*. *bioRxiv*. p. 2022.03.20.485034.
204. Martínez-Jiménez, F., et al., 2020, *A compendium of mutational cancer driver genes*. *Nature Reviews Cancer*. **20**(10): p. 555-572.
205. Martin, F.J., et al., 2022, *Ensembl 2023*. *Nucleic Acids Research*. **51**(D1): p. D933-D941.
206. Landrum, M.J., et al., 2020, *ClinVar: improvements to accessing data*. *Nucleic Acids Res*. **48**(D1): p. D835-d844.
207. Tate, J.G., et al., 2018, *COSMIC: the Catalogue Of Somatic Mutations In Cancer*. *Nucleic Acids Research*. **47**(D1): p. D941-D947.
208. Song, N., et al., 2023, *USP9X deubiquitinates and stabilizes CDC123 to promote breast carcinogenesis through regulating cell cycle*. *Molecular Carcinogenesis*. **62**(10): p. 1487-1503.
209. Krishnan, A., et al., 2020, *Proteogenomics analysis unveils a TFG-RET gene fusion and druggable targets in papillary thyroid carcinomas*. *Nature Communications*. **11**(1): p. 2056.
210. Zhang, J., et al., 2019, *Deubiquitinase USP9X regulates the invasion of prostate cancer cells by regulating the ERK pathway and mitochondrial dynamics*. *Oncology Reports*. **41**(6): p. 3292-3304.
211. Aryapour, E. and T. Kietzmann, 2022, *Mitochondria, mitophagy, and the role of deubiquitinases as novel therapeutic targets in liver pathology*. *Journal of Cellular Biochemistry*. **123**(10): p. 1634-1646.
212. Shen, G., et al., 2014, *MicroRNA-26b inhibits epithelial-mesenchymal transition in hepatocellular carcinoma by targeting USP9X*. *BMC Cancer*. **14**(1): p. 393.
213. Potu, H., et al., 2017, *Usp9x regulates Ets-1 ubiquitination and stability to control NRAS expression and tumorigenicity in melanoma*. *Nature Communications*. **8**(1): p. 14449.
214. Hiemcke-Jiwa, L.S., et al., 2020, *USP6-associated neoplasms: a rapidly expanding family of lesions*. *International Journal of Surgical Pathology*. **28**(8): p. 816-825.
215. Teramura, Y., et al., 2019, *Case of mesenchymal tumor with the PPP6R3-USP6 fusion, possible nodular fasciitis with malignant transformation*. *Pathology International*. **69**(12): p. 706-709.
216. Nakamura, N. and S. Hirose, 2008, *Regulation of Mitochondrial Morphology by USP30, a Deubiquitinating Enzyme Present in the Mitochondrial Outer Membrane*. *Molecular Biology of the Cell*. **19**(5): p. 1903-1911.
217. Hou, J., M. Eldeeb, and X. Wang, 2017, *Beyond deubiquitylation: USP30-mediated regulation of mitochondrial homeostasis*. *Mitochondrial DNA and diseases*. p. 133-148.

218. Lima, A.R., et al., 2021, *S616-p-DRP1 associates with locally invasive behavior of follicular cell-derived thyroid carcinoma*. *Endocrine*. **73**(1): p. 85-97.
219. Chou, C.-K., et al., 2017, *The Regulations of Deubiquitinase USP15 and Its Pathophysiological Mechanisms in Diseases*. *International Journal of Molecular Sciences*. **18**(3): p. 483.
220. Cornelissen, T., et al., 2014, *The deubiquitinase USP15 antagonizes Parkin-mediated mitochondrial ubiquitination and mitophagy*. *Human Molecular Genetics*. **23**(19): p. 5227-5242.
221. Maurer, B., et al., 2021, *Inducible deletion of CDK4 and CDK6 - deciphering CDK4/6 inhibitor effects in the hematopoietic system*. *Haematologica*. **106**(10): p. 2624-2632.
222. Shah, M., M.R. Nunes, and V. Stearns, 2018, *CDK4/6 Inhibitors: Game Changers in the Management of Hormone Receptor-Positive Advanced Breast Cancer?* *Oncology (Williston Park)*. **32**(5): p. 216-22.
223. Miller, C., 2000, *An overview of the potassium channel family*. *Genome biology*. **1**(4).
224. Bates, E., 2015, *Ion Channels in Development and Cancer*. *Annual Review of Cell and Developmental Biology*. **31**(1): p. 231-247.
225. Kuang, Q., P. Purhonen, and H. Hebert, 2015, *Structure of potassium channels*. *Cellular and molecular life sciences : CMLS*. **72**(19): p. 3677-3693.
226. Ranjan, R., et al., 2019, *A kinetic map of the homomeric voltage-gated potassium channel (Kv) family*. *Frontiers in Cellular Neuroscience*. **13**: p. 358.
227. Kim, D.M. and C.M. Nimigean, 2016, *Voltage-gated potassium channels: a structural examination of selectivity and gating*. *Cold Spring Harbor perspectives in biology*. **8**(5): p. a029231.
228. Bocksteins, E. and D.J. Snyders, 2012, *Electrically silent Kv subunits: their molecular and functional characteristics*. *Physiology (Bethesda)*. **27**(2): p. 73-84.
229. Xu, Z., et al., 2022, *Pentameric assembly of the Kv2.1 tetramerization domain*. *Acta Crystallogr D Struct Biol*. **78**(Pt 6): p. 792-802.
230. Strang, C., et al., 2001, *A central role for the T1 domain in voltage-gated potassium channel formation and function*. *Journal of Biological Chemistry*. **276**(30): p. 28493-28502.
231. Minor, D.L., et al., 2000, *The Polar T1 Interface Is Linked to Conformational Changes that Open the Voltage-Gated Potassium Channel*. *Cell*. **102**(5): p. 657-670.
232. González, C., et al., 2012, *K⁺ channels: function-structural overview*. *Comprehensive physiology*. **2**(3): p. 2087-2149.
233. Zhang, P., et al., 2016, *Inhibition of SK4 Potassium Channels Suppresses Cell Proliferation, Migration and the Epithelial-Mesenchymal Transition in Triple-Negative Breast Cancer Cells*. *PLoS One*. **11**(4): p. e0154471.
234. Menéndez, S.T., et al., 2010, *Expression and clinical significance of the Kv3.4 potassium channel subunit in the development and progression of head and neck squamous cell carcinomas*. *The Journal of Pathology*. **221**(4): p. 402-410.
235. Rao, V.R., et al., 2015, *Voltage-gated ion channels in cancer cell proliferation*. *Cancers (Basel)*. **7**(2): p. 849-75.
236. Abbott, G.W., 2014, *Biology of the KCNQ1 Potassium Channel*. *New Journal of Science*. **2014**: p. 26.
237. Abbott, G.W., 2015, *The KCNE2 K⁺ channel regulatory subunit: Ubiquitous influence, complex pathobiology*. *Gene*. **569**(2): p. 162-172.
238. Purtell, K., et al., 2012, *The KCNQ1-KCNE2 K⁺ channel is required for adequate thyroid I⁻ uptake*. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. **26**(8): p. 3252-3259.
239. Boelaert, K., 2009, *The association between serum TSH concentration and thyroid cancer*. *Endocr Relat Cancer*. **16**(4): p. 1065-72.

240. Ma, Y., X. Zhang, and Y. Wang, 2017, *Reactivity of thyroid papillary carcinoma cells to thyroid stimulating hormone-dominated endocrine therapy*. *Oncol Lett.* **14**(6): p. 7405-7409.
241. Lloyd, R., et al., 2017, *WHO Classification of Tumours of Endocrine Organs*, 4th edn IARC Press, Lyon.
242. Agrawal, N., et al., 2014, *Integrated Genomic Characterization of Papillary Thyroid Carcinoma*. *Cell.* **159**(3): p. 676-690.
243. Jung, S.-H., et al., 2016, *Genetic progression of high grade prostatic intraepithelial neoplasia to prostate cancer*. *European urology.* **69**(5): p. 823-830.
244. Boßelmann, C.M., et al., 2022, *Predicting the functional effects of voltage-gated potassium channel missense variants with multi-task learning*. *EBioMedicine.* **81**.
245. Waters, M.F., et al., 2006, *Mutations in voltage-gated potassium channel KCNC3 cause degenerative and developmental central nervous system phenotypes*. *Nat Genet.* **38**(4): p. 447-51.
246. Simons, C., et al., 2015, *Mutations in the voltage-gated potassium channel gene KCNH1 cause Temple-Baraitser syndrome and epilepsy*. *Nature Genetics.* **47**(1): p. 73-77.
247. Giudicessi, J.R. and M.J. Ackerman, 2012, *Potassium-channel mutations and cardiac arrhythmias--diagnosis and therapy*. *Nat Rev Cardiol.* **9**(6): p. 319-32.
248. Dehghani-Samani, A., S. Madreseh-Ghahfarokhi, and A. Dehghani-Samani, 2019, *Mutations of Voltage-Gated Ionic Channels and Risk of Severe Cardiac Arrhythmias*. *Acta Cardiol Sin.* **35**(2): p. 99-110.
249. Zheng, Z. and Y. Song, 2023, *Integrated analysis of the voltage-gated potassium channel-associated gene KCNH2 across cancers*. *BMC Bioinformatics.* **24**(1): p. 51.
250. Zhao, Q., M. Li, and Y. Zhang, 2023, *Comprehensive pan-cancer analysis of potassium voltage-gated channel Q4 (KCNQ4) gene across multiple human malignant tumors*. *Scientific Reports.* **13**(1): p. 18608.
251. Chow, L.W., et al., 2018, *Voltage-gated K(+) channels promote BT-474 breast cancer cell migration*. *Chin J Cancer Res.* **30**(6): p. 613-622.
252. Park, H.W., et al., 2021, *The role of the voltage-gated potassium channel, Kv2.1 in prostate cancer cell migration*. *BMB Rep.* **54**(2): p. 130-135.
253. Barbirou, M., et al., 2020, *KCNB1 gene polymorphisms and related indel as predictor biomarkers of treatment response for colorectal cancer – toward a personalized medicine*. *Tumor Biology.* **42**(6): p. 1010428320925237.
254. Farah, A., et al., 2020, *Selective expression of KCNA5 and KCNB1 genes in gastric and colorectal carcinoma*. *BMC Cancer.* **20**(1): p. 1179.
255. Wang, H.-Y., et al., 2017, *Role of KCNB1 in the prognosis of gliomas and autophagy modulation*. *Scientific Reports.* **7**(1): p. 14.
256. de Kovel, C.G.F., et al., 2017, *Neurodevelopmental Disorders Caused by De Novo Variants in KCNB1 Genotypes and Phenotypes*. *JAMA Neurol.* **74**(10): p. 1228-1236.
257. Cornen, S., et al., 2014, *Candidate luminal B breast cancer genes identified by genome, gene expression and DNA methylation profiling*. *PLoS One.* **9**(1): p. e81843.
258. Fan, J.J., et al., 2022, *In vivo functional genomics identifies essentiality of potassium homeostasis in medulloblastoma*. *bioRxiv.* p. 2022.07. 23.501234.
259. Stelzer, G., et al., 2016, *The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses*. *Current Protocols in Bioinformatics.* **54**(1): p. 1.30.1-1.30.33.
260. Hönigsperger, C., M.J. Nigro, and J.F. Storm, 2017, *Physiological roles of Kv2 channels in entorhinal cortex layer II stellate cells revealed by Guangxitoxin-1E*. *J Physiol.* **595**(3): p. 739-757.
261. Herrington, J., et al., 2006, *Blockers of the delayed-rectifier potassium current in pancreatic β -cells enhance glucose-dependent insulin secretion*. *Diabetes.* **55**(4): p. 1034-1042.

262. Thul, P.J., et al., 2017, *A subcellular map of the human proteome*. Science. **356**(6340): p. eaal3321.
263. Haymart, M.R., 2021, *Progress and Challenges in Thyroid Cancer Management*. Endocrine Practice. **27**(12): p. 1260-1263.
264. Pova, A.A., et al., 2020, *Clinicopathological features as prognostic predictors of poor outcome in papillary thyroid carcinoma*. cancers. **12**(11): p. 3186.
265. Sezer, A., et al., 2017, *Relationship between lymphovascular invasion and clinicopathological features of papillary thyroid carcinoma*. Bosn J Basic Med Sci. **17**(2): p. 144-151.
266. Yoon, S., J. Lee, and E. Soh, 2019, *Which Clinicopathological Factors Are Related to Tumor Size in Papillary Thyroid Cancer?* J Endocr Surg. **19**(4): p. 95-105.
267. Xing, M., et al., 2005, *BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer*. J Clin Endocrinol Metab. **90**(12): p. 6373-9.
268. Xing, M., et al., 2013, *Association Between BRAF V600E Mutation and Mortality in Patients With Papillary Thyroid Cancer*. JAMA. **309**(14): p. 1493-1501.
269. Wang, F., et al., 2018, *BRAF V600E Confers Male Sex Disease-Specific Mortality Risk in Patients With Papillary Thyroid Cancer*. Journal of Clinical Oncology. **36**(27): p. 2787-2795.
270. Shen, X., et al., 2018, *Patient Age–Associated Mortality Risk Is Differentiated by BRAF V600E Status in Papillary Thyroid Cancer*. Journal of Clinical Oncology. **36**(5): p. 438-445.
271. Vuong, H.G., et al., 2017, *A meta-analysis of prognostic roles of molecular markers in papillary thyroid carcinoma*. Endocrine connections. **6**(3): p. R8-R17.
272. Xing, M., 2016, *Clinical utility of RAS mutations in thyroid cancer: a blurred picture now emerging clearer*. BMC Med. **14**: p. 12.
273. Sun, W., et al., 2015, *Risk Factors for Central Lymph Node Metastasis in CNO Papillary Thyroid Carcinoma: A Systematic Review and Meta-Analysis*. PLOS ONE. **10**(10): p. e0139021.
274. Tran, B., et al., 2018, *The Prognostic Impact of Tumor Size in Papillary Thyroid Carcinoma is Modified by Age*. Thyroid. **28**(8): p. 991-996.
275. Kim, J.-Y., et al., 2015, *Impact of tumor size on subclinical central lymph node metastasis in papillary thyroid microcarcinoma depends on age*. World Journal of Surgical Oncology. **13**(1): p. 88.
276. Page, C., et al., 2009, *'Aggressive papillary' thyroid microcarcinoma*. European Archives of Oto-Rhino-Laryngology. **266**(12): p. 1959-1963.
277. Ardito, G., et al., 2013, *Aggressive Papillary Thyroid Microcarcinoma: Prognostic Factors and Therapeutic Strategy*. Clinical Nuclear Medicine. **38**(1).
278. Aguiar, P.H., et al., 2001, *Unusual Brain Metastases from Papillary Thyroid Carcinoma: Case Report*. Neurosurgery. **49**(4).
279. Amado, A., et al., 2023, *Subcentimetric papillary thyroid carcinoma with extensive lymph node and brain metastasis: case report and review of literature*. Endocrinol Diabetes Metab Case Rep. **2023**(4).
280. Ramalho, D., et al., 2023, *Subcentimetric Papillary Thyroid Carcinoma: Does the Diagnosis Kind Impact Prognosis?* Cureus. **15**(11).
281. Vaisman, F., et al., 2011, *Initial therapy with either thyroid lobectomy or total thyroidectomy without radioactive iodine remnant ablation is associated with very low rates of structural disease recurrence in properly selected patients with differentiated thyroid cancer*. Clinical Endocrinology. **75**(1): p. 112-119.
282. Kamani, T., et al., 2022, *Genetic susceptibility to hereditary non-medullary thyroid cancer*. Hereditary Cancer in Clinical Practice. **20**(1): p. 9.
283. Klubo-Gwiedzinska, J., et al., 2017, *Results of screening in familial non-medullary thyroid cancer*. Thyroid. **27**(8): p. 1017-1024.

- 284. Freeman Laura, E.B., et al., 2011, *Atrazine and Cancer Incidence Among Pesticide Applicators in the Agricultural Health Study (1994–2007)*. Environmental Health Perspectives. **119**(9): p. 1253-1259.
- 285. Zhang, C., et al., 2019, *Association of exposure to multiple metals with papillary thyroid cancer risk in China*. Environmental Science and Pollution Research. **26**(20): p. 20560-20572.
- 286. Zhou, Z., et al., 2017, *Higher urinary bisphenol A concentration and excessive iodine intake are associated with nodular goiter and papillary thyroid carcinoma*. Bioscience Reports. **37**(4).
- 287. Martinez-Pinna, J., et al., 2019, *Oestrogen receptor β mediates the actions of bisphenol-A on ion channel expression in mouse pancreatic beta cells*. Diabetologia. **62**(9): p. 1667-1680.
- 288. Awada, Z., et al., 2019, *DNA methylome-wide alterations associated with estrogen receptor-dependent effects of bisphenols in breast cancer*. Clin Epigenetics. **11**(1): p. 138.

APPENDIX

Appendix

ARTICLE VI

Thyroid cancer incidence has been continuously increasing over the past decades [71]. Despite the well-established overdiagnosis effect already discussed in chapter 2.3.2.4., exposure to environmental pollutants, such as endocrine disrupting chemicals, has been pointed as a risk factor for thyroid cancer and therefore a cause for that increase [72, 103]. Our team has explored the association of endocrine disrupting chemicals with six endocrine-related tumors. Despite the emphasis of this thesis is the study of thyroid tumorigenesis related factors, we also explore tumors of the breast, prostate, uterus, testis, ovary.



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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 assessed 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 EDCs 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–

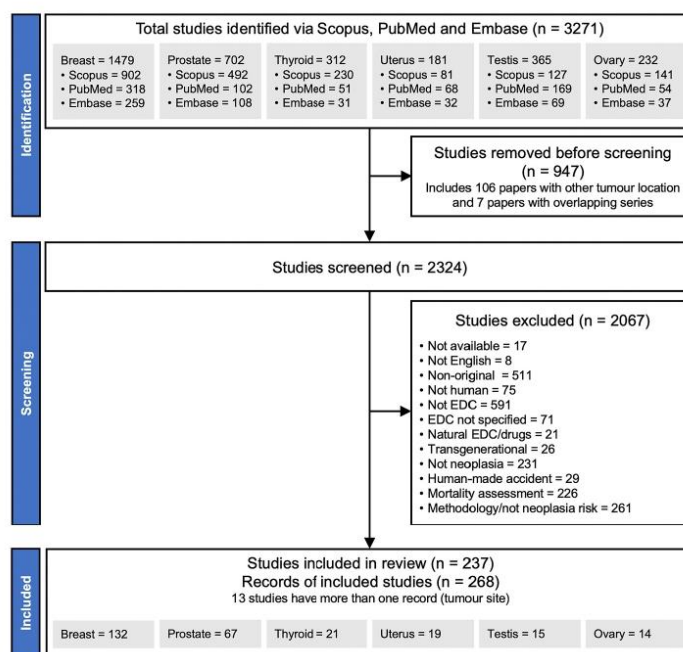


Fig. 1. PRISMA flowchart of the paper selection process according to Page et al. (2021) (Page et al., 2021).

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	1
	Asia	8	6.1	7	10.4	5	23.8	5	26.3	0	0.0	2	14.3
	America	87	65.9	46	68.7	11	52.4	8	42.1	5	33.3	6	42.9
	Europe	31	23.5	13	19.4	5	23.8	6	31.6	10	66.7	6	42.9
	Oceania	5	3.8	1	1.5	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
	2001-2011	42	31.8	33	49.3	4	19.0	4	21.1	8	53.3	5	35.7
	2012-2021	66	50.0	30	44.8	17	81.0	14	73.7	4	26.7	8	57.1
Study duration	5-	55	41.7	13	19.4	10	47.6	9	47.4	7	46.7	3	21.4
	5+	73	55.3	51	76.1	11	52.4	9	47.4	7	46.7	11	78.6
	Not reported	4	3.0	3	4.5	0	0	1	5.3	1	6.7	0	0
Type of study	Case control	102	77.3	37	55.2	12	57.1	10	52.6	13	86.7	5	35.7
	Cohort	21	15.9	27	40.3	6	28.6	7	36.8	1	6.7	5	35.7
	Others*	9	6.8	3	4.5	3	14.3	2	10.5	1	6.7	4	28.6
Sample size	100-	2	1.5	4	6.0	2	9.5	1	5.3	2	13.3	1	7.1
	100+	61	46.2	14	20.9	10	47.6	7	36.8	5	33.3	2	14.3
	1,000+	42	31.8	22	32.8	2	9.5	5	26.3	6	40.0	2	14.3
	10,000+	18	13.6	25	37.3	4	19.0	4	21.1	0	0	5	35.7
	100,000+	9	6.8	2	3.0	3	14.3	2	10.5	2	13.3	4	28.6
Sex	Male	3	2.3	67	100	2	9.5	n.a.	n.a.	15	100	n.a.	87
	Female	126	95.5	n.a.	n.a.	5	23.8	19	100	n.a.	n.a.	14	100
	Both	3	2.3	n.a.	n.a.	14	66.7	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.
	STD	9	n.a.	9	n.a.	7	n.a.	14	n.a.	14	n.a.	14	n.a.
Laboratory tests	Yes	72	54.5	22	32.8	13	61.9	11	57.9	6	40.0	4	28.6
	No	60	45.5	45	67.2	8	38.1	8	42.1	9	60.0	10	71.4
Initial confidence	Very low	3	2.3	0	0.0	2	9.5	0	0	1	6.7	2	14.3
	Low	48	36.4	10	14.9	9	42.9	7	36.8	5	33.3	1	7.1
	Moderate	81	61.4	57	85.1	10	47.6	12	63.2	9	60.0	11	78.6
	High	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
	Low	45	34.1	29	43.3	8	38.1	7	36.8	4	26.7	3	21.4
	Moderate	52	39.4	19	28.4	8	38.1	7	36.8	10	66.7	8	57.1
	High	23	17.4	11	16.4	2	9.5	1	5.3	0	0	1	7.1
Paper with positive effect	Yes	73	55.3	37	55.2	14	66.7	12	63.2	8	53.3	6	42.9
	No	59	44.7	30	44.8	7	33.3	7	36.8	7	46.7	8	57.1

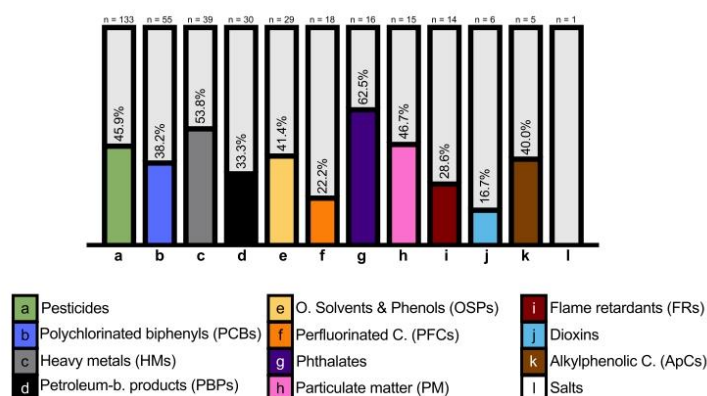


Fig. 2. Percentage of reports of EDCs with increased risk of endocrine-related neoplasia (all tumour sites). Colours and percentages in each bar represent the proportion of records with a positive effect. (For interpretation of the references to colour in this figure legend, the reader is referred to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

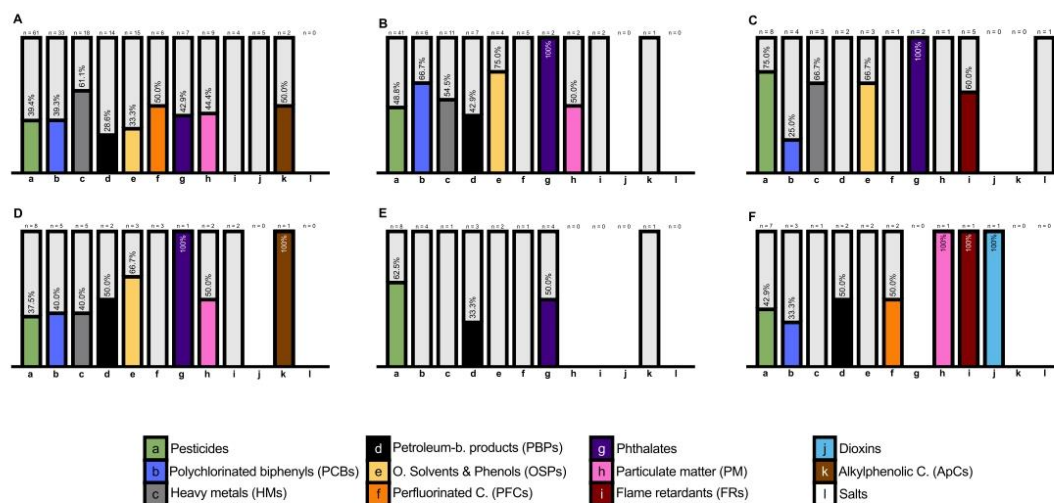


Fig. 3. Percentage of reports of EDCs with increased risk of endocrine-related neoplasia in (A) Breast, (B) Prostate, (C) Thyroid, (D) Uterus, (E) Testis, and (F) Ovary. Colours and percentages in each bar represent the proportion of records with a positive effect. (For interpretation of the references 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 tumorigenesis, 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 tumorigenic 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 tumorigenic 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 (1.220–109.40)] (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, met-alaxyl, 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 (ER α and ER β) 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 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 Voutsas, 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 Voutsas, 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 tumorigenesis.

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>.

References

- AHS. *Agricultural Health Study (AHS)*, 2022. Available from: <https://aghealth.nih.gov/about/index.html>.
- Akash, M.M., Pawade, U.V., Nikam, A.V., 2018. Classification of pesticides: a review. *Int. J. Res. Ayurveda Pharm.* 9 (4), 144–150. <https://doi.org/10.7897/2277-4343.094131>.
- Alavanja, M.C., Ross, M.K., Bonner, M.R., 2013. Increased cancer burden among pesticide applicators and others due to pesticide exposure. *Ca - Cancer J. Clin.* 63 (2), 120–142. <https://doi.org/10.3322/caac.21170>.
- Alsen, M., et al., 2021. Endocrine disrupting chemicals and thyroid cancer: an overview. *Toxics* 9 (1). <https://doi.org/10.3390/toxics9010014>.
- Armstrong, B.G., Kazzantzis, G., 1985. Prostatic cancer and chronic respiratory and renal disease in British cadmium workers: a case control study. *Br. J. Ind. Med.* 42 (8), 540–545. <https://doi.org/10.1136/oem.42.8.540>.
- Aronson, K.J., et al., 2010. Plasma organochlorine levels and prostate cancer risk. *J. Expo. Sci. Environ. Epidemiol.* 20 (5), 434–445. <https://doi.org/10.1038/jes.2009.33>.
- Arrebola, J.P., et al., 2015. Risk of female breast cancer and serum concentrations of organochlorine pesticides and polychlorinated biphenyls: a case-control study in Tunisia. *Sci. Total Environ.* 520, 106–113. <https://doi.org/10.1016/j.scitotenv.2015.03.045>.
- Behrens, T., et al., 2012. Exposure to metal-working fluids in the automobile industry and the risk of male germ cell tumours. *Occup. Environ. Med.* 69 (3), 224–226. <https://doi.org/10.1136/oemed-2011-100070>.
- Blanc-Lapierre, A., Sauve, J.F., Parent, M.E., 2018. Occupational exposure to benzene, toluene, xylene and styrene and risk of prostate cancer in a population-based study. *Occup. Environ. Med.* 75 (8), 562–572. <https://doi.org/10.1136/oemed-2018-105058>.
- Brauner, E.V., et al., 2021. Endocrine disrupting chemicals and risk of testicular cancer: a systematic review and meta-analysis. *J. Clin. Endocrinol. Metab.* 106 (12), e4834–e4860. <https://doi.org/10.1210/clinem/dgab523>.
- Briffa, J., Sinagra, E., Blundell, R., 2020. Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon* 6 (9). <https://doi.org/10.1016/j.heliyon.2020.e04691>.
- Bullman, T.A., Watanabe, K.K., Kano, H.K., 1994. Risk of testicular cancer associated with surrogate measures of agent orange exposure among Vietnam veterans on the agent orange registry. *Ann. Epidemiol.* 4 (1), 11–16. [https://doi.org/10.1016/1047-2797\(94\)90037-x](https://doi.org/10.1016/1047-2797(94)90037-x).
- Byrne, C., et al., 2013. Metals and breast cancer. *J. Mammary Gland Biol. Neoplasia* 18 (1), 63–73. <https://doi.org/10.1007/s10911-013-9273-9>.
- Calaf, G.M., et al., 2020. Endocrine disruptors from the environment affecting breast cancer. *Oncol. Lett.* 20 (1), 19–32. <https://doi.org/10.3892/ol.2020.11566>.
- Chang, W.H., et al., 2018. Oxidative damage in patients with benign prostatic hyperplasia and prostate cancer co-exposed to phthalates and to trace elements. *Environ. Int.* 121 (Pt 2), 1179–1184. <https://doi.org/10.1016/j.envint.2018.10.034>.
- Charlier, C., et al., 2004. Environmental dichlorodiphenyltrichloroethane or hexachlorobenzene exposure and breast cancer: is there a risk? *Clin. Chem. Lab. Med.* 42 (2), 222–227. <https://doi.org/10.1515/CCLM.2004.040>.
- Chen, L.J., et al., 2012. Urinary strontium and the risk of breast cancer: a case-control study in Guangzhou, China. *Environ. Res.* 112, 212–217. <https://doi.org/10.1016/j.envres.2011.11.005>.
- Chia, V.M., et al., 2010. Effect modification of endocrine disruptors and testicular germ cell tumour risk by hormone-metabolizing genes. *Int. J. Androl.* 33 (4), 588–596. <https://doi.org/10.1111/j.1365-2605.2009.00975.x>.
- Christensen, C.H., et al., 2010. Coumaphos exposure and incident cancer among male participants in the Agricultural Health Study (AHS). *Environ. Health Perspect.* 118 (1), 92–96. <https://doi.org/10.1289/ehp.0800446>.
- Christensen, K.Y., et al., 2013. Risk of selected cancers due to occupational exposure to chlorinated solvents in a case-control study in Montreal. *J. Occup. Environ. Med.* 55 (2), 198–208. <https://doi.org/10.1097/JOM.0b013e3182728eab>.
- Chuang, S.C., et al., 2020. Phthalate exposure and prostate cancer in a population-based nested case-control study. *Environ. Res.* 181, 108902. <https://doi.org/10.1016/j.envres.2019.108902>.
- Cohn, B.A., et al., 2007. DDT and breast cancer in young women: new data on the significance of age at exposure. *Environ. Health Perspect.* 115 (10), 1406–1414. <https://doi.org/10.1289/ehp.10260>.
- Cohn, B.A., Cirillo, P.M., Terry, M.B., 2019. DDT and breast cancer: prospective study of induction time and susceptibility windows. *J. Natl. Cancer Inst.* 111 (8), 803–810. <https://doi.org/10.1093/jnci/djy198>.
- Combarnous, Y., Nguyen, T.M.D., 2019. Comparative overview of the mechanisms of action of hormones and endocrine disruptor compounds. *Toxics* 7 (1). <https://doi.org/10.3390/toxics7010005>.
- Corti, M., et al., 2022. Endocrine disruptors and prostate cancer. *Int. J. Mol. Sci.* 23 (3). <https://doi.org/10.3390/ijms23031216>.
- Del Pup, L., et al., 2016. Carcinogenic mechanisms of endocrine disruptors in female cancers (Review). *Oncol. Rep.* 36 (2), 603–612. <https://doi.org/10.3892/or.2016.4886>.
- Demeneix, B., Slama, R., 2019a. Endocrine disruptors: from scientific evidence to human health protection. In: PETI Committee European Parliament.
- Demeneix, B., Slama, R., 2019b. Endocrine Disruptors: from Scientific Evidence to Human Health Protection. European Parliament, Brussels.
- Demers, A., et al., 2000. Risk and aggressiveness of breast cancer in relation to plasma organochlorine concentrations. *Cancer Epidemiol. Biomarkers Prev.* 9 (2), 161–166.
- Dieye, M., et al., 2014. Geographic variations and temporal trends in prostate cancer in Martinique over a 25-year period. *BMC Res. Notes* 7, 262. <https://doi.org/10.1186/1756-0500-7-262>.
- EFSA, 2013. Scientific Opinion on the hazard assessment of endocrine disruptors: scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment. EFSA J.
- Eldakroory, S.A., et al., 2017. Correlation between toxic organochlorine pesticides and breast cancer. *Hum. Exp. Toxicol.* 36 (12), 1326–1334. <https://doi.org/10.1177/0960327116685887>.
- Emeville, E., et al., 2015. Associations of plasma concentrations of dichlorodiphenyldichloroethylene and polychlorinated biphenyls with prostate cancer: a case-control study in Guadeloupe (French West Indies). *Environ. Health Perspect.* 123 (4), 317–323. <https://doi.org/10.1289/ehp.1408407>.
- Encarnacao, T., et al., 2019. Endocrine disrupting chemicals: impact on human health, wildlife and the environment. *Sci. Prog.* 102 (1), 3–42. <https://doi.org/10.1177/0036850419826802>.
- Eurostat, 2019. Chemicals Production and Consumption Statistics [cited 2021 November 23rd, 2021]. Available from: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Chemicals_production_and_consumption_statistics#Production_of_chemicals_hazardous_to_health.
- Fernandez-Martinez, N.F., et al., 2020. Relationship between exposure to mixtures of persistent, bioaccumulative, and toxic chemicals and cancer risk: a systematic review. *Environ. Res.* 188, 109787. <https://doi.org/10.1016/j.envres.2020.109787>.
- Freeman, L.E., et al., 2011. Atrazine and cancer incidence among pesticide applicators in the agricultural health study (1994–2007). *Environ. Health Perspect.* 119 (9), 1253–1259. <https://doi.org/10.1289/ehp.1103561>.
- Gallagher, C.M., Chen, J.J., Kovach, J.S., 2010. Environmental cadmium and breast cancer risk. *Aging (Albany NY)* 2 (11), 804–814. <https://doi.org/10.18632/aging.100226>.
- Gammon, M.D., et al., 2002. Environmental toxins and breast cancer on Long Island. II. Organochlorine compound levels in blood. *Cancer Epidemiol. Biomarkers Prev.* 11 (8), 686–697.
- Gerhardsson, L., Hou, L., Pettersson, K., 2021. Work-related exposure to organic solvents and the risk for multiple sclerosis: a systematic review. *Int. Arch. Occup. Environ. Health* 94 (2), 221–229. <https://doi.org/10.1007/s00420-020-01564-z>.
- Ghisari, M., et al., 2017. Polymorphism in xenobiotic and estrogen metabolizing genes, exposure to perfluorinated compounds and subsequent breast cancer risk: a nested case-control study in the Danish National Birth Cohort. *Environ. Res.* 154, 325–333. <https://doi.org/10.1016/j.envres.2017.01.020>.
- Giannandrea, F., et al., 2011. Pesticide exposure and serum organochlorine residuals among testicular cancer patients and healthy controls. *J. Environ. Sci. Health B* 46 (8), 780–787. <https://doi.org/10.1080/03601234.2012.597704>.
- Giulivo, M., et al., 2016. Human exposure to endocrine disrupting compounds: their role in reproductive systems, metabolic syndrome and breast cancer. A review. *Environ. Res.* 151, 251–264. <https://doi.org/10.1016/j.envres.2016.07.011>.
- Gore, A., et al., 2014. Introduction to Endocrine Disrupting Chemicals (EDCs) A Guide for Public Interest Organizations and Policy-Makers. Endocrine Society. <https://www.endocrine.org/-/media/endosociety/files/advocacy-and-outreach/important-documents/introduction-to-endocrine-disrupting-chemicals.pdf>.
- Gore, A.C., et al., 2015. Executive summary to EDC-2: the endocrine society's second scientific statement on endocrine-disrupting chemicals. *Endocr. Rev.* 36 (6), 593–602. <https://doi.org/10.1210/er.2015-1093>.
- Han, M.A., Kim, J.H., Song, H.S., 2019a. Persistent organic pollutants, pesticides, and the risk of thyroid cancer: systematic review and meta-analysis. *Eur. J. Cancer Prev.* 28 (4), 344–349. <https://doi.org/10.1097/CEJ.0000000000000481>.

- Han, X., et al., 2019b. Associations between exposure to persistent organic pollutants and thyroid function in a case-control study of east China. *Environ. Sci. Technol.* 53 (16), 9866–9875. <https://doi.org/10.1021/acs.est.9b02810>.
- Hardell, L., Ohlsson, C.-G., Fredrikson, M., 1997. Occupational exposure to polyvinyl chloride as a risk factor for testicular cancer evaluated in a case-control study. *Int. J. Cancer* 73 (6), 828–830. [https://doi.org/10.1002/\(sici\)1097-0215\(19971210\)73:6<828::Aid-ijc10>3.0.Co;2-o](https://doi.org/10.1002/(sici)1097-0215(19971210)73:6<828::Aid-ijc10>3.0.Co;2-o).
- Hardell, L., et al., 1998. Case-control study on risk factors for testicular cancer. *Int. J. Oncol.* 13 (6), 1299–1303. <https://doi.org/10.3892/ijo.13.6.1299>.
- Hardell, L., et al., 2004. Testicular cancer and occupational exposure to polyvinyl chloride plastics: a case-control study. *Int. J. Cancer* 109 (3), 425–429. <https://doi.org/10.1002/ijc.11709>.
- Hardell, L., et al., 2006. Adipose tissue concentrations of persistent organic pollutants and the risk of prostate cancer. *J. Occup. Environ. Med.* 48 (7), 700–707. <https://doi.org/10.1097/01.jom.0000205989.46603.43>.
- Heindel, J.J., Newbold, R., Schug, T.T., 2015. Endocrine disruptors and obesity. *Nat. Rev. Endocrinol.* 11 (11), 653–661. <https://doi.org/10.1038/nrendo.2015.163>.
- Hoffman, K., et al., 2017a. Exposure to flame retardant chemicals and occurrence and severity of papillary thyroid cancer: a case-control study. *Environ. Int.* 107, 235–242. <https://doi.org/10.1016/j.envint.2017.06.021>.
- Hoffman, K., et al., 2017b. Temporal trends in exposure to organophosphate flame retardants in the United States. *Environ. Sci. Technol. Lett.* 4 (3), 112–118. <https://doi.org/10.1021/acs.estlett.6b00475>.
- Hopenhayn-Rich, C., Stump, M.L., Browning, S.R., 2002. Regional assessment of atrazine exposure and incidence of breast and ovarian cancers in Kentucky. *Arch. Environ. Contam. Toxicol.* 42 (1), 127–136. <https://doi.org/10.1007/s002440010300>.
- Houston, T.J., Ghosh, R., 2020. Untangling the association between environmental endocrine disruptive chemicals and the etiology of male genitourinary cancers. *Biochem. Pharmacol.* 172, 113743. <https://doi.org/10.1016/j.bcp.2019.113743>.
- Hoyer, A.P., et al., 2000. Repeated measurements of organochlorine exposure and breast cancer risk (Denmark). *Cancer Causes Control* 11 (2), 177–184. <https://doi.org/10.1023/a:1008926219539>.
- Hoyer, A.P., et al., 2001. Organochlorine exposures influence on breast cancer risk and survival according to estrogen receptor status: a Danish cohort-nested case-control study. *BMC Cancer* 1, 8. <https://doi.org/10.1186/1471-2407-1-8>.
- Huang, H., et al., 2020. Polybrominated diphenyl ethers, polybrominated biphenyls, and risk of papillary thyroid cancer: a nested case-control study. *Am. J. Epidemiol.* 189 (2), 120–132. <https://doi.org/10.1093/aje/kwz229>.
- Inoue-Choi, M., et al., 2016. Atrazine in public water supplies and risk of ovarian cancer among postmenopausal women in the Iowa Women's Health Study. *Occup. Environ. Med.* 73 (9), 582–587. <https://doi.org/10.1136/oemed-2016-103575>.
- Johnstone, E.B., et al., 2014. Increased urinary cobalt and whole blood concentrations of cadmium and lead in women with uterine leiomyomata: findings from the ENDO Study. *Reprod. Toxicol.* 49, 27–32. <https://doi.org/10.1016/j.reprotox.2014.06.007>.
- Kabir, E.R., Rahman, M.S., Rahman, I., 2015. A review on endocrine disruptors and their possible impacts on human health. *Environ. Toxicol. Pharmacol.* 40 (1), 241–258. <https://doi.org/10.1016/j.etap.2015.06.009>.
- Kahn, L.G., et al., 2020. Endocrine-disrupting chemicals: implications for human health. *Lancet Diabetes Endocrinol.* 8 (8), 703–718. [https://doi.org/10.1016/S2213-8587\(20\)30129-7](https://doi.org/10.1016/S2213-8587(20)30129-7).
- Kim, Y.A., et al., 2016. Increased urinary phthalate levels in women with uterine leiomyoma: a case-control study. *Int. J. Environ. Res. Publ. Health* 13 (12). <https://doi.org/10.3390/ijerph13121247>.
- Kodavanti, P.R.S., et al., 2017. Polychlorinated biphenyls, polybrominated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans. In: *Reproductive and Developmental Toxicology*, pp. 711–743.
- Koutros, S., et al., 2010. Pesticide use modifies the association between genetic variants on chromosome 8q24 and prostate cancer. *Cancer Res.* 70 (22), 9224–9233. <https://doi.org/10.1158/0008-5472.CAN-10-1078>.
- Koutros, S., et al., 2013. Risk of total and aggressive prostate cancer and pesticide use in the Agricultural Health Study. *Am. J. Epidemiol.* 177 (1), 59–74. <https://doi.org/10.1093/aje/kws225>.
- Kresovich, J.K., et al., 2019. Metallic air pollutants and breast cancer heterogeneity. *Environ. Res.* 177, 108639. <https://doi.org/10.1016/j.envres.2019.108639>.
- La Merrill, M.A., et al., 2020. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nat. Rev. Endocrinol.* 16 (1), 45–57. <https://doi.org/10.1038/s41574-019-0273-8>.
- Lacouture, A., et al., 2022. Impacts of endocrine-disrupting chemicals on prostate function and cancer. *Environ. Res.* 204 (Pt B), 112085. <https://doi.org/10.1016/j.envres.2021.112085>.
- Lambertino, A., et al., 2011. Uterine leiomyomata in a cohort of Great Lakes sport fish consumers. *Environ. Res.* 111 (4), 565–572. <https://doi.org/10.1016/j.envres.2011.01.006>.
- Lauretta, R., et al., 2019. Endocrine disrupting chemicals: effects on endocrine glands. *Front. Endocrinol.* 10, 178. <https://doi.org/10.3389/fendo.2019.00178>.
- Lerro, C.C., et al., 2015a. Organophosphate insecticide use and cancer incidence among spouses of pesticide applicators in the Agricultural Health Study. *Occup. Environ. Med.* 72 (10), 736–744. <https://doi.org/10.1136/oemed-2014-102798>.
- Lerro, C.C., et al., 2015b. Use of acetochlor and cancer incidence in the agricultural health study. *Int. J. Cancer* 137 (5), 1167–1175. <https://doi.org/10.1002/ijc.29416>.
- Lerro, C.C., et al., 2018. A nested case-control study of polychlorinated biphenyls, organochlorine pesticides, and thyroid cancer in the Janus Serum Bank cohort. *Environ. Res.* 165, 125–132. <https://doi.org/10.1016/j.envres.2018.04.012>.
- Lerro, C.C., et al., 2021. Pesticide exposure and incident thyroid cancer among male pesticide applicators in agricultural health study. *Environ. Int.* 146, 106187. <https://doi.org/10.1016/j.envint.2020.106187>.
- Li, F.X., Squartoff, L., Lamm, S.H., 2001. Prevalence of thyroid diseases in Nevada counties with respect to perchlorate in drinking water. *J. Occup. Environ. Med.* 43 (7), 630–634. <https://doi.org/10.1097/00043764-200107000-00010>.
- Lim, J.E., et al., 2017. Serum persistent organic pollutants (POPs) and prostate cancer risk: a case-cohort study. *Int. J. Hyg Environ. Health* 220 (5), 849–856. <https://doi.org/10.1016/j.ijheh.2017.03.014>.
- Liu, C., et al., 2020. Urinary biomarkers of phthalates exposure and risks of thyroid cancer and benign nodule. *J. Hazard Mater.* 383, 121189. <https://doi.org/10.1016/j.jhazmat.2019.121189>.
- Lope, V., et al., 2009. Occupational exposure to chemicals and risk of thyroid cancer in Sweden. *Int. Arch. Occup. Environ. Health* 82 (2), 267–274. <https://doi.org/10.1007/s00420-008-0314-4>.
- Mantovani, A., 2016. Endocrine disruptors and the safety of food chains. *Horm Res Paediatr* 86 (4), 279–288. <https://doi.org/10.1159/000441496>.
- Marciniak, W., et al., 2020. Blood arsenic levels and the risk of familial breast cancer in Poland. *Int. J. Cancer* 146 (10), 2721–2727. <https://doi.org/10.1002/ijc.32595>.
- Marotta, V., et al., 2019. Human exposure to bisphenol AF and diethylhexylphthalate increases susceptibility to develop differentiated thyroid cancer in patients with thyroid nodules. *Chemosphere* 218, 885–894. <https://doi.org/10.1016/j.chemosphere.2018.11.084>.
- McCready, D., et al., 2004. Breast tissue organochlorine levels and metabolic genotypes in relation to breast cancer risk Canada. *Cancer Causes Control* 15 (4), 399–418. <https://doi.org/10.1023/B:CACO.0000027505.32564.c2>.
- McElroy, J.A., et al., 2017. Cadmium exposure and endometrial cancer risk: a large midwestern U.S. population-based case-control study. *PLoS One* 12 (7), e0179360. <https://doi.org/10.1371/journal.pone.0179360>.
- McGlynn, K.A., et al., 2008. Persistent organochlorine pesticides and risk of testicular germ cell tumors. *J. Natl. Cancer Inst.* 100 (9), 663–671. <https://doi.org/10.1093/jnci/djn101>.
- McKinlay, R., et al., 2008. Endocrine disrupting pesticides: implications for risk assessment. *Environ. Int.* 34 (2), 168–183. <https://doi.org/10.1016/j.envint.2007.07.013>.
- Miao, H., et al., 2020. Associations of urinary phthalate metabolites with risk of papillary thyroid cancer. *Chemosphere* 241, 125093. <https://doi.org/10.1016/j.chemosphere.2019.125093>.
- Miao, Y., et al., 2021. Serum concentrations of organochlorine pesticides, biomarkers of oxidative stress, and risk of breast cancer. *Environ. Pollut.* 286, 117386. <https://doi.org/10.1016/j.envpol.2021.117386>.
- Nagata, C., et al., 2013. Cadmium exposure and the risk of breast cancer in Japanese women. *Breast Cancer Res. Treat.* 138 (1), 235–239. <https://doi.org/10.1007/s10549-013-2414-4>.
- National Toxicology Program, 2019. Handbook for conducting a literature-based health assessment using OHAT Approach for systematic review and evidence integration. In: *Office of Health Assessment and Translation*. National Institute of Environmental Health Sciences.
- Nyqvist, F., et al., 2017. Increased cancer incidence in the local population around metal-contaminated glassworks sites. *J. Occup. Environ. Med.* 59 (5), e84–e90. <https://doi.org/10.1097/JOM.00000000000001003>.
- Olaya-Contreras, P., et al., 1998. Organochlorine exposure and breast cancer risk in Colombian women. *Cad. Saúde Pública* 14 (Suppl. 3), 125–132. <https://doi.org/10.1590/s0102-311x1998000700013>.
- Omoike, O.E., et al., 2021. A cross-sectional study of the association between perfluorinated chemical exposure and cancers related to deregulation of estrogen receptors. *Environ. Res.* 196, 110329. <https://doi.org/10.1016/j.envres.2020.110329>.
- Page, M.J., et al., 2021. PRISMA 2020 explanation and elaboration: updated guidance and exemplars for reporting systematic reviews. *BMJ* 372, n160. <https://doi.org/10.1136/bmj.n160>.
- Pantelaki, I., Voutsas, D., 2019. Organophosphate flame retardants (OPFRs): a review on analytical methods and occurrence in wastewater and aquatic environment. *Sci. Total Environ.* 649, 247–263. <https://doi.org/10.1016/j.scitotenv.2018.08.286>.
- Paoli, D., et al., 2015. Exposure to polychlorinated biphenyls and hexachlorobenzene, semen quality and testicular cancer risk. *J. Endocrinol. Invest.* 38 (7), 745–752. <https://doi.org/10.1007/s40618-015-0251-5>.
- Park, E., et al., 2021. Environmental exposure to cadmium and risk of thyroid cancer from national industrial complex areas: a population-based cohort study. *Chemosphere* 268, 128819. <https://doi.org/10.1016/j.chemosphere.2020.128819>.
- Parron, T., et al., 2014. Environmental exposure to pesticides and cancer risk in multiple human organ systems. *Toxicol. Lett.* 230 (2), 157–165. <https://doi.org/10.1016/j.toxlet.2013.11.009>.
- Peng, L., et al., 2015. Cadmium exposure and the risk of breast cancer in Chaoshan population of southeast China. *Environ. Sci. Pollut. Res.* 22 (24), 19870–19878. <https://doi.org/10.1007/s11356-015-5212-1>.
- Peremiquel-Trillas, P., et al., 2019. Alkylphenolic compounds and risk of breast and prostate cancer in the MCC-Spain study. *Environ. Int.* 122, 389–399. <https://doi.org/10.1016/j.envint.2018.12.007>.
- Pi, N., et al., 2016. Associations of serum organohalogen levels and prostate cancer risk: results from a case-control study in Singapore. *Chemosphere* 144, 1505–1512. <https://doi.org/10.1016/j.chemosphere.2015.10.020>.
- Pierozan, P., Cattani, D., Karlsson, O., 2020. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) induce epigenetic alterations and promote human breast cell carcinogenesis in vitro. *Arch. Toxicol.* 94 (11), 3893–3906. <https://doi.org/10.1007/s00204-020-02848-6>.

- Platz, E.A., et al., 2002. Prediagnostic toenail cadmium and zinc and subsequent prostate cancer risk. *Prostate* 52 (4), 288–296. <https://doi.org/10.1002/pros.10115>.
- Plotteau, S., et al., 2017. Associations between internal exposure levels of persistent organic pollutants in adipose tissue and deep infiltrating endometriosis with or without concurrent ovarian endometrioma. *Environ. Int.* 108, 195–203. <https://doi.org/10.1016/j.envint.2017.08.019>.
- Recio-Vega, R., et al., 2011. Serum levels of polychlorinated biphenyls in Mexican women and breast cancer risk. *J. Appl. Toxicol.* 31 (3), 270–278. <https://doi.org/10.1002/jat.1672>.
- Rocha, P.R.S., et al., 2021. Exposure to endocrine disruptors and risk of breast cancer: a systematic review. *Crit. Rev. Oncol. Hematol.* 161 <https://doi.org/10.1016/j.critrevonc.2021.103330>.
- Rodgers, K.M., et al., 2018. Environmental chemicals and breast cancer: an updated review of epidemiological literature informed by biological mechanisms. *Environ. Res.* 160, 152–182. <https://doi.org/10.1016/j.envres.2017.08.045>.
- Roman, M.D., et al., 2018. Do exposure to arsenic, occupation and diet have synergistic effects on prostate cancer risk? *Asian Pac. J. Cancer Prev. APJCP* 19 (6), 1495–1501. <https://doi.org/10.22034/APJCP.2018.19.6.1495>.
- Romanik capital A, C., et al., 2017. Heavy metals effect on breast cancer progression. *J. Occup. Med. Toxicol.* 12, 32. <https://doi.org/10.1186/s12995-017-0178-1>.
- Romieu, I., et al., 2000. Breast cancer, lactation history, and serum organochlorines. *Am. J. Epidemiol.* 152 (4), 363–370. <https://doi.org/10.1093/aje/152.4.363>.
- Salamanca-Fernandez, E., et al., 2021. Bisphenol-A exposure and risk of breast and prostate cancer in the Spanish European Prospective Investigation into Cancer and Nutrition study. *Environ. Health* 20 (1), 88. <https://doi.org/10.1186/s12940-021-00779-y>.
- Schettler, T., 2006. Human exposure to phthalates via consumer products. *Int. J. Androl.* 29 (1), 134–139. <https://doi.org/10.1111/j.1365-2605.2005.00567.x>; discussion 181–5.
- Schug, T.T., et al., 2016. Minireview: endocrine disruptors: past lessons and future directions. *Mol. Endocrinol.* 30 (8), 833–847. <https://doi.org/10.1210/me.2016-1096>.
- Silva, J.F., et al., 2016. Exposure to pesticides and prostate cancer: systematic review of the literature. *Rev. Environ. Health* 31 (3), 311–327. <https://doi.org/10.1515/revehl-2016-0001>.
- Stockholm Convention on Persistent Organic Pollutants (POPs), 2019. United Nations Environment Programme (UNEP) Chemicals, Geneva, Switzerland. Stockholm Convention Secretariat.
- Strumylaite, L., et al., 2019. Cadmium exposure and risk of breast cancer by histological and tumor receptor subtype in white caucasian women: a hospital-based case-control study. *Int. J. Mol. Sci.* 20 (12) <https://doi.org/10.3390/ijms20123029>.
- Sung, H., et al., 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca - Cancer J. Clin.* 71 (3), 209–249. <https://doi.org/10.3322/caac.21660>.
- Tang, M., et al., 2014. Assessing the underlying breast cancer risk of Chinese females contributed by dietary intake of residual DDT from agricultural soils. *Environ. Int.* 73, 208–215. <https://doi.org/10.1016/j.envint.2014.08.001>.
- Trabert, B., et al., 2015. Persistent organic pollutants (POPs) and fibroids: results from the ENDO study. *J. Expo. Sci. Environ. Epidemiol.* 25 (3), 278–285. <https://doi.org/10.1038/jes.2014.31>.
- Tse, L.A., et al., 2018. Environmental risk factors of prostate cancer: a case-control study. *Hong Kong Med. J.* 24 (4), 30–33. Suppl 4.
- van der Gulden, J.W., Kolk, J.J., Verbeek, A.L., 1995. Work environment and prostate cancer risk. *Prostate* 27 (5), 250–257. <https://doi.org/10.1002/pros.2990270504>.
- van Gerwen, M., et al., 2022. The role of heavy metals in thyroid cancer: a meta-analysis. *J. Trace Elem. Med. Biol.* 69, 126900 <https://doi.org/10.1016/j.jtemb.2021.126900>.
- Vella, V., et al., 2017. Recent views of heavy metals as possible risk factors and potential preventive and therapeutic agents in prostate cancer. *Mol. Cell. Endocrinol.* 457, 57–72. <https://doi.org/10.1016/j.mce.2016.10.020>.
- Vinceti, M., et al., 2007. Case-control study of toenail cadmium and prostate cancer risk in Italy. *Sci. Total Environ.* 373 (1), 77–81. <https://doi.org/10.1016/j.scitotenv.2006.11.005>.
- Wan, M.L.Y., Co, V.A., El-Nezami, H., 2021. Endocrine Disrupting Chemicals and Breast Cancer: a Systematic Review of Epidemiological Studies. *Crit Rev Food Sci Nutr*, pp. 1–27. <https://doi.org/10.1080/10408398.2021.1903382>.
- Weiderpass, E., et al., 2000. Organochlorines and endometrial cancer risk. *Cancer Epidemiol. Biomarkers Prev.* 9 (5), 487–493.
- Wen, H.J., et al., 2020. Exposure to endocrine disruptor alkylphenols and the occurrence of endometrial cancer. *Environ. Pollut.* 267, 115475 <https://doi.org/10.1016/j.envpol.2020.115475>.
- Wernli, K.J., et al., 2008a. Occupational risk factors for endometrial cancer among textile workers in Shanghai, China. *Am. J. Ind. Med.* 51 (9), 673–679. <https://doi.org/10.1002/ajim.20614>.
- Wernli, K.J., et al., 2008b. Occupational exposures and ovarian cancer in textile workers. *Epidemiology* 19 (2), 244–250. <https://doi.org/10.1097/EDE.0b013e31816339f9>.
- Westberg, H.B., et al., 2005. On the use of different measures of exposure-experiences from a case-control study on testicular cancer and PVC exposure. *J. Occup. Environ. Hyg.* 2 (7), 351–356. <https://doi.org/10.1080/15459620590969046>.
- White, A.J., et al., 2013. Exposure to fogger trucks and breast cancer incidence in the Long Island Breast Cancer Study Project: a case-control study. *Environ. Health* 12, 24. <https://doi.org/10.1186/1476-069X-12-24>.
- Woolcott, C.G., et al., 2001. Organochlorines and breast cancer risk by receptor status, tumor size, and grade (Canada). *Cancer Causes Control* 12 (5), 395–404. <https://doi.org/10.1023/a:1011289905751>.
- World Health, O., et al., 2013. State of the Science of Endocrine Disrupting Chemicals 2012: Summary for Decision-Makers. World Health Organization, Geneva.
- Yoon, K., et al., 2014. Estrogenic endocrine-disrupting chemicals: molecular mechanisms of actions on putative human diseases. *J. Toxicol. Environ. Health B Crit. Rev.* 17 (3), 127–174. <https://doi.org/10.1080/10937404.2014.882194>.
- Young, H.A., et al., 2005. Triazine herbicides and epithelial ovarian cancer risk in central California. *J. Occup. Environ. Med.* 47 (11), 1148–1156. <https://doi.org/10.1097/01.jom.0000177044.43959.e8>.
- Zhang, X., Ho, S.M., 2011. Epigenetics meets endocrinology. *J. Mol. Endocrinol.* 46 (1), R11–R32. <https://doi.org/10.1677/jme-10-0053>.
- Zhang, C., et al., 2019a. Association of exposure to multiple metals with papillary thyroid cancer risk in China. *Environ. Sci. Pollut. Res. Int.* 26 (20), 20560–20572. <https://doi.org/10.1007/s11356-019-04733-x>.
- Zhang, Z., et al., 2019b. The relationship between exposure to particulate matter and breast cancer incidence and mortality: a meta-analysis. *Medicine (Baltim.)* 98 (50), e18349. <https://doi.org/10.1097/MD.00000000000018349>.
- Zhou, Z., et al., 2017. Higher urinary bisphenol A concentration and excessive iodine intake are associated with nodular goiter and papillary thyroid carcinoma. *Biosci. Rep.* 37 (4) <https://doi.org/10.1042/BSR20170678>.
- Zoeller, R.T., et al., 2012. Endocrine-disrupting chemicals and public health protection: a statement of principles from the Endocrine Society. *Endocrinology* 153 (9), 4097–4110. <https://doi.org/10.1210/en.2012-1422>.

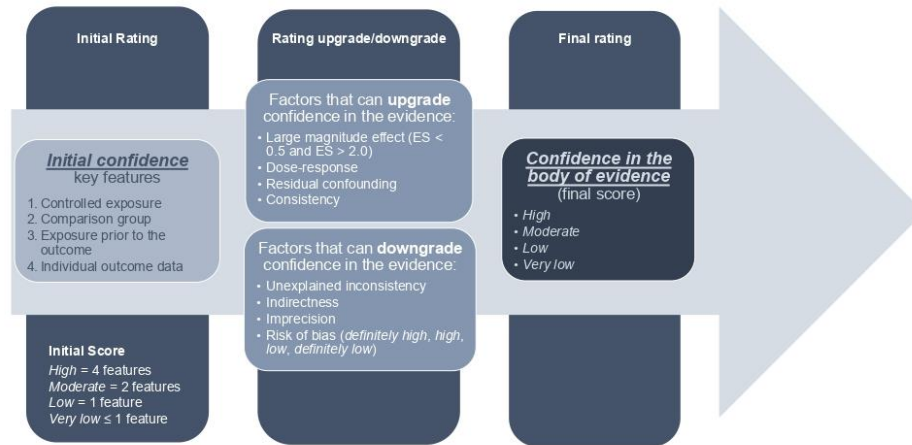
Supplementary Data

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

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

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

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.

MA s	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** diethylstilbestrol, **EDC** endocrine disrupting chemical, **MA** mechanism of action, **PBDEs** polybrominated diphenyl ethers, **PCBs** polychlorinated biphenyls. 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 <i>in vitro</i> 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 eds 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 eds 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 eds 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 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))

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 assessment	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
Salemo C., et al. [30]	2016	a	Italy	2002-2009	C.-Control	-	Moderate	D. High	Low
Schechter 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
Milikan 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
Ghisani 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	Guadeloupe	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 Zealand	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
Salemo 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
Shekarzifard 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
Salemo 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 (PBP), 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
Salemo 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
Salemo 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 (PBP), e organic solvents & phenols (OSP), 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.

References

1. National Toxicology Program 2019. *Handbook for Conducting a Literature-Based Health Assessment Using OHAT Approach for Systematic Review and Evidence Integration*. Office of Health Assessment and Translation. National Institute of Environmental Health Sciences.
2. La Merrill, M.A., et al., *Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification*. *Nat Rev Endocrinol*, 2020. **16**(1): p. 45-57. <http://doi.org/10.1038/s41574-019-0273-8>.
3. Sung, H., et al., *Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries*. *CA Cancer J Clin*, 2021. **71**(3): p. 209-249. <http://doi.org/10.3322/caac.21660>.
4. Aschengrau, A., et al., *Cancer risk and residential proximity to cranberry cultivation in Massachusetts*. *Am J Public Health*, 1996. **86**(9): p. 1289-96. <http://doi.org/10.2105/ajph.86.9.1289>.
5. Ashley-Martin, J., et al., *Breast cancer risk, fungicide exposure and CYP1A1*2A gene-environment interactions in a province-wide case control study in Prince Edward Island, Canada*. *Int J Environ Res Public Health*, 2012. **9**(5): p. 1846-58. <http://doi.org/10.3390/ijerph9051846>.
6. Freeman, L.E., et al., *Atrazine and cancer incidence among pesticide applicators in the agricultural health study (1994-2007)*. *Environ Health Perspect*, 2011. **119**(9): p. 1253-9. <http://doi.org/10.1289/ehp.1103561>.
7. Brody, J.G., et al., *Breast cancer risk and historical exposure to pesticides from wide-area applications assessed with GIS*. *Environ Health Perspect*, 2004. **112**(8): p. 889-97. <http://doi.org/10.1289/ehp.6845>.
8. Charlier, C., et al., *Environmental dichlorodiphenyltrichlorethane or hexachlorobenzene exposure and breast cancer: is there a risk?* *Clin Chem Lab Med*, 2004. **42**(2): p. 222-7. <http://doi.org/10.1515/CCLM.2004.040>.
9. Cohn, B.A., et al., *DDT and breast cancer in young women: new data on the significance of age at exposure*. *Environ Health Perspect*, 2007. **115**(10): p. 1406-14. <http://doi.org/10.1289/ehp.10260>.
10. Cohn, B.A., P.M. Cirillo, and M.B. Terry, *DDT and Breast Cancer: Prospective Study of Induction Time and Susceptibility Windows*. *J Natl Cancer Inst*, 2019. **111**(8): p. 803-810. <http://doi.org/10.1093/jnci/djy198>.
11. Duell, E.J., et al., *A population-based case-control study of farming and breast cancer in North Carolina*. *Epidemiology*, 2000. **11**(5): p. 523-31. <http://doi.org/10.1097/00001648-200009000-00007>.
12. El-Zaemey, S., J. Heyworth, and L. Fritschi, *Noticing pesticide spray drift from agricultural pesticide application areas and breast cancer: a case-control study*. *Aust N Z J Public Health*, 2013. **37**(6): p. 547-55. <http://doi.org/10.1111/1753-6405.12111>.
13. El-Zaemey, S., et al., *Household and occupational exposure to pesticides and risk of breast cancer*. *Int J Environ Health Res*, 2014. **24**(2): p. 91-102. <http://doi.org/10.1080/09603123.2013.800958>.
14. Eldakroory, S.A., et al., *Correlation between toxic organochlorine pesticides and breast cancer*. *Hum Exp Toxicol*, 2017. **36**(12): p. 1326-1334. <http://doi.org/10.1177/0960327116685887>.

15. Engel, L.S., et al., *Insecticide Use and Breast Cancer Risk among Farmers' Wives in the Agricultural Health Study*. Environ Health Perspect, 2017. **125**(9): p. 097002. <http://doi.org/10.1289/EHP1295>.
16. Espejo-Herrera, N., et al., *Ingested Nitrate and Breast Cancer in the Spanish Multicase-Control Study on Cancer (MCC-Spain)*. Environ Health Perspect, 2016. **124**(7): p. 1042-9. <http://doi.org/10.1289/ehp.1510334>.
17. Farooq, U., et al., *Self-reported exposure to pesticides in residential settings and risk of breast cancer: a case-control study*. Environ Health, 2010. **9**: p. 30. <http://doi.org/10.1186/1476-069X-9-30>.
18. Hopenhayn-Rich, C., M.L. Stump, and S.R. Browning, *Regional assessment of atrazine exposure and incidence of breast and ovarian cancers in Kentucky*. Arch Environ Contam Toxicol, 2002. **42**(1): p. 127-36. <http://doi.org/10.1007/s002440010300>.
19. Ibarluzea Jm, J., et al., *Breast cancer risk and the combined effect of environmental estrogens*. Cancer Causes Control, 2004. **15**(6): p. 591-600. <http://doi.org/10.1023/B:CACO.0000036167.51236.86>.
20. Khanjani, N., D.R. English, and M.R. Sim, *An ecological study of organochlorine pesticides and breast cancer in rural Victoria, Australia*. Arch Environ Contam Toxicol, 2006. **50**(3): p. 452-61. <http://doi.org/10.1007/s00244-004-7217-5>.
21. Lerro, C.C., et al., *Organophosphate insecticide use and cancer incidence among spouses of pesticide applicators in the Agricultural Health Study*. Occup Environ Med, 2015. **72**(10): p. 736-44. <http://doi.org/10.1136/oemed-2014-102798>.
22. Lopez-Carrillo, L., et al., *Dichlorodiphenyltrichloroethane serum levels and breast cancer risk: a case-control study from Mexico*. Cancer Res, 1997. **57**(17): p. 3728-32.
23. McElroy, J.A., et al., *Risk of breast cancer for women living in rural areas from adult exposure to atrazine from well water in Wisconsin*. J Expo Sci Environ Epidemiol, 2007. **17**(2): p. 207-14. <http://doi.org/10.1038/sj.jes.7500511>.
24. Mills, P.K., et al., *Agricultural Exposures and Breast Cancer Among Latina in the San Joaquin Valley of California*. J Occup Environ Med, 2019. **61**(7): p. 552-558. <http://doi.org/10.1097/JOM.0000000000001598>.
25. Niehoff, N.M., et al., *Childhood and Adolescent Pesticide Exposure and Breast Cancer Risk*. Epidemiology, 2016. **27**(3): p. 326-33. <http://doi.org/10.1097/EDE.0000000000000451>.
26. Olaya-Contreras, P., et al., *Organochlorine exposure and breast cancer risk in Colombian women*. Cad Saude Publica, 1998. **14 Suppl 3**: p. 125-32. <http://doi.org/10.1590/s0102-311x1998000700013>.
27. Parron, T., et al., *Environmental exposure to pesticides and cancer risk in multiple human organ systems*. Toxicol Lett, 2014. **230**(2): p. 157-65. <http://doi.org/10.1016/j.toxlet.2013.11.009>.
28. Reynolds, P., et al., *Residential proximity to agricultural pesticide use and incidence of breast cancer in the California Teachers Study cohort*. Environ Res, 2004. **96**(2): p. 206-18. <http://doi.org/10.1016/j.envres.2004.03.001>.
29. Romieu, I., et al., *Breast cancer, lactation history, and serum organochlorines*. Am J Epidemiol, 2000. **152**(4): p. 363-70. <http://doi.org/10.1093/aje/152.4.363>.
30. Salerno, C., et al., *An Italian population-based case-control study on the association between farming and cancer: Are pesticides a plausible risk factor?* Arch Environ Occup Health, 2016. **71**(3): p. 147-56. <http://doi.org/10.1080/19338244.2015.1027808>.

31. Schecter, A., et al., *Blood levels of DDT and breast cancer risk among women living in the north of Vietnam*. Arch Environ Contam Toxicol, 1997. **33**(4): p. 453-6. <http://doi.org/10.1007/s002449900276>.
32. Tang, M., et al., *Assessing the underlying breast cancer risk of Chinese females contributed by dietary intake of residual DDT from agricultural soils*. Environ Int, 2014. **73**: p. 208-15. <http://doi.org/10.1016/j.envint.2014.08.001>.
33. Teitelbaum, S.L., et al., *Reported residential pesticide use and breast cancer risk on Long Island, New York*. Am J Epidemiol, 2007. **165**(6): p. 643-51. <http://doi.org/10.1093/aje/kwk046>.
34. van't Veer, P., et al., *DDT (dicophane) and postmenopausal breast cancer in Europe: case-control study*. BMJ, 1997. **315**(7100): p. 81-5. <http://doi.org/10.1136/bmj.315.7100.81>.
35. White, A.J., et al., *Exposure to fogger trucks and breast cancer incidence in the Long Island Breast Cancer Study Project: a case-control study*. Environ Health, 2013. **12**: p. 24. <http://doi.org/10.1186/1476-069X-12-24>.
36. Xu, X., et al., *Associations of serum concentrations of organochlorine pesticides with breast cancer and prostate cancer in U.S. adults*. Environ Health Perspect, 2010. **118**(1): p. 60-6. <http://doi.org/10.1289/ehp.0900919>.
37. Zota, A.R., et al., *Self-reported chemicals exposure, beliefs about disease causation, and risk of breast cancer in the Cape Cod Breast Cancer and Environment Study: a case-control study*. Environ Health, 2010. **9**: p. 40. <http://doi.org/10.1186/1476-069X-9-40>.
38. Aronson, K.J., et al., *Breast adipose tissue concentrations of polychlorinated biphenyls and other organochlorines and breast cancer risk*. Cancer Epidemiol Biomarkers Prev, 2000. **9**(1): p. 55-63.
39. Arrebola, J.P., et al., *Risk of female breast cancer and serum concentrations of organochlorine pesticides and polychlorinated biphenyls: a case-control study in Tunisia*. Sci Total Environ, 2015. **520**: p. 106-13. <http://doi.org/10.1016/j.scitotenv.2015.03.045>.
40. Demers, A., et al., *Risk and aggressiveness of breast cancer in relation to plasma organochlorine concentrations*. Cancer Epidemiol Biomarkers Prev, 2000. **9**(2): p. 161-6.
41. Gammon, M.D., et al., *Environmental toxins and breast cancer on Long Island. II. Organochlorine compound levels in blood*. Cancer Epidemiol Biomarkers Prev, 2002. **11**(8): p. 686-97.
42. Gatto, N.M., et al., *Serum organochlorines and breast cancer: a case-control study among African-American women*. Cancer Causes Control, 2007. **18**(1): p. 29-39. <http://doi.org/10.1007/s10552-006-0070-2>.
43. Hoyer, A.P., et al., *Repeated measurements of organochlorine exposure and breast cancer risk (Denmark)*. Cancer Causes Control, 2000. **11**(2): p. 177-84. <http://doi.org/10.1023/a:1008926219539>.
44. Hoyer, A.P., et al., *Organochlorine exposures influence on breast cancer risk and survival according to estrogen receptor status: a Danish cohort-nested case-control study*. BMC Cancer, 2001. **1**: p. 8. <http://doi.org/10.1186/1471-2407-1-8>.
45. Krieger, N., et al., *Breast cancer and serum organochlorines: a prospective study among white, black, and Asian women*. J Natl Cancer Inst, 1994. **86**(8): p. 589-99. <http://doi.org/10.1093/jnci/86.8.589>.

46. Laden, F., et al., *Plasma organochlorine levels and the risk of breast cancer: an extended follow-up in the Nurses' Health Study*. Int J Cancer, 2001. **91**(4): p. 568-74. [http://doi.org/10.1002/1097-0215\(200002\)9999:9999<::aid-ijc1081>3.0.co;2-w](http://doi.org/10.1002/1097-0215(200002)9999:9999<::aid-ijc1081>3.0.co;2-w).
47. Lopez-Carrillo, L., et al., *Serum levels of beta-hexachlorocyclohexane, hexachlorobenzene and polychlorinated biphenyls and breast cancer in Mexican women*. Eur J Cancer Prev, 2002. **11**(2): p. 129-35. <http://doi.org/10.1097/00008469-200204000-00004>.
48. McCready, D., et al., *Breast tissue organochlorine levels and metabolic genotypes in relation to breast cancer risk Canada*. Cancer Causes Control, 2004. **15**(4): p. 399-418. <http://doi.org/10.1023/B:CACO.0000027505.32564.c2>.
49. Millikan, R., et al., *Dichlorodiphenyldichloroethene, polychlorinated biphenyls, and breast cancer among African-American and white women in North Carolina*. Cancer Epidemiology Biomarkers & Prevention, 2000. **9**(11): p. 1233-1240.
50. Moysich, K.B., et al., *Environmental organochlorine exposure and postmenopausal breast cancer risk*. Cancer Epidemiol Biomarkers Prev, 1998. **7**(3): p. 181-8.
51. Moysich, K.B., et al., *Polychlorinated biphenyls, cytochrome P4501A1 polymorphism, and postmenopausal breast cancer risk*. Cancer Epidemiol Biomarkers Prev, 1999. **8**(1): p. 41-4.
52. Pavuk, M., et al., *Case-control study of PCBs, other organochlorines and breast cancer in Eastern Slovakia*. J Expo Anal Environ Epidemiol, 2003. **13**(4): p. 267-75. <http://doi.org/10.1038/sj.jea.7500277>.
53. O, R.-N., et al., *Adipose organochlorine concentrations and risk of breast cancer among postmenopausal Danish women*. Cancer Epidemiol Biomarkers Prev, 2005. **14**(1): p. 67-74.
54. Stellman, S.D., et al., *Breast cancer risk in relation to adipose concentrations of organochlorine pesticides and polychlorinated biphenyls in Long Island, New York*. Cancer Epidemiol Biomarkers Prev, 2000. **9**(11): p. 1241-9.
55. Ward, E.M., et al., *Serum organochlorine levels and breast cancer: a nested case-control study of Norwegian women*. Cancer Epidemiol Biomarkers Prev, 2000. **9**(12): p. 1357-67.
56. Wolff, M.S., et al., *Risk of breast cancer and organochlorine exposure*. Cancer Epidemiol Biomarkers Prev, 2000. **9**(3): p. 271-7.
57. Woolcott, C.G., et al., *Organochlorines and breast cancer risk by receptor status, tumor size, and grade (Canada)*. Cancer Causes Control, 2001. **12**(5): p. 395-404. <http://doi.org/10.1023/a:1011289905751>.
58. Zheng, T., et al., *Breast cancer risk associated with congeners of polychlorinated biphenyls*. Am J Epidemiol, 2000. **152**(1): p. 50-8. <http://doi.org/10.1093/aje/152.1.50>.
59. Holmes, A.K., et al., *Case-control study of breast cancer and exposure to synthetic environmental chemicals among Alaska Native women*. International Journal of Circumpolar Health, 2014. **73**(1). <http://doi.org/10.3402/ijch.v73.25760>.
60. Acheampong, T., et al., *Occupational exposure to endocrine disrupting substances and the risk of breast Cancer: the Singapore Chinese health study*. BMC Public Health, 2018. **18**(1): p. 929. <http://doi.org/10.1186/s12889-018-5862-2>.
61. Ekenga, C.C., C.G. Parks, and D.P. Sandler, *Chemical exposures in the workplace and breast cancer risk: A prospective cohort study*. Int J Cancer, 2015. **137**(7): p. 1765-74. <http://doi.org/10.1002/ijc.29545>.

62. Engel, L.S., et al., *Pesticide use and breast cancer risk among farmers' wives in the agricultural health study*. Am J Epidemiol, 2005. **161**(2): p. 121-35. <http://doi.org/10.1093/aje/kwi022>.
63. Mills, P.K. and R. Yang, *Breast cancer risk in Hispanic agricultural workers in California*. Int J Occup Environ Health, 2005. **11**(2): p. 123-31. <http://doi.org/10.1179/oeh.2005.11.2.123>.
64. O'Leary, E.S., et al., *Pesticide exposure and risk of breast cancer: a nested case-control study of residentially stable women living on Long Island*. Environ Res, 2004. **94**(2): p. 134-44. <http://doi.org/10.1016/j.envres.2003.08.001>.
65. Bencko, V., et al., *Human exposure to polyhalogenated hydrocarbons and incidence of selected malignancies -central European experience*. Neoplasma, 2009. **56**(4): p. 353-7. http://doi.org/10.4149/neo_2009_04_353.
66. Charlier, C.J., et al., *Polychlorinated biphenyls contamination in women with breast cancer*. Clin Chim Acta, 2004. **347**(1-2): p. 177-81. <http://doi.org/10.1016/j.cccn.2004.04.025>.
67. Cohn, B.A., et al., *Exposure to polychlorinated biphenyl (PCB) congeners measured shortly after giving birth and subsequent risk of maternal breast cancer before age 50*. Breast Cancer Res Treat, 2012. **136**(1): p. 267-75. <http://doi.org/10.1007/s10549-012-2257-4>.
68. Donat-Vargas, C., et al., *Dietary exposure to polychlorinated biphenyls and risk of breast, endometrial and ovarian cancer in a prospective cohort*. Br J Cancer, 2016. **115**(9): p. 1113-1121. <http://doi.org/10.1038/bjc.2016.282>.
69. Holford, T.R., et al., *Joint effects of nine polychlorinated biphenyl (PCB) congeners on breast cancer risk*. Int J Epidemiol, 2000. **29**(6): p. 975-82. <http://doi.org/10.1093/ije/29.6.975>.
70. Laden, F., et al., *Polychlorinated biphenyls, cytochrome P450 1A1, and breast cancer risk in the Nurses' Health Study*. Cancer Epidemiol Biomarkers Prev, 2002. **11**(12): p. 1560-5.
71. Li, Y., et al., *Polychlorinated biphenyls, cytochrome P450 1A1 (CYP1A1) polymorphisms, and breast cancer risk among African American women and white women in North Carolina: a population-based case-control study*. Breast Cancer Res, 2005. **7**(1): p. R12-8. <http://doi.org/10.1186/bcr941>.
72. Recio-Vega, R., et al., *Serum levels of polychlorinated biphenyls in Mexican women and breast cancer risk*. J Appl Toxicol, 2011. **31**(3): p. 270-8. <http://doi.org/10.1002/jat.1672>.
73. Rusiecki, J.A., et al., *Polychlorinated biphenyls and breast cancer risk by combined estrogen and progesterone receptor status*. Eur J Epidemiol, 2004. **19**(8): p. 793-801. <http://doi.org/10.1023/b:ejep.0000036580.05471.31>.
74. Zhang, Y., et al., *Serum polychlorinated biphenyls, cytochrome P-450 1A1 polymorphisms, and risk of breast cancer in Connecticut women*. Am J Epidemiol, 2004. **160**(12): p. 1177-83. <http://doi.org/10.1093/aje/kwh346>.
75. Morgan, M., et al., *Environmental estrogen-like endocrine disrupting chemicals and breast cancer*. Mol Cell Endocrinol, 2017. **457**: p. 89-102. <http://doi.org/10.1016/j.mce.2016.10.003>.
76. Amadou, A., et al., *Chronic long-term exposure to cadmium air pollution and breast cancer risk in the French E3N cohort*. Int J Cancer, 2020. **146**(2): p. 341-351. <http://doi.org/10.1002/ijc.32257>.

77. Chen, L.J., et al., *Urinary strontium and the risk of breast cancer: a case-control study in Guangzhou, China*. Environ Res, 2012. **112**: p. 212-7. <http://doi.org/10.1016/j.envres.2011.11.005>.
78. Gallagher, C.M., J.J. Chen, and J.S. Kovach, *Environmental cadmium and breast cancer risk*. Aging (Albany NY), 2010. **2**(11): p. 804-14. <http://doi.org/10.18632/aging.100226>.
79. Garland, M., et al., *Toenail trace element levels and breast cancer: a prospective study*. Am J Epidemiol, 1996. **144**(7): p. 653-60. <http://doi.org/10.1093/oxfordjournals.aje.a008977>.
80. Gaudet, M.M., et al., *Blood levels of cadmium and lead in relation to breast cancer risk in three prospective cohorts*. Int J Cancer, 2019. **144**(5): p. 1010-1016. <http://doi.org/10.1002/ijc.31805>.
81. Kresovich, J.K., et al., *Metallic air pollutants and breast cancer heterogeneity*. Environ Res, 2019. **177**: p. 108639. <http://doi.org/10.1016/j.envres.2019.108639>.
82. Lopez-Carrillo, L., et al., *Arsenic methylation capacity is associated with breast cancer in northern Mexico*. Toxicol Appl Pharmacol, 2014. **280**(1): p. 53-9. <http://doi.org/10.1016/j.taap.2014.07.013>.
83. Lopez-Carrillo, L., et al., *Inorganic arsenic methylation capacity and breast cancer by immunohistochemical subtypes in northern Mexican women*. Environ Res, 2020. **184**: p. 109361. <http://doi.org/10.1016/j.envres.2020.109361>.
84. Marciniak, W., et al., *Blood arsenic levels and the risk of familial breast cancer in Poland*. Int J Cancer, 2020. **146**(10): p. 2721-2727. <http://doi.org/10.1002/ijc.32595>.
85. Nagata, C., et al., *Cadmium exposure and the risk of breast cancer in Japanese women*. Breast Cancer Res Treat, 2013. **138**(1): p. 235-9. <http://doi.org/10.1007/s10549-013-2414-4>.
86. Peng, L., et al., *Cadmium exposure and the risk of breast cancer in Chaoshan population of southeast China*. Environ Sci Pollut Res Int, 2015. **22**(24): p. 19870-8. <http://doi.org/10.1007/s11356-015-5212-1>.
87. Thompson, D., et al., *Occupational exposure to metalworking fluids and risk of breast cancer among female autoworkers*. Am J Ind Med, 2005. **47**(2): p. 153-60. <http://doi.org/10.1002/ajim.20132>.
88. Wei, Y. and J. Zhu, *Blood levels of endocrine-disrupting metals and prevalent breast cancer among US women*. Med Oncol, 2019. **37**(1): p. 1. <http://doi.org/10.1007/s12032-019-1328-3>.
89. White, A.J., et al., *Metallic Air Pollutants and Breast Cancer Risk in a Nationwide Cohort Study*. Epidemiology, 2019. **30**(1): p. 20-28. <http://doi.org/10.1097/EDE.0000000000000917>.
90. Hart, J.E., et al., *Exposure to hazardous air pollutants and risk of incident breast cancer in the nurses' health study II*. Environ Health, 2018. **17**(1): p. 28. <http://doi.org/10.1186/s12940-018-0372-3>.
91. Strumylaite, L., et al., *Cadmium Exposure and Risk of Breast Cancer by Histological and Tumor Receptor Subtype in White Caucasian Women: A Hospital-Based Case-Control Study*. Int J Mol Sci, 2019. **20**(12). <http://doi.org/10.3390/ijms20123029>.
92. Bonner, M.R., et al., *Breast cancer risk and exposure in early life to polycyclic aromatic hydrocarbons using total suspended particulates as a proxy measure*. Cancer Epidemiology Biomarkers & Prevention, 2005. **14**(1): p. 53-60.

93. DeBono, N.L., et al., *Cancer surveillance among workers in plastics and rubber manufacturing in Ontario, Canada*. *Occup Environ Med*, 2020. **77**(12): p. 847-856. <http://doi.org/10.1136/oemed-2020-106581>.
94. Hansen, J., *Elevated risk for male breast cancer after occupational exposure to gasoline and vehicular combustion products*. *American Journal of Industrial Medicine*, 2000. **37**(4): p. 349-352. [http://doi.org/10.1002/\(sici\)1097-0274\(200004\)37:4<349::Aid-ajim4>3.0.Co;2-l](http://doi.org/10.1002/(sici)1097-0274(200004)37:4<349::Aid-ajim4>3.0.Co;2-l).
95. Lee, D.G., et al., *Women's occupational exposure to polycyclic aromatic hydrocarbons and risk of breast cancer*. *Occup Environ Med*, 2019. **76**(1): p. 22-29. <http://doi.org/10.1136/oemed-2018-105261>.
96. Mordukhovich, I., et al., *Polymorphisms in DNA repair genes, traffic-related polycyclic aromatic hydrocarbon exposure and breast cancer incidence*. *Int J Cancer*, 2016. **139**(2): p. 310-21. <http://doi.org/10.1002/ijc.30079>.
97. Mordukhovich, I., et al., *Vehicular Traffic-Related Polycyclic Aromatic Hydrocarbon Exposure and Breast Cancer Incidence: The Long Island Breast Cancer Study Project (LIBCSP)*. *Environ Health Perspect*, 2016. **124**(1): p. 30-8. <http://doi.org/10.1289/ehp.1307736>.
98. Pedersen, J.E., et al., *Occupational exposure to specific organic solvents and risk of subtypes of breast cancer in a large population of Danish women, 1964-2016*. *Occup Environ Med*, 2020. <http://doi.org/10.1136/oemed-2020-106865>.
99. Rai, R., et al., *Occupational exposures to engine exhausts and other PAHs and breast cancer risk: A population-based case-control study*. *Am J Ind Med*, 2016. **59**(6): p. 437-44. <http://doi.org/10.1002/ajim.22592>.
100. Laouali, N., et al., *Occupational exposure to organic solvents and risk of male breast cancer: a European multicenter case-control study*. *Scand J Work Environ Health*, 2018. **44**(3): p. 310-322. <http://doi.org/10.5271/sjweh.3717>.
101. Petralia, S.A., et al., *Risk of premenopausal breast cancer in association with occupational exposure to polycyclic aromatic hydrocarbons and benzene*. *Scand J Work Environ Health*, 1999. **25**(3): p. 215-21. <http://doi.org/10.5271/sjweh.426>.
102. Ekenga, C.C., et al., *Breast cancer risk after occupational solvent exposure: the influence of timing and setting*. *Cancer Res*, 2014. **74**(11): p. 3076-83. <http://doi.org/10.1158/0008-5472.CAN-13-2430>.
103. Gallagher, L.G., et al., *Risk of breast cancer following exposure to tetrachloroethylene-contaminated drinking water in Cape Cod, Massachusetts: reanalysis of a case-control study using a modified exposure assessment*. *Environ Health*, 2011. **10**: p. 47. <http://doi.org/10.1186/1476-069X-10-47>.
104. Glass, D.C., et al., *Occupational exposure to solvents and risk of breast cancer*. *Am J Ind Med*, 2015. **58**(9): p. 915-22. <http://doi.org/10.1002/ajim.22478>.
105. Parada, H., Jr., et al., *Urinary concentrations of environmental phenols and their associations with breast cancer incidence and mortality following breast cancer*. *Environ Int*, 2019. **130**: p. 104890. <http://doi.org/10.1016/j.envint.2019.05.084>.
106. Pedersen, J.E., et al., *Breast cancer among Danish women occupationally exposed to diesel exhaust and polycyclic aromatic hydrocarbons, 1964-2016*. *Scand J Work Environ Health*, 2021. **47**(2): p. 154-162. <http://doi.org/10.5271/sjweh.3923>.
107. Peplonska, B., et al., *Occupational exposure to organic solvents and breast cancer in women*. *Occup Environ Med*, 2010. **67**(11): p. 722-9. <http://doi.org/10.1136/oem.2009.046557>.

108. Ruckart, P.Z., et al., *Evaluation of contaminated drinking water and male breast cancer at Marine Corps Base Camp Lejeune, North Carolina: a case control study*. Environ Health, 2015. **14**: p. 74. <http://doi.org/10.1186/s12940-015-0061-4>.
109. Ghisari, M., et al., *Polymorphism in xenobiotic and estrogen metabolizing genes, exposure to perfluorinated compounds and subsequent breast cancer risk: A nested case-control study in the Danish National Birth Cohort*. Environ Res, 2017. **154**: p. 325-333. <http://doi.org/10.1016/j.envres.2017.01.020>.
110. Hurley, S., et al., *Breast cancer risk and serum levels of per- and poly-fluoroalkyl substances: a case-control study nested in the California Teachers Study*. Environ Health, 2018. **17**(1): p. 83. <http://doi.org/10.1186/s12940-018-0426-6>.
111. Mancini, F.R., et al., *Perfluorinated alkylated substances serum concentration and breast cancer risk: Evidence from a nested case-control study in the French E3N cohort*. Int J Cancer, 2020. **146**(4): p. 917-928. <http://doi.org/10.1002/ijc.32357>.
112. Omoike, O.E., et al., *A cross-sectional study of the association between perfluorinated chemical exposure and cancers related to deregulation of estrogen receptors*. Environ Res, 2021. **196**: p. 110329. <http://doi.org/10.1016/j.envres.2020.110329>.
113. Tsai, M.S., et al., *A case-control study of perfluoroalkyl substances and the risk of breast cancer in Taiwanese women*. Environ Int, 2020. **142**: p. 105850. <http://doi.org/10.1016/j.envint.2020.105850>.
114. Vieira, V.M., et al., *Perfluorooctanoic acid exposure and cancer outcomes in a contaminated community: a geographic analysis*. Environ Health Perspect, 2013. **121**(3): p. 318-23. <http://doi.org/10.1289/ehp.1205829>.
115. Lopez-Carrillo, L., et al., *Exposure to phthalates and breast cancer risk in northern Mexico*. Environ Health Perspect, 2010. **118**(4): p. 539-44. <http://doi.org/10.1289/ehp.0901091>.
116. Merida-Ortega, A., et al., *Phthalate exposure, flavonoid consumption and breast cancer risk among Mexican women*. Environ Int, 2016. **96**: p. 167-172. <http://doi.org/10.1016/j.envint.2016.08.023>.
117. Parada, H., Jr., et al., *Urinary Phthalate Metabolite Concentrations and Breast Cancer Incidence and Survival following Breast Cancer: The Long Island Breast Cancer Study Project*. Environ Health Perspect, 2018. **126**(4): p. 047013. <http://doi.org/10.1289/EHP2083>.
118. Aschengrau, A., et al., *Occupational exposure to estrogenic chemicals and the occurrence of breast cancer: an exploratory analysis*. Am J Ind Med, 1998. **34**(1): p. 6-14. [http://doi.org/10.1002/\(sici\)1097-0274\(199807\)34:1<6::aid-ajim2>3.0.co;2-x](http://doi.org/10.1002/(sici)1097-0274(199807)34:1<6::aid-ajim2>3.0.co;2-x).
119. Amaral, A., et al., *Chronic exposure to volcanic environments and cancer incidence in the Azores, Portugal*. Sci Total Environ, 2006. **367**(1): p. 123-8. <http://doi.org/10.1016/j.scitotenv.2006.01.024>.
120. Bai, L., et al., *Exposure to ambient air pollution and the incidence of lung cancer and breast cancer in the Ontario Population Health and Environment Cohort*. Int J Cancer, 2020. **146**(9): p. 2450-2459. <http://doi.org/10.1002/ijc.32575>.
121. Crouse, D.L., et al., *Postmenopausal breast cancer is associated with exposure to traffic-related air pollution in Montreal, Canada: a case-control study*. Environ Health Perspect, 2010. **118**(11): p. 1578-83. <http://doi.org/10.1289/ehp.1002221>.
122. Goldberg, M.S., et al., *The association between the incidence of postmenopausal breast cancer and concentrations at street-level of nitrogen dioxide and ultrafine*

- particles. Environ Res, 2017. **158**: p. 7-15. <http://doi.org/10.1016/j.envres.2017.05.038>.
123. Hart, J.E., et al., *Long-term Particulate Matter Exposures during Adulthood and Risk of Breast Cancer Incidence in the Nurses' Health Study II Prospective Cohort*. Cancer Epidemiol Biomarkers Prev, 2016. **25**(8): p. 1274-6. <http://doi.org/10.1158/1055-9965.EPI-16-0246>.
 124. Hystad, P., et al., *Exposure to traffic-related air pollution and the risk of developing breast cancer among women in eight Canadian provinces: a case-control study*. Environ Int, 2015. **74**: p. 240-8. <http://doi.org/10.1016/j.envint.2014.09.004>.
 125. Shekarzifard, M., et al., *Investigating the role of transportation models in epidemiologic studies of traffic related air pollution and health effects*. Environ Res, 2015. **140**: p. 282-91. <http://doi.org/10.1016/j.envres.2015.04.002>.
 126. White, A.J., et al., *Air Pollution, Clustering of Particulate Matter Components, and Breast Cancer in the Sister Study: A U.S.-Wide Cohort*. Environ Health Perspect, 2019. **127**(10): p. 107002. <http://doi.org/10.1289/EHP5131>.
 127. Hurley, S., et al., *Adipose levels of polybrominated diphenyl ethers and risk of breast cancer*. Breast Cancer Res Treat, 2011. **129**(2): p. 505-11. <http://doi.org/10.1007/s10549-011-1481-7>.
 128. Hurley, S., et al., *A breast cancer case-control study of polybrominated diphenyl ether (PBDE) serum levels among California women*. Environ Int, 2019. **127**: p. 412-419. <http://doi.org/10.1016/j.envint.2019.03.043>.
 129. Mancini, F.R., et al., *Plasma concentration of brominated flame retardants and postmenopausal breast cancer risk: a nested case-control study in the French E3N cohort*. Environ Health, 2020. **19**(1): p. 54. <http://doi.org/10.1186/s12940-020-00607-9>.
 130. Danjou, A.M., et al., *Estimated dietary dioxin exposure and breast cancer risk among women from the French E3N prospective cohort*. Breast Cancer Res, 2015. **17**: p. 39. <http://doi.org/10.1186/s13058-015-0536-9>.
 131. Danjou, A.M.N., et al., *Long-term airborne dioxin exposure and breast cancer risk in a case-control study nested within the French E3N prospective cohort*. Environ Int, 2019. **124**: p. 236-248. <http://doi.org/10.1016/j.envint.2019.01.001>.
 132. Hardell, L., et al., *Increased concentrations of octachlorodibenzo-p-dioxin in cases with breast cancer--results from a case-control study*. Eur J Cancer Prev, 1996. **5**(5): p. 351-7. <http://doi.org/10.1097/00008469-199610000-00007>.
 133. Viel, J.F., et al., *Dioxin emissions from a municipal solid waste incinerator and risk of invasive breast cancer: a population-based case-control study with GIS-derived exposure*. Int J Health Geogr, 2008. **7**(1): p. 4. <http://doi.org/10.1186/1476-072X-7-4>.
 134. VoPham, T., et al., *Dioxin exposure and breast cancer risk in a prospective cohort study*. Environ Res, 2020. **186**: p. 109516. <http://doi.org/10.1016/j.envres.2020.109516>.
 135. Peremiquel-Trillas, P., et al., *Alkylphenolic compounds and risk of breast and prostate cancer in the MCC-Spain study*. Environ Int, 2019. **122**: p. 389-399. <http://doi.org/10.1016/j.envint.2018.12.007>.
 136. Barry, K.H., et al., *Genetic variation in base excision repair pathway genes, pesticide exposure, and prostate cancer risk*. Environ Health Perspect, 2011. **119**(12): p. 1726-32. <http://doi.org/10.1289/ehp.1103454>.

137. Barry, K.H., et al., *Methyl bromide exposure and cancer risk in the Agricultural Health Study*. *Cancer Causes Control*, 2012. **23**(6): p. 807-18. <http://doi.org/10.1007/s10552-012-9949-2>.
138. Bonner, M.R., et al., *Occupational exposure to terbufos and the incidence of cancer in the Agricultural Health Study*. *Cancer Causes Control*, 2010. **21**(6): p. 871-7. <http://doi.org/10.1007/s10552-010-9514-9>.
139. Christensen, C.H., et al., *Coumaphos exposure and incident cancer among male participants in the Agricultural Health Study (AHS)*. *Environ Health Perspect*, 2010. **118**(1): p. 92-6. <http://doi.org/10.1289/ehp.0800446>.
140. Cockburn, M., et al., *Prostate cancer and ambient pesticide exposure in agriculturally intensive areas in California*. *Am J Epidemiol*, 2011. **173**(11): p. 1280-8. <http://doi.org/10.1093/aje/kwr003>.
141. Dieye, M., et al., *Geographic variations and temporal trends in prostate cancer in Martinique over a 25-year period*. *BMC Res Notes*, 2014. **7**: p. 262. <http://doi.org/10.1186/1756-0500-7-262>.
142. Giri, V.N., et al., *Association between Agent Orange and prostate cancer: a pilot case-control study*. *Urology*, 2004. **63**(4): p. 757-60; discussion 760-1. <http://doi.org/10.1016/j.urology.2003.11.044>.
143. Greenburg, D.L., et al., *Cancer incidence among pesticide applicators exposed to captan in the Agricultural Health Study*. *Cancer Causes Control*, 2008. **19**(10): p. 1401-7. <http://doi.org/10.1007/s10552-008-9187-9>.
144. Hessel, P.A., et al., *A nested case-control study of prostate cancer and atrazine exposure*. *J Occup Environ Med*, 2004. **46**(4): p. 379-85. <http://doi.org/10.1097/01.jom.0000121128.73921.a1>.
145. Hou, L., et al., *Pendimethalin exposure and cancer incidence among pesticide applicators*. *Epidemiology*, 2006. **17**(3): p. 302-7. <http://doi.org/10.1097/01.ede.0000201398.82658.50>.
146. Koutros, S., et al., *Pesticide use modifies the association between genetic variants on chromosome 8q24 and prostate cancer*. *Cancer Res*, 2010. **70**(22): p. 9224-33. <http://doi.org/10.1158/0008-5472.CAN-10-1078>.
147. Koutros, S., et al., *Genetic susceptibility loci, pesticide exposure and prostate cancer risk*. *PLoS One*, 2013. **8**(4): p. e58195. <http://doi.org/10.1371/journal.pone.0058195>.
148. Koutros, S., et al., *Risk of total and aggressive prostate cancer and pesticide use in the Agricultural Health Study*. *Am J Epidemiol*, 2013. **177**(1): p. 59-74. <http://doi.org/10.1093/aje/kws225>.
149. Lerro, C.C., et al., *Use of acetochlor and cancer incidence in the Agricultural Health Study*. *Int J Cancer*, 2015. **137**(5): p. 1167-75. <http://doi.org/10.1002/ijc.29416>.
150. Lynch, S.M., et al., *Cancer incidence among pesticide applicators exposed to cyanazine in the agricultural health study*. *Environ Health Perspect*, 2006. **114**(8): p. 1248-52. <http://doi.org/10.1289/ehp.8997>.
151. Lynch, S.M., et al., *Cancer incidence among pesticide applicators exposed to butylate in the Agricultural Health Study (AHS)*. *Environ Res*, 2009. **109**(7): p. 860-8. <http://doi.org/10.1016/j.envres.2009.06.006>.
152. Mahajan, R., et al., *Fonofos exposure and cancer incidence in the agricultural health study*. *Environ Health Perspect*, 2006. **114**(12): p. 1838-42. <http://doi.org/10.1289/ehp.9301>.

153. Mahajan, R., et al., *Phorate exposure and incidence of cancer in the agricultural health study*. Environ Health Perspect, 2006. **114**(8): p. 1205-9. <http://doi.org/10.1289/ehp.8911>.
154. Mahajan, R., et al., *Carbaryl exposure and incident cancer in the Agricultural Health Study*. Int J Cancer, 2007. **121**(8): p. 1799-805. <http://doi.org/10.1002/ijc.22836>.
155. Meyer, T.E., et al., *A case-control study of farming and prostate cancer in African-American and Caucasian men*. Occup Environ Med, 2007. **64**(3): p. 155-60. <http://doi.org/10.1136/oem.2006.027383>.
156. Mills, P.K. and R. Yang, *Prostate cancer risk in California farm workers*. J Occup Environ Med, 2003. **45**(3): p. 249-58. <http://doi.org/10.1097/01.jom.0000058339.05741.0c>.
157. Mozzachio, A.M., et al., *Chlorothalonil exposure and cancer incidence among pesticide applicator participants in the agricultural health study*. Environ Res, 2008. **108**(3): p. 400-3. <http://doi.org/10.1016/j.envres.2008.07.018>.
158. Multigner, L., et al., *Chlordecone exposure and risk of prostate cancer*. J Clin Oncol, 2010. **28**(21): p. 3457-62. <http://doi.org/10.1200/JCO.2009.27.2153>.
159. Pearce, N.E., R.A. Sheppard, and J. Fraser, *Case-control study of occupation and cancer of the prostate in New Zealand*. J Epidemiol Community Health, 1987. **41**(2): p. 130-2. <http://doi.org/10.1136/jech.41.2.130>.
160. Rusiecki, J.A., et al., *Cancer incidence among pesticide applicators exposed to metolachlor in the Agricultural Health Study*. Int J Cancer, 2006. **118**(12): p. 3118-23. <http://doi.org/10.1002/ijc.21758>.
161. Rusiecki, J.A., et al., *Cancer incidence among pesticide applicators exposed to permethrin in the Agricultural Health Study*. Environ Health Perspect, 2009. **117**(4): p. 581-6. <http://doi.org/10.1289/ehp.11318>.
162. Sawada, N., et al., *Plasma organochlorines and subsequent risk of prostate cancer in Japanese men: a nested case-control study*. Environ Health Perspect, 2010. **118**(5): p. 659-65. <http://doi.org/10.1289/ehp.0901214>.
163. van Bommel, D.M., et al., *S-ethyl-N,N-dipropylthiocarbamate exposure and cancer incidence among male pesticide applicators in the agricultural health study: a prospective cohort*. Environ Health Perspect, 2008. **116**(11): p. 1541-6. <http://doi.org/10.1289/ehp.11371>.
164. Yi, S.W., et al., *Agent Orange exposure and prevalence of self-reported diseases in Korean Vietnam veterans*. J Prev Med Public Health, 2013. **46**(5): p. 213-25. <http://doi.org/10.3961/jpmph.2013.46.5.213>.
165. Aronson, K.J., et al., *Plasma organochlorine levels and prostate cancer risk*. J Expo Sci Environ Epidemiol, 2010. **20**(5): p. 434-45. <http://doi.org/10.1038/jes.2009.33>.
166. Emeville, E., et al., *Associations of plasma concentrations of dichlorodiphenyldichloroethylene and polychlorinated biphenyls with prostate cancer: a case-control study in Guadeloupe (French West Indies)*. Environ Health Perspect, 2015. **123**(4): p. 317-23. <http://doi.org/10.1289/ehp.1408407>.
167. Lim, J.E., et al., *Serum persistent organic pollutants (POPs) and prostate cancer risk: A case-cohort study*. Int J Hyg Environ Health, 2017. **220**(5): p. 849-856. <http://doi.org/10.1016/j.ijheh.2017.03.014>.
168. Hardell, L., et al., *Adipose tissue concentrations of persistent organic pollutants and the risk of prostate cancer*. J Occup Environ Med, 2006. **48**(7): p. 700-7. <http://doi.org/10.1097/01.jom.0000205989.46603.43>.

169. Pi, N., et al., *Associations of serum organohalogen levels and prostate cancer risk: Results from a case-control study in Singapore*. Chemosphere, 2016. **144**: p. 1505-12. <http://doi.org/10.1016/j.chemosphere.2015.10.020>.
170. Boers, D., et al., *The influence of occupational exposure to pesticides, polycyclic aromatic hydrocarbons, diesel exhaust, metal dust, metal fumes, and mineral oil on prostate cancer: a prospective cohort study*. Occup Environ Med, 2005. **62**(8): p. 531-7. <http://doi.org/10.1136/oem.2004.018622>.
171. van der Gulden, J.W., J.J. Kolk, and A.L. Verbeek, *Work environment and prostate cancer risk*. Prostate, 1995. **27**(5): p. 250-7. <http://doi.org/10.1002/pros.2990270504>.
172. Koutros, S., et al., *Xenobiotic-metabolizing gene variants, pesticide use, and the risk of prostate cancer*. Pharmacogenet Genomics, 2011. **21**(10): p. 615-23. <http://doi.org/10.1097/FPC.0b013e3283493a57>.
173. Ruder, A.M., et al., *Cancer incidence among capacitor manufacturing workers exposed to polychlorinated biphenyls*. Am J Ind Med, 2017. **60**(2): p. 198-207. <http://doi.org/10.1002/ajim.22657>.
174. Armstrong, B.G. and G. Kazantzi, *Prostatic cancer and chronic respiratory and renal disease in British cadmium workers: a case control study*. Br J Ind Med, 1985. **42**(8): p. 540-5. <http://doi.org/10.1136/oem.42.8.540>.
175. Neslund-Dudas, C., et al., *Case-only gene-environment interaction between ALAD tagSNPs and occupational lead exposure in prostate cancer*. Prostate, 2014. **74**(6): p. 637-46. <http://doi.org/10.1002/pros.22781>.
176. Nyqvist, F., et al., *Increased Cancer Incidence in the Local Population Around Metal-Contaminated Glassworks Sites*. J Occup Environ Med, 2017. **59**(5): p. e84-e90. <http://doi.org/10.1097/JOM.0000000000001003>.
177. Platz, E.A., et al., *Prediagnostic toenail cadmium and zinc and subsequent prostate cancer risk*. Prostate, 2002. **52**(4): p. 288-96. <http://doi.org/10.1002/pros.10115>.
178. Roh, T., et al., *Low-level arsenic exposure from drinking water is associated with prostate cancer in Iowa*. Environ Res, 2017. **159**: p. 338-343. <http://doi.org/10.1016/j.envres.2017.08.026>.
179. Roman, M.D., et al., *Do Exposure to Arsenic, Occupation and Diet Have Synergistic Effects on Prostate Cancer Risk?* Asian Pac J Cancer Prev, 2018. **19**(6): p. 1495-1501. <http://doi.org/10.22034/APJCP.2018.19.6.1495>.
180. Rousseau, M.C., et al., *Occupational exposure to lead compounds and risk of cancer among men: a population-based case-control study*. Am J Epidemiol, 2007. **166**(9): p. 1005-14. <http://doi.org/10.1093/aje/kwm183>.
181. Vinceti, M., et al., *Case-control study of toenail cadmium and prostate cancer risk in Italy*. Sci Total Environ, 2007. **373**(1): p. 77-81. <http://doi.org/10.1016/j.scitotenv.2006.11.005>.
182. Chang, W.H., et al., *Oxidative damage in patients with benign prostatic hyperplasia and prostate cancer co-exposed to phthalates and to trace elements*. Environ Int, 2018. **121**(Pt 2): p. 1179-1184. <http://doi.org/10.1016/j.envint.2018.10.034>.
183. Nadon, L., et al., *Cancer risk due to occupational exposure to polycyclic aromatic hydrocarbons*. Am J Ind Med, 1995. **28**(3): p. 303-24. <http://doi.org/10.1002/ajim.4700280302>.
184. Rybicki, B.A., et al., *Prostate cancer risk from occupational exposure to polycyclic aromatic hydrocarbons interacting with the GSTP1 Ile105Val polymorphism*. Cancer Detect Prev, 2006. **30**(5): p. 412-22. <http://doi.org/10.1016/j.cdp.2006.09.004>.

185. Weichenthal, S., et al., *Spatial variations in ambient ultrafine particle concentrations and the risk of incident prostate cancer: A case-control study*. Environ Res, 2017. **156**: p. 374-380. <http://doi.org/10.1016/j.envres.2017.03.035>.
186. Blanc-Lapierre, A., J.F. Sauve, and M.E. Parent, *Occupational exposure to benzene, toluene, xylene and styrene and risk of prostate cancer in a population-based study*. Occup Environ Med, 2018. **75**(8): p. 562-572. <http://doi.org/10.1136/oemed-2018-105058>.
187. Christensen, K.Y., et al., *Risk of selected cancers due to occupational exposure to chlorinated solvents in a case-control study in Montreal*. J Occup Environ Med, 2013. **55**(2): p. 198-208. <http://doi.org/10.1097/JOM.0b013e3182728eab>.
188. Tse, L.A., et al., *Environmental risk factors of prostate cancer: a case-control study*. Hong Kong Med J, 2018. **24 Suppl 4**(4): p. 30-33.
189. Eriksen, K.T., et al., *Perfluorooctanoate and perfluorooctanesulfonate plasma levels and risk of cancer in the general Danish population*. J Natl Cancer Inst, 2009. **101**(8): p. 605-9. <http://doi.org/10.1093/jnci/djp041>.
190. Grice, M.M., et al., *Self-reported medical conditions in perfluorooctanesulfonyl fluoride manufacturing workers*. J Occup Environ Med, 2007. **49**(7): p. 722-9. <http://doi.org/10.1097/JOM.0b013e3180582043>.
191. Steenland, K., L. Zhao, and A. Winqvist, *A cohort incidence study of workers exposed to perfluorooctanoic acid (PFOA)*. Occup Environ Med, 2015. **72**(5): p. 373-80. <http://doi.org/10.1136/oemed-2014-102364>.
192. Chuang, S.C., et al., *Phthalate exposure and prostate cancer in a population-based nested case-control study*. Environ Res, 2020. **181**: p. 108902. <http://doi.org/10.1016/j.envres.2019.108902>.
193. Parent, M.E., et al., *Traffic-related air pollution and prostate cancer risk: a case-control study in Montreal, Canada*. Occup Environ Med, 2013. **70**(7): p. 511-8. <http://doi.org/10.1136/oemed-2012-101211>.
194. Zeng, F., et al., *Occupational exposure to pesticides and other biocides and risk of thyroid cancer*. Occup Environ Med, 2017. **74**(7): p. 502-510. <http://doi.org/10.1136/oemed-2016-103931>.
195. Deziel, N.C., et al., *Exposure to polychlorinated biphenyls and organochlorine pesticides and thyroid cancer in connecticut women*. Environ Res, 2021. **192**: p. 110333. <http://doi.org/10.1016/j.envres.2020.110333>.
196. Lerro, C.C., et al., *A nested case-control study of polychlorinated biphenyls, organochlorine pesticides, and thyroid cancer in the Janus Serum Bank cohort*. Environ Res, 2018. **165**: p. 125-132. <http://doi.org/10.1016/j.envres.2018.04.012>.
197. Han, X., et al., *Associations between Exposure to Persistent Organic Pollutants and Thyroid Function in a Case-Control Study of East China*. Environ Sci Technol, 2019. **53**(16): p. 9866-9875. <http://doi.org/10.1021/acs.est.9b02810>.
198. Lerro, C.C., et al., *Pesticide exposure and incident thyroid cancer among male pesticide applicators in agricultural health study*. Environ Int, 2021. **146**: p. 106187. <http://doi.org/10.1016/j.envint.2020.106187>.
199. Park, E., et al., *Environmental exposure to cadmium and risk of thyroid cancer from national industrial complex areas: A population-based cohort study*. Chemosphere, 2021. **268**: p. 128819. <http://doi.org/10.1016/j.chemosphere.2020.128819>.

200. Zhang, C., et al., *Association of exposure to multiple metals with papillary thyroid cancer risk in China*. *Environ Sci Pollut Res Int*, 2019. **26**(20): p. 20560-20572. <http://doi.org/10.1007/s11356-019-04733-x>.
201. Lope, V., et al., *Occupational exposure to chemicals and risk of thyroid cancer in Sweden*. *Int Arch Occup Environ Health*, 2009. **82**(2): p. 267-74. <http://doi.org/10.1007/s00420-008-0314-4>.
202. Zhou, Z., et al., *Higher urinary bisphenol A concentration and excessive iodine intake are associated with nodular goiter and papillary thyroid carcinoma*. *Biosci Rep*, 2017. **37**(4). <http://doi.org/10.1042/BSR20170678>.
203. Marotta, V., et al., *Human exposure to bisphenol AF and diethylhexylphthalate increases susceptibility to develop differentiated thyroid cancer in patients with thyroid nodules*. *Chemosphere*, 2019. **218**: p. 885-894. <http://doi.org/10.1016/j.chemosphere.2018.11.084>.
204. Miao, H., et al., *Associations of urinary phthalate metabolites with risk of papillary thyroid cancer*. *Chemosphere*, 2020. **241**: p. 125093. <http://doi.org/10.1016/j.chemosphere.2019.125093>.
205. Aschebrook-Kilfoy, B., et al., *Polybrominated diphenyl ethers and thyroid cancer risk in the Prostate, Colorectal, Lung, and Ovarian Cancer Screening Trial cohort*. *Am J Epidemiol*, 2015. **181**(11): p. 883-8. <http://doi.org/10.1093/aje/kwu358>.
206. Deziel, N.C., et al., *Exposure to Polybrominated Diphenyl Ethers and a Polybrominated Biphenyl and Risk of Thyroid Cancer in Women: Single and Multi-Pollutant Approaches*. *Cancer Epidemiol Biomarkers Prev*, 2019. **28**(10): p. 1755-1764. <http://doi.org/10.1158/1055-9965.EPI-19-0526>.
207. Hoffman, K., et al., *Exposure to flame retardant chemicals and occurrence and severity of papillary thyroid cancer: A case-control study*. *Environ Int*, 2017. **107**: p. 235-242. <http://doi.org/10.1016/j.envint.2017.06.021>.
208. Huang, H., et al., *Polybrominated Diphenyl Ethers, Polybrominated Biphenyls, and Risk of Papillary Thyroid Cancer: A Nested Case-Control Study*. *Am J Epidemiol*, 2020. **189**(2): p. 120-132. <http://doi.org/10.1093/aje/kwz229>.
209. Li, F.X., L. Squartsoff, and S.H. Lamm, *Prevalence of thyroid diseases in Nevada counties with respect to perchlorate in drinking water*. *J Occup Environ Med*, 2001. **43**(7): p. 630-4. <http://doi.org/10.1097/00043764-200107000-00010>.
210. Weiderpass, E., et al., *Organochlorines and endometrial cancer risk*. *Cancer Epidemiol Biomarkers Prev*, 2000. **9**(5): p. 487-93.
211. Hardell, L., et al., *Adipose tissue concentrations of p,p'-DDE and the risk for endometrial cancer*. *Gynecol Oncol*, 2004. **95**(3): p. 706-11. <http://doi.org/10.1016/j.ygyno.2004.08.022>.
212. Trabert, B., et al., *Persistent organic pollutants (POPs) and fibroids: results from the ENDO study*. *J Expo Sci Environ Epidemiol*, 2015. **25**(3): p. 278-85. <http://doi.org/10.1038/jes.2014.31>.
213. Weiderpass, E., et al., *Occupational exposures and cancers of the endometrium and cervix uteri in Finland*. *Am J Ind Med*, 2001. **39**(6): p. 572-80. <http://doi.org/10.1002/ajim.1056>.
214. Shen, Y., et al., *Environmental exposure and risk of uterine leiomyoma: an epidemiologic survey*. *Eur Rev Med Pharmacol Sci*, 2013. **17**(23): p. 3249-56.

215. Lambertino, A., et al., *Uterine leiomyomata in a cohort of Great Lakes sport fish consumers*. Environ Res, 2011. **111**(4): p. 565-72. <http://doi.org/10.1016/j.envres.2011.01.006>.
216. Betenia, N., S. Costello, and E.A. Eisen, *Risk of cervical cancer among female autoworkers exposed to metalworking fluids*. Scand J Work Environ Health, 2012. **38**(1): p. 78-83. <http://doi.org/10.5271/sjweh.3193>.
217. Johnstone, E.B., et al., *Increased urinary cobalt and whole blood concentrations of cadmium and lead in women with uterine leiomyomata: Findings from the ENDO Study*. Reprod Toxicol, 2014. **49**: p. 27-32. <http://doi.org/10.1016/j.reprotox.2014.06.007>.
218. McElroy, J.A., et al., *Cadmium exposure and endometrial cancer risk: A large midwestern U.S. population-based case-control study*. PLoS One, 2017. **12**(7): p. e0179360. <http://doi.org/10.1371/journal.pone.0179360>.
219. Wernli, K.J., et al., *Occupational risk factors for endometrial cancer among textile workers in Shanghai, China*. Am J Ind Med, 2008. **51**(9): p. 673-9. <http://doi.org/10.1002/ajim.20614>.
220. Rashidi, B.H., et al., *A case-control study of bisphenol A and endometrioma among subgroup of Iranian women*. J Res Med Sci, 2017. **22**: p. 7. <http://doi.org/10.4103/1735-1995.199086>.
221. Kim, Y.A., et al., *Increased Urinary Phthalate Levels in Women with Uterine Leiomyoma: A Case-Control Study*. Int J Environ Res Public Health, 2016. **13**(12). <http://doi.org/10.3390/ijerph13121247>.
222. Wen, H.J., et al., *Exposure to endocrine disruptor alkylphenols and the occurrence of endometrial cancer*. Environ Pollut, 2020. **267**: p. 115475. <http://doi.org/10.1016/j.envpol.2020.115475>.
223. Bullman, T.A., K.K. Watanabe, and H.K. Kano, *Risk of testicular cancer associated with surrogate measures of agent orange exposure among Vietnam veterans on the agent orange registry*. Annals of Epidemiology, 1994. **4**(1): p. 11-16. [http://doi.org/10.1016/1047-2797\(94\)90037-x](http://doi.org/10.1016/1047-2797(94)90037-x).
224. Giannandrea, F., et al., *Pesticide exposure and serum organochlorine residuals among testicular cancer patients and healthy controls*. J Environ Sci Health B, 2011. **46**(8): p. 780-7. <http://doi.org/10.1080/03601234.2012.597704>.
225. McGlynn, K.A., et al., *Persistent organochlorine pesticides and risk of testicular germ cell tumors*. J Natl Cancer Inst, 2008. **100**(9): p. 663-71. <http://doi.org/10.1093/jnci/djn101>.
226. Walschaerts, M., et al., *Environmental, occupational and familial risks for testicular cancer: a hospital-based case-control study*. Int J Androl, 2007. **30**(4): p. 222-9. <http://doi.org/10.1111/j.1365-2605.2007.00805.x>.
227. Chia, V.M., et al., *Effect modification of endocrine disruptors and testicular germ cell tumour risk by hormone-metabolizing genes*. Int J Androl, 2010. **33**(4): p. 588-96. <http://doi.org/10.1111/j.1365-2605.2009.00975.x>.
228. Paoli, D., et al., *Exposure to polychlorinated biphenyls and hexachlorobenzene, semen quality and testicular cancer risk*. J Endocrinol Invest, 2015. **38**(7): p. 745-52. <http://doi.org/10.1007/s40618-015-0251-5>.
229. Hardell, L., et al., *Case-control study on risk factors for testicular cancer*. Int J Oncol, 1998. **13**(6): p. 1299-303. <http://doi.org/10.3892/ijo.13.6.1299>.

230. McGlynn, K.A., et al., *Polychlorinated biphenyls and risk of testicular germ cell tumors*. Cancer Res, 2009. **69**(5): p. 1901-9. <http://doi.org/10.1158/0008-5472.CAN-08-3935>.
231. Behrens, T., et al., *Exposure to metal-working fluids in the automobile industry and the risk of male germ cell tumours*. Occup Environ Med, 2012. **69**(3): p. 224-6. <http://doi.org/10.1136/oemed-2011-100070>.
232. Hardell, L., C.-G. Ohlson, and M. Fredrikson, *Occupational exposure to polyvinyl chloride as a risk factor for testicular cancer evaluated in a case-control study*. International Journal of Cancer, 1997. **73**(6): p. 828-830. [http://doi.org/10.1002/\(sici\)1097-0215\(19971210\)73:6<828::Aid-ijc10>3.0.Co;2-0](http://doi.org/10.1002/(sici)1097-0215(19971210)73:6<828::Aid-ijc10>3.0.Co;2-0).
233. Hardell, L., et al., *Testicular cancer and occupational exposure to polyvinyl chloride plastics: a case-control study*. Int J Cancer, 2004. **109**(3): p. 425-9. <http://doi.org/10.1002/ijc.11709>.
234. Westberg, H.B., et al., *On the use of different measures of exposure-experiences from a case-control study on testicular cancer and PVC exposure*. J Occup Environ Hyg, 2005. **2**(7): p. 351-6. <http://doi.org/10.1080/15459620590969046>.
235. Inoue-Choi, M., et al., *Atrazine in public water supplies and risk of ovarian cancer among postmenopausal women in the Iowa Women's Health Study*. Occup Environ Med, 2016. **73**(9): p. 582-7. <http://doi.org/10.1136/oemed-2016-103575>.
236. Young, H.A., et al., *Triazine herbicides and epithelial ovarian cancer risk in central California*. J Occup Environ Med, 2005. **47**(11): p. 1148-56. <http://doi.org/10.1097/01.iom.0000177044.43959.e8>.
237. Ploteau, S., et al., *Associations between internal exposure levels of persistent organic pollutants in adipose tissue and deep infiltrating endometriosis with or without concurrent ovarian endometrioma*. Environ Int, 2017. **108**: p. 195-203. <http://doi.org/10.1016/j.envint.2017.08.019>.
238. Wernli, K.J., et al., *Occupational exposures and ovarian cancer in textile workers*. Epidemiology, 2008. **19**(2): p. 244-50. <http://doi.org/10.1097/EDE.0b013e31816339f9>.
239. Guo, J., et al., *Risk of esophageal, ovarian, testicular, kidney and bladder cancers and leukemia among finnish workers exposed to diesel or gasoline engine exhaust*. Int J Cancer, 2004. **111**(2): p. 286-92. <http://doi.org/10.1002/ijc.20263>.
240. Shu, X.O., et al., *Population-based case-control study of ovarian cancer in Shanghai*. Cancer Res, 1989. **49**(13): p. 3670-4.

BOOK CHAPTER

Regarding tumor invasion, in the BOOK CHAPTER we provide a review of the evolution of the Thyroid Classification Systems from 1914 to 2022 and perform a reflection on the pathobiology, morphology and clinical significance of minimally invasive tumors. We further characterize the histology and prognostic significance of benign and low-risk tumors approached in chapter 2.3.3.

Chapter 1

Pathobiology, morphology and clinical significance of minimally invasive thyroid neoplasms

Paula Soares, José Manuel Cameselle-Teijeiro, Elisabete Teixeira, Antónia Póvoa, João Magalhães, Isabel Amendoeira, Manuel Sobrinho-Simões

INTRODUCTION

In this chapter, we review the concept, histopathology and clinical significance of “minimally invasive thyroid carcinomas”. In addition, tumours with uncertain malignant potential (UMP) and non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) are addressed in the context of encapsulated/well circumscribed follicular patterned tumours.

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Initially, when the concept of minimally invasive carcinomas was created, it was restricted to follicular thyroid carcinomas (FTC), which were separated into minimally and widely invasive follicular thyroid carcinomas (MI-FTC and WI-FTC, respectively). Once the importance of angioinvasion was recognised, the category of angioinvasive follicular carcinoma was created. This was further subdivided into limited angioinvasive and extensively angioinvasive follicular carcinoma.

Until the second decade of 21st century, the diagnosis of follicular variant of papillary thyroid carcinoma (FVPTC) was made when the nuclear features were those of papillary thyroid carcinoma (PTC), regardless of the growth pattern (encapsulated/well circumscribed or infiltrative), and the presence or absence of invasion signs. More recently, the concept of minimally invasive thyroid carcinomas was expanded towards the inclusion of cases of encapsulated FVPTC in the group of minimally invasive thyroid carcinomas regardless of the nuclear features, as well as oncocytic carcinoma.

IMPORTANCE OF INVASION

The concept of neoplastic invasion in thyroid tumour diagnosis to distinguish follicular thyroid adenomas (follicular adenomas) from follicular carcinomas was introduced by Graham in 1914 [1]. In fact, Graham referred to capsular penetration and/or vascular invasion as major diagnostic features, and these findings were reinforced in subsequent studies in the following 20 years [2] (**Figure 1.1**). The concept of invasion in the diagnosis of follicular tumours was supported in 1929 by Collier [3] and subsequently by Warren [4]. Years later, several studies further expanded the concept to oncocytic (Hürthle cell) carcinoma as another example of invasive follicular patterned thyroid neoplasm [5] (**Figure 1.1**).

Remarkably, the concept of invasion as essential diagnostic parameter was for many years only applied to encapsulated follicular patterned tumours, without recognising the importance of invasion in papillary thyroid carcinomas (PTC). By definition, these have papillary or mixed papillary-follicular architecture and lack encapsulation. Other more recent concepts are minimal invasion, in terms of its identification and definition of the degree of invasion, and the most recent subdivision of angioinvasion (invasion of blood vessels) into limited and extensive vascular invasion [6].

The focus on cancer features that substantiate the diagnostic and prognostic meaning of invasion by thyroid neoplastic cells joins together pathogenesis and diagnosis, in view of the notion that the most important characteristic of any malignant neoplasm is invasiveness, e.g. growth that does not respect tissue frontiers. Evaluation of the presence and degree of invasion in encapsulated/well-circumscribed tumours is crucial to understand the biology of follicular patterned thyroid neoplasms and to classify these lesions by separating the group of low-risk neoplasms, which includes NIFTP, follicular tumour of uncertain malignant potential (FT-UMP) and well-differentiated tumour of uncertain malignant potential (WDT-UMP), from minimally invasive thyroid neoplasms. When adequate and reproducible histopathological guidelines are generally used, this provides relevant information regarding prognosis and treatment. Regarding thyroid tumours, invasion needs to be specified according to site: into the capsule of the tumour, into the peritumoural thyroid parenchyma, and into lymphatic vessels and blood vessels.

In practice, a frequently encountered problem is the presence of questionable signs of capsular and/or vascular invasion in encapsulated, follicular patterned neoplasm

Thyroid Historical Marks

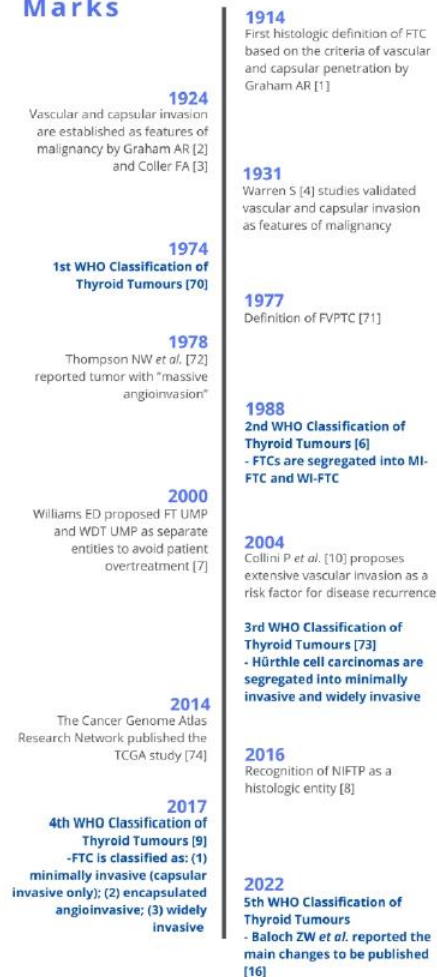


Figure 1.1 Timeline of most relevant thyroid historical marks. This timeline was built based on the following studies [1-10,16,71-76].

(FT, follicular tumour; FTC, follicular thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; MI-FTC, minimally invasive follicular thyroid carcinoma; NIFTP, non-invasive follicular thyroid neoplasms with papillary-like nuclear features; TCGA, The Cancer Genome Atlas; UMP, uncertain malignant potential; WDT, well-differentiated tumour; WI-FTC, widely invasive follicular thyroid carcinoma)

without nuclear features of PTC, which has led to the category of tumours with UMP. This tangentially also addresses the concept of NIFTP [7–9].

Taken together, this approach results in a spectrum of clinico-pathological entities of encapsulated tumours, ranging from NIFTP with an (extremely) low risk of malignancy, through the questionably invasive and minimally invasive thyroid neoplasms, to the highly aggressive extensively angioinvasive carcinomas [10]. The relevance of this detailed spectrum is the prognostic significance of the used terminology.

The concepts of widely invasive carcinoma and of any type of extrathyroidal neoplastic invasion are beyond the scope of this chapter. The same applies to invasion of intratumoural blood vessels, which lacks clinical significance [11]. We will, however, briefly address the pathobiology and clinical significance of invasion of lymphatic vessels by classic PTC.

HISTOPATHOLOGY OF FOLLICULAR PATTERNED NEOPLASMS

Histopathology of encapsulated (capsular and/or venous) invasive, follicular-patterned neoplasms

The fourth edition of the WHO thyroid tumour classification highlighted invasiveness rather than nuclear features as a key feature to define the malignant nature and prognosis of well-differentiated follicular cell neoplasms [9]. Accordingly, invasive encapsulated follicular variants of PTC, FTC, and oncocytic carcinoma are subdivided into the following three histopathological subtypes:

1. Minimally invasive (encapsulated tumours showing only capsular invasion)
2. Encapsulated angioinvasive (encapsulated tumours with venous invasion regardless of whether or not there is capsular invasion)
3. Widely invasive (overtly invasive tumours often accompanied by venous invasion).

Tumour capsular invasion is defined as the complete transgression of the thickness of the tumour fibrous capsule, generally accompanied by expansion of the invasive tongue of the tumour in the form of a mushroom or shark fin (**Figure 1.2**) [9,12]. When capsular invasion does not affect the entire thickness of the capsule, additional sections are necessary to confirm (or exclude) capsular invasion. When one or more tumour nodules adjacent to the capsule of a tumour are observed, sharing a similar microscopic appearance, it is also necessary to perform additional sections to exclude in-depth continuity between any of the nodules and the main tumour, which would provide evidence of capsular invasion [9,12].

Of note, often tumour capsular invasion actually represents capsular-vascular invasion (**Figure 1.3**). Capsular invasion usually occurs in areas of lesser resistance of the tumour capsule, at the point of penetration of the vein that drains the tumour. The cleavage plane between the tumour capsule and the venous wall constitutes an area prone to tumour herniation akin to capsular invasion, and facilitates tumour venous invasion (**Figure 1.4**). In contrast, spread via lymphatic vessels to regional lymph nodes is a very common

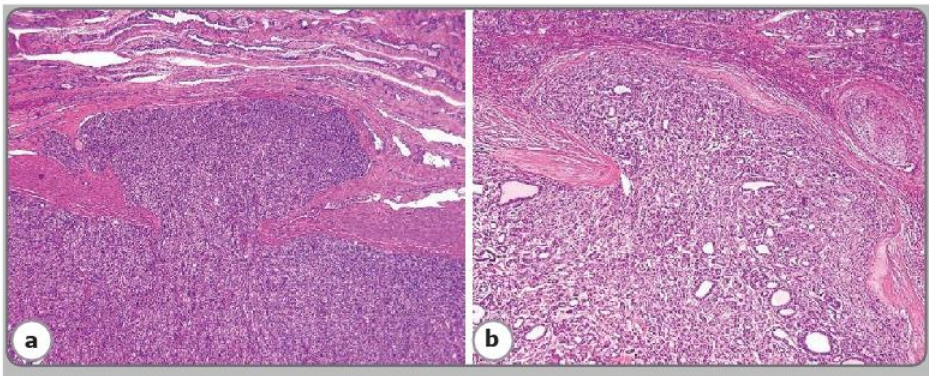


Figure 1.2 Minimally invasive follicular thyroid carcinoma (capsular invasion only). In thyroid carcinomas that show only capsular invasion, tumour cells invade the entire thickness of the tumour capsule, often with mushroom-shaped (a) or shark-fin growth (b).

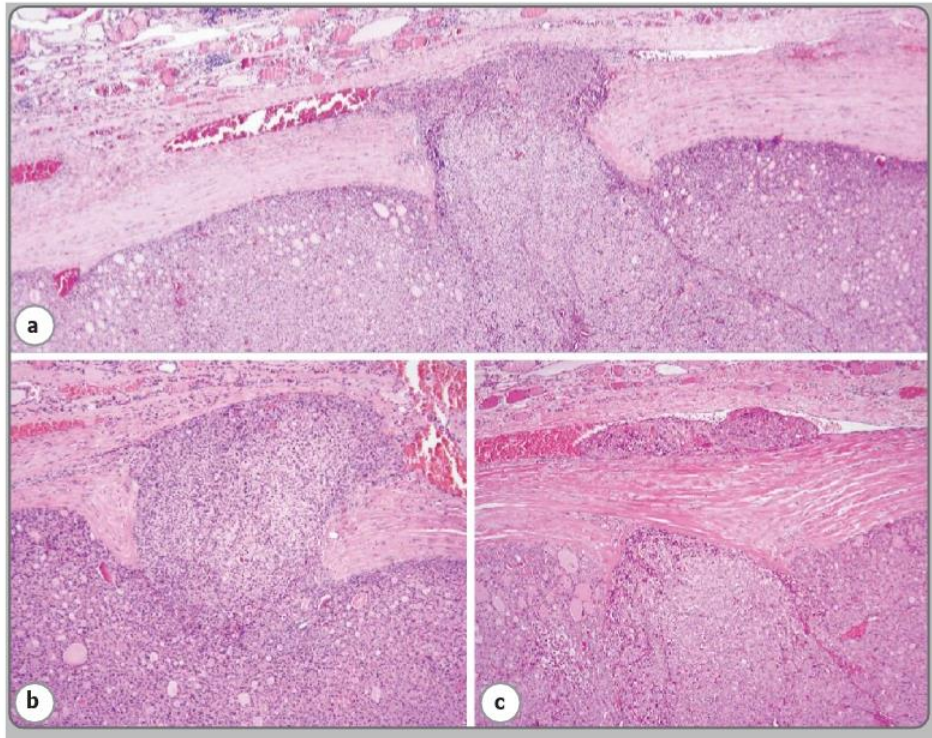


Figure 1.3 Angioinvasive encapsulated thyroid follicular carcinoma. This tumour initially presented with a focus of capsular invasion (a). Deeper sections of the same focus of suspected vascular invasion showed intimate relationship with adjacent veins (b) and finally genuine vascular invasion (c).

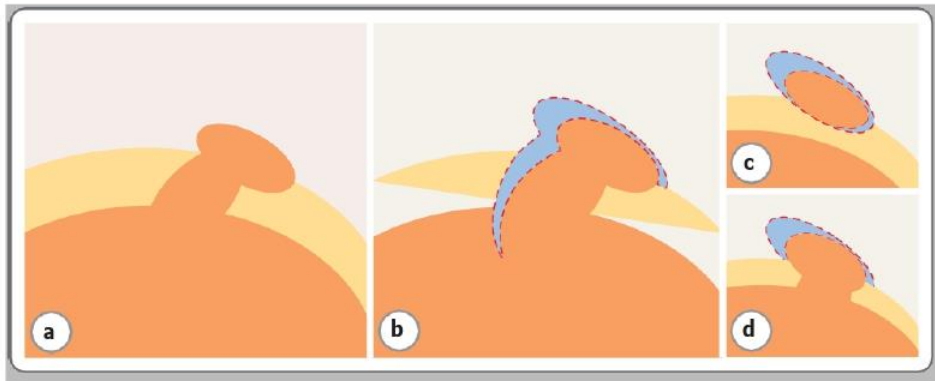


Figure 1.4 Schematic representation of capsular-vascular invasion in thyroid carcinoma. As in the case of figure 1.3, it is very common for capsular invasion to actually represent capsular-vascular invasion (a). Capsular invasion usually occurs in areas of lesser resistance of the tumour capsule at the point of penetration of the vein that drains the tumour (b). These foci of vascular invasion lined by the endothelium of the adjacent vein may represent preinvasive stages (c and d).

Tumour: brown; tumour capsule: yellow; vein lumen: blue; endothelial cells: red.

presentation of PTC and most of its subtypes that can be associated with recurrence, but not with patient mortality [13,14]. For this reason, identifying tumour venous invasion requires the application of strict criteria [9,12]. For the diagnosis of venous invasion only veins inside or outside the tumour capsule may be considered. Furthermore, confirmation of genuine venous invasion also requires that the tumour embolus adheres to the vascular wall and/or is lined by endothelium [12,15,16] (**Figure 1.5**). When the tumour embolus is accompanied by fibrin and/or has infiltrated into the vascular wall the risk of distant metastases increases [11], which is consistent with the notion that some tumour emboli only covered by endothelium may represent a preinvasive stage [17]

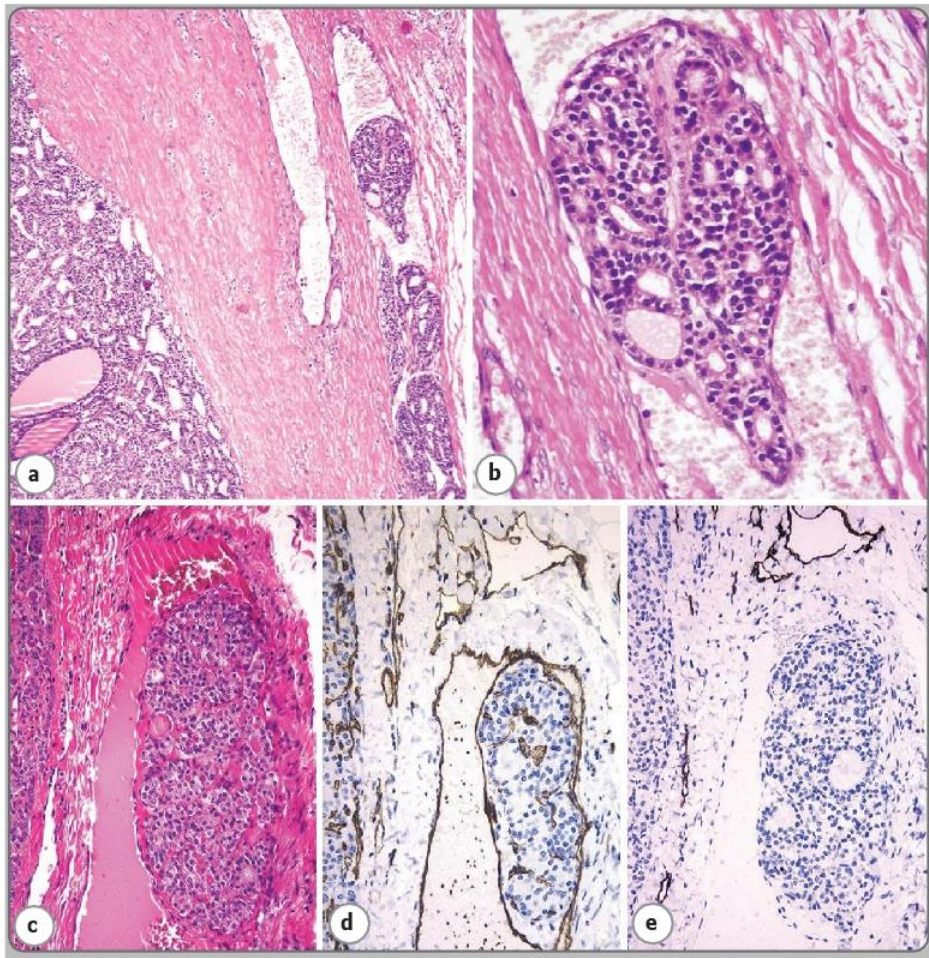


Figure 1.5 Angioinvasive encapsulated follicular carcinoma. The diagnosis of venous invasion is only applicable to veins inside or outside (a) the tumour capsule. The confirmation of genuine venous invasion also requires that the tumour thrombus be adhered to the vascular wall and/or lined by endothelium (b). Distant metastases in follicular carcinoma (c) occur through the veins which are positive for CD31 (d) and negative for lymphatic endothelial markers (D2-40) (e).

(Figure 1.4). It is important to stress that the presence of tumour plugs in subcapsular venous vessels does not count as blood vessel invasion.

Particular care must be taken not to confuse capsular invasion with artefacts secondary to capsule rupture after biopsy with a needle [18], or due to artefactual V-shaped folding of the tumour capsule (tuned into itself) at the edge of a tissue section [19]. Reactive proliferations of endothelial cells projecting into the vascular lumen, intravascular papillary endothelial hyperplasia (Masson-like haemangioma) or Kaposi sarcoma-like endothelial proliferations may mimic vascular invasion by the tumour [12,20]. Although intravascular endothelial hyperplasia is more common in malignant thyroid tumours [21,22], it can also occur in follicular adenoma and adenomatous goitre [23,24] **(Figure 1.6)**. Of note, while distant metastases in follicular carcinoma occur through veins [25], intravascular endothelial hyperplasia usually occurs in lymphatic vessels [22] **(Figure 1.5)**. Cell groups trapped in the capsule with retraction artefact but devoid of endothelium can mimic angioinvasion. Immunostaining for vascular markers, particularly CD31 and/or ERG, and in case of need a marker for lymphatic vessels such as podoplanin (also known as D2-40), may help to establish whether the focus of tumour cells is covered by endothelial cells or to confirm that they are in a blood vessel lumen [12,16,25,26]. The presence of individual tumour cells in the vascular lumen neither adherent to the wall nor covered by endothelial cells is not true vascular invasion [12,15].

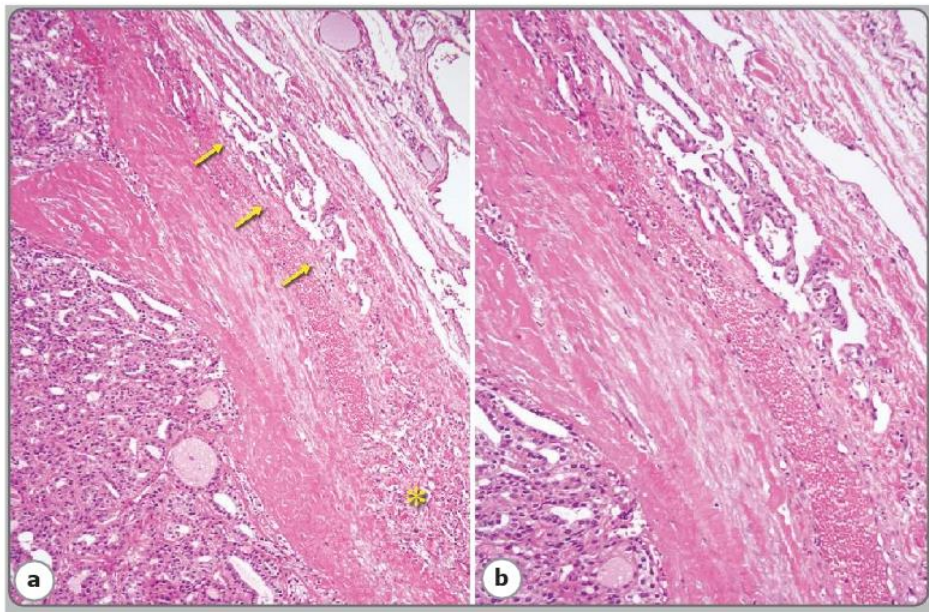


Figure 1.6 Intravascular endothelial hyperplasia. Intravascular endothelial hyperplasia is more common in malignant thyroid tumours but can also occur in benign tumours. Intravascular endothelial hyperplasia usually occurs in lymphatic vessels (a and b) and should not be confused with vascular invasion.

Histopathology of follicular patterned neoplasms with questionable capsular or vascular invasion

This category addresses tumours with follicular-cell nuclei (FT-UMP) or with equivocal (or even typical) nuclear features of PTC (WDT-UMP). We will also address NIFTP in this section as it belongs to the group of low-risk neoplasms. The concept of FT-UMP was created in 2,000 when pathologists realised that sometimes it was not possible to decide whether or not there was capsular or vascular invasion in encapsulated follicular neoplasms (**Figure 1.7**). In parallel, for tumours of which the nuclear features of the neoplastic cells approached the appearance of PTC nuclei (large, clear and grooved) without fulfilling the criteria for typical PTC nuclei, the category of WDT-UMP was created.

The problem caused by classifying follicular patterned tumours as papillary carcinomas, exclusively based on the PTC-like appearance of the nuclei, was overtreatment of

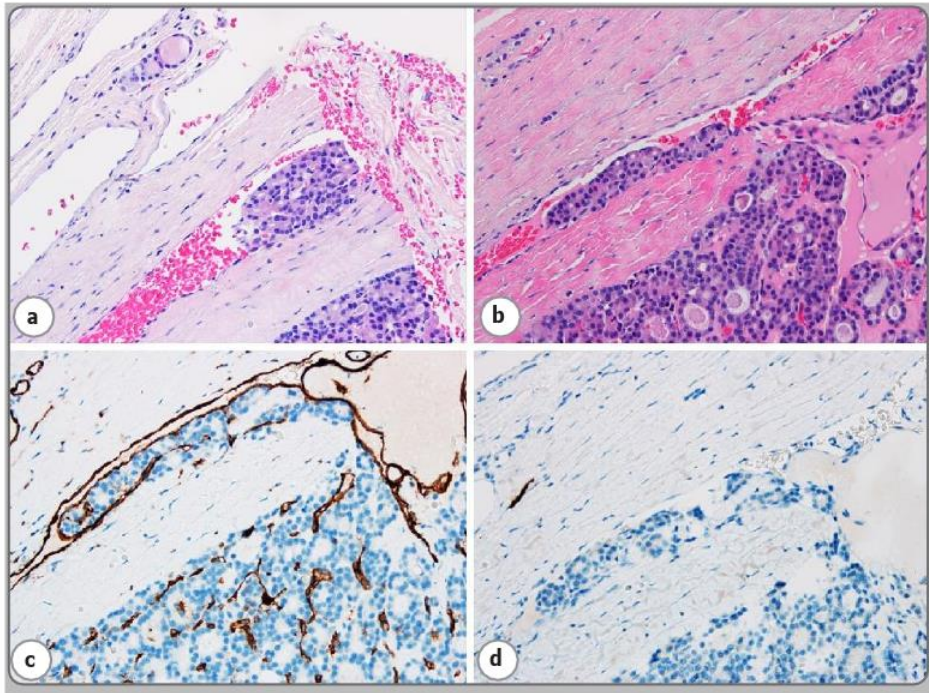


Figure 1.7 Follicular tumour of uncertain malignant potential (FT, UMP). (a) This tumour presented a focus of suggestive vascular invasion in a blood vessel in the capsule. However, there is not enough evidence to diagnose vascular invasion because the tumour plug was not lined by endothelial cells and there is not also a fibrin thrombus. This finding raises the possibility one is dealing with an artefact [haematoxylin and eosin (H&E)]. A thorough study was performed using numerous additional sections. (b) Just in one recut we observed an image suggestive of vascular invasion in the capsule of the tumour (H&E). (c and d) The confirmation of vascular invasion requires the presence of endothelial cells lining the blood vessel and the neoplastic cells using CD31 and D2-40, respectively. In this particular case, we were not able to identify unequivocal signs of vascular invasion and we kept the diagnosis of FT, UMP based upon questionable vascular invasion.

patients with encapsulated/well-circumscribed tumours. In an attempt to avoid this, the aforementioned WDT-UMP category was created. A diagnosis of WDT-UMP implies close follow-up after hemithyroidectomy, instead of total thyroidectomy plus radioiodine therapy, provided there are no signs of invasion. Follow-up of patients with encapsulated, non-invasive, follicular patterned tumours displaying PTC nuclei demonstrated that their prognosis is excellent, regardless of the type of surgery (**Figure 1.8**). In contrast, infiltrative (predominantly) follicular patterned PTC carries a worse prognosis as it follows a natural history similar to that of classic PTC, with frequent lymph node metastases. Recently, Ito et al (2022) described distant recurrences in 1% of patients with a radioiodine-avid lesion classified as FT-UMP, with questionable capsular invasion but without vascular invasion [27]. They recommend re-examination of histological material in case of questionable capsular invasion and/or vascular invasion as well as long-term post-surgery active patient surveillance. Hysek et al (2019) reported a 5% recurrence rate of distant metastases in FT-UMP [28], suggesting that a fraction of these lesions may harbour malignant potential [28]. This finding once more emphasises the need for exhaustive examination of the surgical specimen.

The clinico-pathological entity non-invasive follicular thyroid neoplasm with papillary-like nuclear features (with as uncomfortable acronym NIFTP) was carved out from WDT-UMP and encapsulated, non-invasive FVPTC in 2016 [8]. Its definition lacks in precision and hence it is somewhat heterogeneous. As an example, there is no consensus as to the percentage of true papillae allowed in NIFTP: <1% papillae or no well-formed papillae at all [16]. This is important as a diagnosis of NIFTP should exclude any type of more aggressive neoplasm, notably classic PTC [29].

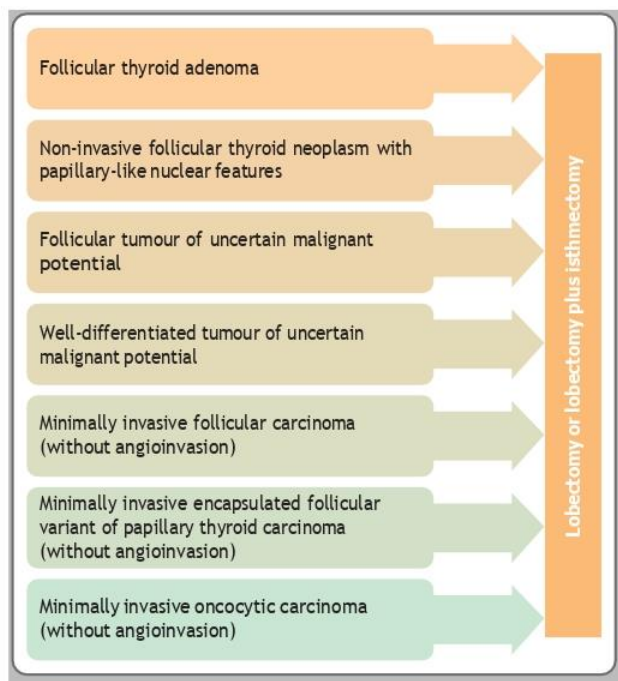


Figure 1.8 Thyroid tumours treated by lobectomy or lobectomy plus isthmectomy.

The risk of lymph node metastasis remains a matter of debate. In two studies of NIFTP, with as criterium for papillae <1%, no nodal metastases were present at diagnosis [30,31] but lymph node status was not verified as in these studies prophylactic lymphadenectomy was not performed. In contrast, in NIFTP studies, using as criterium absence of papillae, Paniza et al (2019) did not find any metastasis at diagnosis [32] while Cho et al (2017) found micrometastases in central neck lymph nodes at diagnosis [33]. In several follow-up studies, no cases with residual or local/distant recurrent disease were reported, regardless of the used criterium for papillae (absence or <1%) [30,33,34]. Metastases at follow-up to regional lymph nodes [29,35] and distant metastases [29] were reported by few groups in cases with ≤1% papillary growth. A limiting factor of most of these studies is the question how exhaustively the specimens were studied regarding the presence of papillary structures, as a higher percentage of papillary structures would have supported a diagnosis of classic PTC.

Taken together, when strict criteria for a diagnosis of NIFTP are applied, metastases are not observed during follow-up [36]. Even large NIFTP (≥4 cm) show indolent behaviour [36] and have a very low recurrence rate [32] despite some isolated cases with biochemical persistence [34]. Nonetheless, the few cases with adverse outcome [29,35], argue against classification of NIFTP as a benign lesion, even when strict inclusion and exclusion criteria are applied [36].

The treatment of choice for NIFTP is hemithyroidectomy followed by clinical and radiologic surveillance [16]. Full thyroidectomy and radioactive iodine are not required treatments for this diagnostic category [33] and intense follow-up is not needed [32].

NEOPLASMS WITH MINIMAL CAPSULAR AND/OR VASCULAR INVASION

Definition

For intrathyroidal encapsulated follicular cell tumours, neoplasms with a very low risk of recurrence after a complete excision which includes benign (follicular adenoma), low-risk (FT-UMP, WDT-UMP, and NIFTP) and minimally invasive neoplasms (FTC, encapsulated FVPTC, and oncocytic carcinoma), hemithyroidectomy alone is curative (**Figure 1.8**) [37,41]. These subtypes guide risk stratification in terms of local recurrence, lymph node and distant metastases, which are important parameters determining prognosis and allowing individualised surgical strategies [42,43].

The category MI-FCT emerged in 1988, when FTC was subdivided into minimally invasive (MI-FTC) and widely invasive (WI-FTC) in the 2nd edition of the WHO Classification (1988) [6]. Data on prognosis and treatment of patients with minimally invasive thyroid neoplasms are mostly based on cases diagnosed according to this classification, which somewhat limits their relevance. While WI-FTCs are grossly invasive tumours infiltrating into blood vessels and/or adjacent thyroid tissue and often lack complete encapsulation, MI-FTC is more difficult to define. In the 1988 classification, MI-FCT includes neoplasms displaying limited or extensive angioinvasion, which goes beyond the current concept of minimally invasive thyroid carcinoma, defined by capsular invasion without vascular invasion. In current classifications, the presence of either capsular or vascular invasion separates benign from malignant tumours [44]. In view of the lacunar definition of MI-FCT, the prognostic significance of minimal invasion remains debatable [42].

This lack of clarity in diagnostic criteria resulted in their modification in subsequent years. Many studies, especially those prior to the 4th edition of WHO Classification (2017) [9] minimal invasion remained defined as capsular and/or vascular invasion, with a cut-off for diagnosing venous invasion at ≥ 4 venous invasive foci [45,46]. In the 4th and 5th edition of the WHO Classification [9,16], a major change was introduced resulting into the subdivision of invasive encapsulated neoplasms into three categories:

1. Minimally invasive (classified as tumours with capsular invasion only)
2. Angioinvasive
3. Widely invasive

Prognostic factors

Although MI-FTC carries an overall good prognosis and is mostly considered to be an indolent tumour, some patients present with metastatic disease [46] and an aggressive clinical course [47–49]. In a cohort of 481 patients with 16 years of follow-up, disease-free survival of MI-FTC was similar to that of follicular adenoma (100% disease-free), with as implication that treatment might be the same for both entities [50]. However, a study on 195 MI-FTC patients reported distant metastases in about 8% of patients and half of these already presented distant metastases upon initial diagnosis [51].

An important question is which pathological variables, i.e. extent of capsular invasion, tumour size, and extent of angioinvasion, are predictive of the clinical course. In general, no significant association is found between capsular invasion and tumour recurrence [42,43,51,61].

Significance of tumour size is still debated: some studies reported that tumours >4 cm have an increased risk of recurrence [51,57,58], increased incidence of distant metastases and shorter distant metastases-free survival [55]. Another cohort comprising MI-FTC and WI-FTC showed that only tumour size (>4 cm) was an indicator of shorter disease-free survival [57]. Others did not find a significant association between tumour size and metastatic disease [59].

Recognition of venous invasion is particularly important, given that while disease-free survival at 40 months is 97% in minimally invasive carcinomas, it drops to 81 and 45% in encapsulated angioinvasive and widely invasive carcinomas, respectively [62,63]. Extensive angioinvasion (≥ 4 foci) has been reported as the most powerful risk factor for distant metastases [51,58,62,64].

Vascular invasion has consistently been associated with thyroid carcinoma prognosis and its presence has been included in the American Thyroid Association (ATA) guidelines for patient-risk assessment [44,61]. According to ATA guidelines, minimally invasive thyroid tumours are low-risk tumours with an expected recurrence rate of $<1\%$, if an excellent response to treatment is achieved [61]. Patients with ≥ 4 foci of vascular invasion tumours have decreased recurrence-free survival [42,43,54]. When evaluating capsular and vascular invasion presence alone, no significant changes in patient prognosis were found; however, combined capsular and vascular invasion showed a significant impact on patient prognosis [54].

Taken together, the available data indicate that the risk of distant metastases and disease-specific mortality in MI-FTC increases with progression from capsular invasion alone to limited vascular invasion (<4 blood vessels) and afterwards to extensively invasive vascular invasion (≥ 4 blood vessels) [60].

Clinical variables also impact on the prognosis of MI-FTC, most notably older age which increases the risk of recurrent disease [53], decreases cancer-specific and distant metastasis-free survival [54–56], and is associated with distant recurrence [53]. Gender is not associated with outcome [42,52]. A study including 251 patients reported age as the most powerful prognostic factor but any association with vascular invasion and distant metastases was not found [55].

In encapsulated oncocytic carcinomas [65], it is the extent of vascular invasion rather than the mere presence of vascular invasion that denotes an aggressive behaviour [43,64], while extensive capsular invasion, gender, or age was not associated with a higher recurrence rate [43].

Treatment

MI-FTC diagnosis is as a rule established through histopathological study of the hemithyroidectomy specimen. The question to be answered is whether full thyroidectomy and radioiodine therapy are necessary, or postoperative surveillance suffices. Completion thyroidectomy is not uniformly recommended [50,53,56], particularly for minimally invasive (capsular invasion only) and <50 mm encapsulated, limited vascular invasive tumours (<4 vessels) [60]. The European Society of Endocrine Surgeons (ESES) recommends [42]:

- Hemithyroidectomy for:
 - Younger than 45-year-old patients with minimally invasive (capsule invasion only)
 - Less than 40-mm tumours, without vascular invasion, lymph node metastases or distant metastases.
- Thyroidectomy for:
 - Older than or equal to 45-year-old patients with ≥ 40 -mm tumours with evidence of vascular invasion, lymph node metastases or distant metastases.
- Radioiodine therapy for:
 - Patients that underwent total thyroidectomy and whose tumours have extensive vascular invasion and/or lymph node metastases and/or distant metastases and/or recurrent disease [42,61].

Immunohistochemical and molecular markers in encapsulated neoplasms

We only briefly summarise the practical importance of molecular markers in low-risk malignant lesions (NIFTP, FT-UMP, and WDT-UMP) and minimally invasive carcinomas (**Figure 1.9**). All lesions with a *BRAF V600E* mutation, a *TERT* promoter mutation, or a mutation of *TP53* or *PIK3CA* should be considered as high risk [16,37–40]. Notably, morphologically low-risk lesions such as NIFTP, FT-UMP or WDT-UMP should not be classified as low-risk malignancy when any of these high-risk mutations are found (even though the clinical significance of these molecular findings remain to be clarified) [16].

In view of the importance of invasion in the pathobiology of low-risk thyroid neoplasms, much attention has been paid to expression of cell-adhesion (such as E-cadherin), cytoskeletal (vimentin) and extracellular matrix molecules (such as fibronectin, collagens and matrix metalloproteinases) or markers of epithelial-to-mesenchymal transition in the context of invasive capacity in thyroid tumours, by immunohistochemistry on human tissue specimens or in model systems. Most studies based upon human tissue specimens

What is the best way to diagnose parenchymatous/vascular invasion?	
Cytopathology	No
Histopathology	Yes
Conventional molecular pathology and high through put approaches (including next-generation sequencing)	No
Detection of biomarkers in the plasma/blood (DNA, circulating tumour cells, exosomes)	Maybe

Figure 1.9 Ways to diagnose parenchymatous/vascular invasion.

(focussing on MI-FTC, WI-FTC minimally invasive FTC, FVPTC or oncocytic carcinoma) have not reported robust findings regarding expression of such proteins as helpful to establish capsular invasion or lymphatic/venous invasion [50,54,67–69]. The same holds true for MI-FTC studies [51,69]. Further studies in this field are required to better understand the role played by tumour microenvironment, including extracellular matrix and epithelial-mesenchymal transition, in the behaviour of encapsulated thyroid neoplasms.

ENCAPSULATED DIFFERENTIATED THYROID CARCINOMAS WITH HIGH-GRADE FEATURES

Encapsulated follicular cell-derived thyroid tumours (FTC, encapsulated FVPTC, and oncocytic carcinoma) usually carry a good prognosis [66,67], but occasionally they present so-called high-grade features, such as tumour necrosis and/or high-mitotic rate [66]. These tumours have been clustered in the newly created category of high-grade differentiated thyroid carcinomas [16], even though this category also contains highly invasive neoplasms.

The clinical importance of this new category remains to be clarified. Thompson et al performed a study comprising 130 patients with MI-FTC and segregated those patients in two-grade groups according to the histologic features large vessel invasion, extension into parenchyma, and tumour necrosis [68]. Of the patients, 95 patients were classified as MI-FTC and the remaining 35 patients as “not low grade”. No significant differences in prognosis were found between the two groups. Also, Heffness et al found no significant differences in patient outcome based on the presence of necrosis or mitotic rate [52]. This was furthermore confirmed by Rivera et al [66] who reported that encapsulated, non-invasive follicular cell-derived thyroid tumours displaying high-grade features including extensive tumour necrosis had an indolent behaviour. In addition, tumours with high-grade features but lacking vascular invasion carried an excellent prognosis regardless of the presence of tumour necrosis. This suggests that high-grade features in a differentiated carcinoma, not accompanied by vascular invasion, are not predictive of aggressive tumour behaviour. However, this issue can only be resolved by long-term follow-up studies of such cases [66].

INFILTRATIVE TUMOURS (MAINLY CLASSIC PTC)

In case of a thyroid neoplasm with an infiltrative growth pattern, invasive carcinoma is the diagnosis and as a rule invasiveness is not graded unless extrathyroidal invasion and/or invasion of venous vessels is encountered. The use of the category of minimal invasion is restricted to well-circumscribed/encapsulated follicular patterned neoplasms (FTC and FVPTC and oncocytic carcinoma).

The clinical significance of lymphatic invasion in infiltrative forms of PTC is an important point since lymphatic invasion and even regional lymph node metastases do not convey meaningful prognostic information. The umbrella term prognosis encompasses local recurrence, regional recurrence, as well as survival. In classic PTC lymph node metastases do not necessarily predict poor survival. Even in the presence of regional lymph node metastases, classic PTC often follows a (very) benign course in terms of mortality. In such cases, any attempt to grade lymphatic invasion using minimal invasion as a distinct category would be futile as histological confirmation of lymphatic invasion, regardless of the number and size of the lymphatic vessels, would immediately classify the case as lymphatic invasive [70–73].

It is important to emphasise that lymphatic invasion is difficult to establish in most cases of infiltrative PTC, regardless of the type of growth pattern [classic PTC and (predominantly) FVPTC] [74–75], even when using D2-40 immunohistochemistry, and as a rule this will not be mentioned in the pathology report. In case of dissemination of neoplastic cells and/or psammoma bodies throughout the thyroid parenchyma,

lymphatic invasion might be mentioned in the report, even when not confirmed by D2-40 immunohistochemistry.

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Key points for clinical practice

Glossary

- *Invasion*: It is defined in the context of thyroid tumours by the infiltration by neoplastic cells of the surrounding (peritumoural) parenchyma, and/or lymphatic and blood vessels, and/or total penetration of the capsule whenever the tumour is encapsulated. The term “lymphovascular invasion”, tentatively separating lymphatic invasion from invasion of blood vessel, should be avoided.
- *Minimal invasion*: This term is used to define minimally invasive neoplasms. In the WHO classification of thyroid tumours (2017, 2023), the term is restricted to encapsulated, follicular patterned neoplasms demonstrating complete transcapsular penetration by the neoplastic cells without signs of vascular invasion. This includes minimally invasive follicular carcinoma, minimally invasive oncocytic carcinoma and minimally invasive follicular variant of papillary carcinoma.
- *Follicular adenoma*: Benign encapsulated, follicular/trabecular/solid neoplasm without nuclear features of papillary thyroid carcinoma and without signs of capsular and/or vascular invasion. This includes adenomas with papillary architecture which are often hyperfunctional (papillary adenoma, without nuclear features of papillary thyroid carcinoma) and oncocytic adenoma.
- *Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP)* encapsulated, follicular-patterned neoplasm with nuclear features of papillary thyroid carcinoma, without signs of capsular and/or vascular invasion.
- *Follicular tumour of uncertain malignant/potential (FT-UMP)* encapsulated, follicular-patterned neoplasm, with questionable signs of capsular and/or vascular invasion, without nuclear features of papillary thyroid carcinoma.
- *Well-differentiated tumour of uncertain malignant potential (WDT-UMP)* encapsulated, follicular-patterned neoplasm, with nuclear features of papillary thyroid carcinoma and questionable signs of capsular and/or vascular invasion.

What should not be called minimal invasion?

For the term minimal invasion to have full diagnostic and prognostic significance, its use should be strictly defined. The following situations clarify when the term should not be used.

- Incomplete transcapsular penetration and absence of signs of vascular invasion. This leads to a diagnosis of follicular adenoma, oncocytic adenoma or NIFTP (when the nuclei display PTC-like features).
- Questionable transcapsular penetration and/or questionable vascular invasion. This leads to a diagnosis of follicular tumour of uncertain malignant potential (FT-UMP) or, if the nuclei display PTC-like features, well-differentiated tumour of uncertain malignant potential (WDT-UMP).
- When invasion of capillaries and/or venous vessels by neoplastic cells is observed inside the tumour, or in a subcapsular location, this should not be considered minimal invasion, and the tumour under examination should be classified as benign.
- The presence of tumour plugs in subcapsular vessels does not count as vascular invasion. The putative biological meaning of this observation is unknown, but this histopathological feature does not carry clinical significance.
- When in an encapsulated, angioinvasive thyroid neoplasm less than four vessels are invaded, this is classified as limited vascular invasion, not as minimal vascular invasion.

- Regularly, several foci of extrathyroidal invasion by neoplastic cells may be found and these might show different degrees of invasion. In this context the least aggressive form is classified as microscopic extrathyroidal invasion, not as minimal invasion.
- Minimal invasion is not used for PTC with an infiltrative growth pattern, regardless of the tumour being a classic PTC or any subtype including follicular-patterned infiltrative PTC. This means that the term minimal invasion should not be used when dealing with a very small PTC (which was formerly classified as micro-PTC).

REFERENCES

1. Graham A. Malignant epithelial tumours of the thyroid with special reference to invasion of blood vessels. *Surg Gynecol Obstet* 1924; 39:781-790.
2. Graham A. Malignant tumours of the thyroid: Epithelial types. *Ann Surg* 1925; 82:30-44.
3. Collier FA. Adenoma and cancer of the thyroid: a study of their relation in ninety epithelial neoplasms of the thyroid. *J Am Med Assoc* 1929; 92:457-463.
4. Warren S. The significance of invasion of blood vessel in adenoma of the thyroid gland. *Arch Pathol* 1931; 11:255-7.
5. Goldstein NS, Czako P, Neill JS. Metastatic minimally invasive (encapsulated) follicular and Hurthle cell thyroid carcinoma: a study of 34 patients. *Modern Pathol* 2000; 13:123-130.
6. Hedinger C. Histological Classification of Thyroid Tumours. In: Hedinger C [Ed]. *Histological Typing of Thyroid Tumours*. Berlin, Heidelberg: Springer Berlin Heidelberg, 1988.
7. Williams ED. Guest Editorial: Two proposals regarding the terminology of thyroid tumors. *Int J Surg Pathol*. 2000; 8:181-183.
8. Nikiforov YE, Seethala RR, Tallini G, et al. Nomenclature revision for encapsulated follicular variant of papillary thyroid carcinoma: a paradigm shift to reduce overtreatment of indolent tumors. *JAMA Oncol* 2016; 2:1023-1029.
9. Lloyd R, Osamura R, Klöppel G, Rosai J. WHO classification of tumours of the thyroid gland. Lyon, France: International Agency for Research on Cancer (IARC), 2017.
10. Collini P, Sampietro G, Pilotti S. Extensive vascular invasion is a marker of risk of relapse in encapsulated non-Hürthle cell follicular carcinoma of the thyroid gland: a clinicopathological study of 18 consecutive cases from a single institution with a 11-year median follow-up. *Histopathology* 2004; 44:35-39.
11. Mete O, Asa SL. Pathological definition and clinical significance of vascular invasion in thyroid carcinomas of follicular epithelial derivation. *Modern Pathol* 2011; 24:1545-1552.
12. Cameselle-Teijeiro JM, Bella Cueto MR, Eloy C, et al. Tumores de la glándula tiroidea. Propuesta para el manejo y estudio de las muestras de pacientes con neoplasias tiroideas. *Rev Esp Patol*. 2020; 53:27-36.
13. Leboulleux S, Rubino C, Baudin E, et al. Prognostic factors for persistent or recurrent disease of papillary thyroid carcinoma with neck lymph node metastases and/or tumour extension beyond the thyroid capsule at initial diagnosis. *J Clin Endocrinol Metabol* 2005; 90:5723-5729.
14. Hay ID, Johnson TR, Kaggal S, et al. Papillary thyroid carcinoma (PTC) in children and adults: comparison of initial presentation and long-term postoperative outcome in 4432 patients consecutively treated at the Mayo Clinic during eight decades (1936-2015). *World J Surg* 2018; 42:329-342.
15. Sobrinho-Simoes M, Eloy C, Magalhaes J, Lobo C, Amaro T. Follicular thyroid carcinoma. *Modern Pathol* 2011; 24:S10-S18.
16. Baloch ZW, Asa SL, Barletta JA, et al. Overview of the 2022 who classification of thyroid neoplasms. *Endocrine Pathol* 2022; 33:27-63.
17. Aida N, Yamada N, Asano G, Tanaka S. 3-D analysis of vascular and capsular invasion in thyroid follicular carcinoma. *Pathol Int* 2001; 51:425-430.

18. LiVolsi VA, Merino MJ. Worrisome histologic alterations following fine-needle aspiration of the thyroid (WHAFET). *Pathol Annu* 1994; 29:99-120.
19. Rosai J, Kuhn E, Carcangiu M. Pitfalls in thyroid tumour pathology. *Histopathology* 2006; 49:107-120.
20. Cameselle-Teijeiro JM, Eloy C, Sobrinho-Simões M. Rare tumours of the thyroid gland: diagnosis and WHO classification. Berlin, Germany: Springer, 2018.
21. Tse L, Chan I, Chan J. Capsular intravascular endothelial hyperplasia: a peculiar form of vasoproliferative lesion associated with thyroid carcinoma. *Histopathology* 2001; 39:463-468.
22. Schmitz BA, Singh C, Gulbahce HE, Manivel JC, Pambuccian SE. Florid capsular and pericapsular papillary endothelial proliferation associated with poorly differentiated thyroid carcinoma. *Int J Surg Pathol* 2011; 19:110-112.
23. Sapino A, Papotti M, Macri L, Satolli MA, Bussolati G. Intranodular reactive endothelial hyperplasia in adenomatous goitre. *Histopathology* 1995; 26:457-462.
24. Papotti M, Arrondini M, Tavaglione V, Veltri A, Volante M. Diagnostic controversies in vascular proliferations of the thyroid gland. *Endocr Pathol* 2008; 19:175-183.
25. Lin X, Zhu B, Liu Y, Silverman JF. Follicular thyroid carcinoma invades venous rather than lymphatic vessels. *Diagn Pathol* 2010; 5:8.
26. Liu J, Tao L-L, Yu G-Y, et al. Diagnostic significance of CyclinD1 and D2-40 expression for follicular neoplasm of the thyroid. *Pathol Res Pract* 2022; 229:153739.
27. Ito Y, Hirokawa M, Hayashi T, et al. Clinical outcomes of follicular tumour of uncertain malignant potential of the thyroid: real-world data. *Endocr J* 2022; EJ21-0723.
28. Hysek M, Paulsson JO, Jatta K, et al. Clinical routine TERT promoter mutational screening of follicular thyroid tumours of uncertain malignant potential (FT-UMPs): a useful predictor of metastatic disease. *Cancers* 2019; 11:1443.
29. Parente DN, Kluijfhout WP, Bongers PJ, et al. Clinical safety of renaming encapsulated follicular variant of papillary thyroid carcinoma: is NIFTP truly benign? *World J Surg* 2018; 42:321-326.
30. Paja M, Zafón C, Iglesias C, Ugalde A, et al. Rate of non-invasive follicular thyroid neoplasms with papillary-like nuclear features depends on pathologist's criteria: A multicentre retrospective Southern European study with prolonged follow-up. *Endocrine* 2021; 73:131-140.
31. Sowder AM, Witt BL, Hunt JP. An update on the risk of lymph node metastasis for the follicular variant of papillary thyroid carcinoma with the new diagnostic paradigm. *Head Neck Pathol* 2018; 12:105-109.
32. de Jesus Paniza AC, Mendes TB, Viana MDB, et al. Revised criteria for diagnosis of NIFTP reveals a better correlation with tumour biological behavior. *Endocr Connect* 2019; 8:1529-1538.
33. Cho U, Mete O, Kim MH, Bae JS, Jung CK. Molecular correlates and rate of lymph node metastasis of non-invasive follicular thyroid neoplasm with papillary-like nuclear features and invasive follicular variant papillary thyroid carcinoma: the impact of rigid criteria to distinguish non-invasive follicular thyroid neoplasm with papillary-like nuclear features. *Modern Pathol* 2017; 30:810-825.
34. Rosario PW, Mourão GF, Nunes MB, Nunes MS, Calsolari MR. Noninvasive follicular thyroid neoplasm with papillary-like nuclear features. *Endocrine-Related Cancer* 2016; 23:893-897.
35. Kim MJ, Won JK, Jung KC, et al. Clinical characteristics of subtypes of follicular variant papillary thyroid carcinoma. *Thyroid* 2018; 28:311-318.
36. Zajkowska K, Kopczyński J, Gózdź S, Kowalska A. Noninvasive follicular thyroid neoplasm with papillary-like nuclear features: a problematic entity. *Endocr Connect* 2020; 9:R47-R58.
37. Póvoa AA, Teixeira E, Bella-Cueto MR, et al. Genetic determinants for prediction of outcome of patients with papillary thyroid carcinoma. *Cancers* 2021; 13:2048.
38. Penna GC, Vaisman F, Vaisman M, Sobrinho-Simões M, Soares P. Molecular Markers Involved in Tumorigenesis of Thyroid Carcinoma: Focus on Aggressive Histotypes. *Cytogenetic Genome Res* 2016; 150:194-207.
39. García-Rostán G, Costa AM, Pereira-Castro I, et al. Mutation of the PIK3CA gene in anaplastic thyroid cancer. *Cancer Res* 2005; 65:10199-207.
40. Nishida T, Nakao K, Hamaji M, Nakahara M-A, Tsujimoto M. Overexpression of p53 protein and DNA content are important biologic prognostic factors for thyroid cancer. *Surgery* 1996; 119:568-575.
41. Kakudo K, Bychkov A, Bai Y, et al. The new 4th edition World Health Organization classification for thyroid tumours, Asian perspectives. United States: Wiley Online Library; 2018.

42. Dionigi G, Kraimps JL, Schmid KW, et al. Minimally invasive follicular thyroid cancer (MIFTC)—a consensus report of the European Society of Endocrine Surgeons (ESES). *Langenbeck's Arch Surg* 2014; 399:165-184.
43. Ghossein RA, Hiltzik DH, Carlson DL, et al. Prognostic factors of recurrence in encapsulated Hurthle cell carcinoma of the thyroid gland. *Cancer* 2006; 106:1669-1676.
44. Xu B, Teplov A, Ibrahim K, et al. Detection and assessment of capsular invasion, vascular invasion and lymph node metastasis volume in thyroid carcinoma using microCT scanning of paraffin tissue blocks (3D whole block imaging): a proof of concept. *Modern Pathol* 2020; 33:2449-2457.
45. Poma AM, Giannini R, Piaggi P, et al. A six-gene panel to label follicular adenoma, low- and high-risk follicular thyroid carcinoma. *Endocr Connect* 2018; 7:124-132.
46. Lee Y-M, Park JH. A Case of Minimally Invasive Follicular Thyroid Carcinoma Relapsed as a Large Cervical Lymphadenopathy and Multiple Lung Metastases. *J Endocr Surg* 2020; 20:21-25.
47. Fozzatti L, Cheng SY. Tumour Cells and Cancer-Associated Fibroblasts: A Synergistic Crosstalk to Promote Thyroid Cancer. *Endocrinol Metab (Seoul)* 2020; 35:673-680.
48. Khoo ACH, Chen SL. A rare case of minimally invasive follicular thyroid cancer with intraluminal superior vena cava and right atrium metastases. *World J Nucl Med* 2019; 18:301-303.
49. Gao H, Wu S, Zhang X, Xie T. Minimally invasive follicular thyroid carcinoma mimicking pituitary adenoma: a case report. *Int J Clin Experiment Pathol* 2019; 12:3949.
50. Robinson A, Schneider D, Sippel R, Chen H. Minimally invasive follicular thyroid cancer: treat as a benign or malignant lesion? *J Surg Res* 2017; 207:235-240.
51. Lee Y-M, Sung T-Y, Yoon JH, Chung K-W, Hong SJ. Long term outcome of minimally invasive follicular thyroid carcinoma according to risk group stratification. *J Endocr Surg* 2018; 18:183-190.
52. Heffess CS, Thompson LD. Minimally invasive follicular thyroid carcinoma. *Endocr Pathol* 2001; 12:417-422.
53. Ito Y, Onoda N, Okamoto T. The revised clinical practice guidelines on the management of thyroid tumours by the Japan Associations of Endocrine Surgeons: Core questions and recommendations for treatments of thyroid cancer. *Endocr J* 2020; 67:669-717.
54. Stenson G, Nilsson I-L, Mu N, et al. Minimally invasive follicular thyroid carcinomas: prognostic factors. *Endocrine* 2016; 53:505-511.
55. Sugino K, Kameyama K, Ito K, et al. Outcomes and Prognostic Factors of 251 Patients with Minimally Invasive Follicular Thyroid Carcinoma. *Thyroid* 2012; 22:798-804.
56. Yamazaki H, Sugino K, Katoh R, et al. Outcomes for Minimally Invasive Follicular Thyroid Carcinoma in Relation to the Change in Age Stratification in the AJCC 8th Edition. *Ann Surg Oncol* 2021; 28:3576-3583.
57. Podda M, Saba A, Porru F, Reccia I, Pisanu A. Follicular thyroid carcinoma: differences in clinical relevance between minimally invasive and widely invasive tumours. *World J Surg Oncol* 2015; 13:193.
58. Ito Y, Hirokawa M, Masuoka H, et al. Prognostic factors of minimally invasive follicular thyroid carcinoma: Extensive vascular invasion significantly affects patient prognosis. *Endocr J* 2013; 60:637-642.
59. Ban EJ, Andrabi A, Grodski S, et al. Follicular thyroid cancer: minimally invasive tumours can give rise to metastases. *ANZ J Surg* 2012; 82:136-139.
60. Daniels GH. Follicular thyroid carcinoma: a perspective. *Thyroid* 2018; 28:1229-1242.
61. Haugen BR, Alexander EK, Bible KC, et al. 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer. *Thyroid* 2016; 26:1-133.
62. D'Avanzo A, Treseler P, Ituarte PH, et al. Follicular thyroid carcinoma: histology and prognosis. *Cancer* 2004; 100:1123-1129.

63. O'Neill CJ, Vaughan L, Learoyd DL, et al. Management of follicular thyroid carcinoma should be individualised based on degree of capsular and vascular invasion. *Eur J Surg Oncol* 2011; 37:181-185.
64. Xu B, Wang L, Tuttle RM, Ganly I, Ghossein R. Prognostic impact of extent of vascular invasion in low-grade encapsulated follicular cell-derived thyroid carcinomas: a clinicopathologic study of 276 cases. *Hum Pathol* 2015; 46:1789-1798.
65. Xu B, Ghossein R. Evolution of the histologic classification of thyroid neoplasms and its impact on clinical management. *Eur J Surg Oncol* 2018; 44:338-347.
66. Rivera M, Ricarte-Filho J, Patel S, et al. Encapsulated thyroid tumours of follicular cell origin with high grade features (high mitotic rate/tumour necrosis): a clinicopathologic and molecular study. *Hum Pathol* 2010; 41:172-180.
67. Liu J, Singh B, Tallini G, et al. Follicular variant of papillary thyroid carcinoma. *Cancer* 2006; 107:1255-1264.
68. Thompson LD, Wieneke JA, Paal E, et al. A clinicopathologic study of minimally invasive follicular carcinoma of the thyroid gland with a review of the English literature. *Cancer* 2001; 91:505-524.
69. Chow TL, Tam SW, Choi CY, Kwan WW. Hemithyroidectomy for low-risk follicular carcinoma of the thyroid: results from a regional hospital. *Singapore Med J* 2018; 59:311-315.
70. Hedinger C, Sobin LH. Histological typing of thyroid tumours—International Histological Classification of Tumours. Geneva, Switzerland: World Health Organization, 1974.
71. Chen K, Rosai J. Follicular variant of thyroid papillary carcinoma: a clinicopathologic study of six cases. *Am J Surg Pathol* 1977; 1:123-130.
72. Thompson NW, Brown J, Orringer M, Sisson J, Nishiyama R. Follicular carcinoma of the thyroid with massive angioinvasion: extension of tumour thrombus to the heart. *Surgery* 1978; 83:451-457.
73. DeLellis RA. Pathology and genetics of tumours of endocrine organs. France: IARC, 2004.
74. The Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. *Cell* 2014; 159:676-690.
75. Tallini G, Tuttle RM, Ghossein RA. The history of the follicular variant of papillary thyroid carcinoma. *J Clin Endocrinol Metabol* 2017; 102:15-22.

