

MESTRADO
MEDICINA E ONCOLOGIA MOLECULAR

Evaluation of the expression of ACE-2, TMPRSS2, and furin in endocrine lesions

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M

2022



Dissertação de candidatura ao grau de Mestre em Medicina e Oncologia Molecular,
apresentada à Faculdade de Medicina da Universidade do Porto

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"The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day."

Albert Einstein

Agradecimentos

Depois desta etapa, existem várias pessoas a quem muito tenho a agradecer.

Começo pela minha Orientadora, Professora Doutora Paula Soares que permitiu que esta aventura se iniciasse. Obrigado por toda a sua orientação e dedicação, mas sobretudo pela oportunidade que me deu de aprender e crescer na Ciência.

À Sofia, ao Tiago, à Elisa e à Carolina, obrigada por estarem ao meu lado desde o início desta caminhada, tenho-vos a agradecer do fundo do coração por toda a vossa orientação, paciência, persistência e dedicação. Obrigada por me fazem ver o lado bom das coisas e por me fazerem rir mesmo depois de eu ter acabado de chorar. Obrigado por me fazerem acreditar que tudo é possível.

À Bárbara e à Luísa, as minhas companheiras de mestrado, não podia pedir melhores pessoas para partilhar esta etapa, obrigado por todo o vosso apoio.

Um obrigada gigantesco à minha família, obrigada por acreditarem em mim e nunca me deixarem desistir, obrigado por serem o meu suporte nos bons e maus momentos. Gosto muito de vocês!

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Abbreviations

A

aa	Amino acids
ACE	Angiotensin converting enzyme
ACE-2	Angiotensin converting enzyme 2
ACS	American Cancer Society
ADAM 10	Disintegrin and metalloproteinase domain-containing protein 10
ADAM 17	Disintegrin and metalloproteinase domain-containing protein 17
AHS	Asparagine-Histidine-Serine
AITD	Autoimmune thyroid disorders
AJCC	American Joint Committee on Cancer
Ang 1-7	Angiotensin 1-7
Ang 1-9	Angiotensin 1-9
Ang I	Angiotensin I
Ang II	Angiotensin II
AT	Adjacent thyroid
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
ATA	American Thyroid Association
ATC	Anaplastic thyroid carcinoma

B

BL	Benign lesions
----	----------------

C

C cell	Parafollicular cell
cAMP	Cyclic adenosine monophosphate
CCVPTC	Clear cell variant of papillary thyroid carcinoma
cDNA	Complementary Deoxyribonucleic acid
COVID-19	Coronavirus disease 2019
cPTC	Classical papillary thyroid carcinoma
Ct	Threshold cycle

D

dNTP	Deoxynucleoside triphosphate
DSVPTC	Diffuse sclerosing variant of papillary thyroid carcinoma

E

EDC	Endocrine disrupting chemical
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ETE	Extra thyroidal extension

F

FC	Follicular Carcinoma
FFPE	Formalin-fixed paraffin-embedded
F-PanNEN	Functioning pancreatic neuroendocrine neoplasm
FTA	Follicular thyroid adenomas
FTC	Follicular thyroid carcinoma
FVPTC	Follicular variant papillary thyroid carcinoma

G

G	Goiter
GD	Grave's disease
Globocan	Global Cancer Observatory
GPCR	G-protein coupled receptor
GTEX	The Genotype-Tissue Expression project

H

HDS	Histidine-Aspartate-Serine
H&E	Hematoxylin and eosin staining
HEXXH	Histidina- Glutamina-X-X-Histidina
HPA	The Human Protein Atlas
HPF	High power fields
HPT	Hypothalamic-pituitary-thyroid axis
HT	Hashimoto's thyroiditis

HVPTC Hobnail variant of papillary thyroid carcinoma

I

IARC International Agency for Research on Cancer

IFN- γ Interferon gamma

IHC Immunohistochemistry

iNOS Inducible nitric oxide synthase

IPATIMUP Instituto de Patologia e Imunologia Molecular da Universidade do Porto

IQR Interquartile range

IRS Immune reactive score

L

LDLRA Low density lipoprotein receptor class A

M

mACE-2 Membranar angiotensin converting enzyme 2

MasR Mitochondrial assembly receptor

Max Maximum

MEN1 Multiple endocrine neoplasia type1

MEN2 Multiple endocrine neoplasia type 2

Min Minimum

mRNA Messenger ribonucleic acid

MTC Medullary thyroid carcinoma

N

NF- PanNEN Non-functioning pancreatic neuroendocrine neoplasm

NF1 Neurofibromatosis type 1

NIFTP Non-invasive follicular thyroid neoplasm with papillary-like nuclear features

NO Nitric oxide

NTC Non template control

O

OTA Oncocytic thyroid adenoma

OTC	Oncocytic thyroid carcinoma
OVPTC	Oncocytic variant of papillary thyroid carcinoma

P

PanNEC	Pancreatic neuroendocrine carcinoma
PanNEN	Pancreatic neuroendocrine neoplasm
PanNET	Pancreatic neuroendocrine tumor
PanNET G	PanNET histological grade
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline - Tween buffer
PC	Papillary carcinoma
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PDTC	Poorly differentiated thyroid carcinomas
PSCSK	Subtilisin/kexin like proprotein convertase
PTC	Papillary thyroid carcinoma
PTMC	Papillary thyroid microcarcinoma

R

RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerase chain reaction

S

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SEER	Surveillance, Epidemiology and End Results Program
SP	Signal peptide
SRCR	Scavenger receptor cysteine rich
RT	Room temperature
SVPTC	Solid variant of papillary thyroid carcinoma

T

T1D	Type 1 diabetes
T2D	Type 2 diabetes

T3	Triiodothyronine
T4	Thyroxine
TBP	TATA-box binding protein
TC	Thyroid carcinoma
TCVPTC	Tall cell variant of papillary thyroid carcinoma
TGN	Trans-Golgi network
TMD	Transmembrane domain
TMPRSS2	Transmembrane serine protease 2
TNF- α	Tumor necrosis factor alpha
TRE	Thyroid hormone response element
TRH	Thyrotropin-releasing hormone
TSC	Tuberous sclerosis complex
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
TTSP	Type II transmembrane serine protease

V

VEGFa	Vascular endothelial growth factor A
VEGFR2	Vascular endothelial growth factor receptor 2
VHL	Von Hippel Lindau
VIP	Vasoactive intestinal peptide

W

WDTC	Well differentiated thyroid carcinoma
WDT-UMP	Well differentiated thyroid tumor of uncertain malignant potential
WHO	World Health Organization

Abstract

Angiotensin converting enzyme 2 (ACE-2), Transmembrane serine protease 2 (TMPRSS2) and furin are described as key players in the mechanism of entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into host cells.

Several cases of thyroid dysfunction, such as thyrotoxicosis and subacute thyroiditis, as well as new onset type 2 diabetes (T2D), have been reported in patients after SARS-CoV-2 infection. However, the expression of these molecules in thyroid lesions and in pancreatic neuroendocrine neoplasms (PanNENs) remains to be established.

The main aim of our project was to characterize the expression pattern of ACE-2, TMPRSS2 and furin in a series of adjacent thyroid tissues, benign and malignant thyroid neoplasms. Additionally, we aimed to characterize the expression pattern of these proteins in a series of PanNENs.

In the thyroid series, we quantified the mRNA expression of these molecules by real-time quantitative PCR (RT-qPCR) in 181 samples: 44 classical papillary thyroid carcinomas (cPTCs), 13 follicular variants of papillary thyroid carcinomas (FVPTCs), 10 follicular thyroid carcinomas (FTCs), 2 oncocytic thyroid carcinomas (OTC), 74 follicular adenomas (FTAs), 2 goiters and 36 adjacent tissues. Next, all the available correspondent formalin-fixed paraffin-embedded (FFPE) tissue samples were included (n=68) to evaluate protein expression and subcellular localization through immunohistochemistry (IHC). FFPE tissues comprehended 26 cPTCs, 11 FVPTCs, 4 FTCs, 2 OTCs, 17 FTAs, 4 goiters and 4 adjacent thyroid tissues. In FFPE slides presenting representative normal thyroid adjacent tissue to the lesion (n=50), the expression was also recorded.

At the mRNA level, the highest ACE-2 expression was observed in the adjacent thyroid tissues; all thyroid lesions (goiters, adenomas, and carcinomas) showed significantly reduced ACE-2 mRNA expression compared to adjacent thyroid tissues. Regarding the TMPRSS2, no statistically significant differences were observed between the mRNA expression of adjacent tissues and thyroid lesions. For furin, a significant increase in mRNA levels was observed in adenomas and carcinomas compared to adjacent thyroid tissues. Significant differences were also observed in relation to FTCs, which showed a lower furin expression compared to adenomas, cPTCs, and FVPTCs. Additionally, goiters presented a significant decrease in furin mRNA expression compared to adenomas.

Regarding the protein expression, the staining pattern observed for ACE-2 was membranous with scarce extension. Positivity was limited to cells in small

vessels. ACE-2 protein expression in adjacent thyroid tissues and papillary carcinomas (i.e., cPTCs and FVPTCs) was significantly reduced compared to benign lesions (i.e., goiters and follicular adenomas). In contrast to ACE-2 protein expression observed in cPTCs, FVPTCs and FTCs presented a significant increase.

The staining pattern in follicular cells for TMPRSS2 and furin was mostly observed in the cytoplasm and occasionally in nuclei. Cytoplasmic and nuclear immune expression of TMPRSS2 and furin were evaluated separately. Concerning the cytoplasmic expression of TMPRSS2, malignant lesions showed a significant decrease in TMPRSS2 expression when compared to the adjacent tissues. A significant decrease was also observed between FVPTCs, and FTCs compared to adjacent thyroid tissues expression. In addition, a significant difference was observed between benign lesions and follicular carcinomas (i.e. FTCs and OTCs). No statistical differences were found in cytoplasmic furin expression. Regarding nuclear staining, no relevant differences were found for TMPRSS2; as for furin, both adjacent thyroid tissues and thyroid carcinomas presented a significant decrease expression in comparison to adenomas. Significant differences were also verified between adenomas and cPTCs.

In addition, some associations between the clinicopathological characteristics of patients were observed: smaller tumors and the presence of lymphocytic infiltration in benign lesions were associated with higher ACE-2 mRNA expression; older patients presented higher TMPRSS2 mRNA levels and male patients with papillary carcinoma were associated with a higher furin mRNA expression. Regarding the protein expression, the absence of lymphocytic infiltrate and presence of capsule, in papillary tumors, presented an association with a higher ACE-2 expression.

No statistically significant correlations were found in the correlation analysis between the IHC score and the mRNA expression of the three transcripts.

Concerning the PanNENs series, IHC for ACE-2 was performed in 104 cases: 74 PanNETs G1, 25 PanNETs G2, 3 PanNETs G3, and 2 PanNECs G3. The ACE-2 immunoprofile observed in these tumors was similar to that observed in thyroid (staining of cells in small vessels). However, additional studies are needed to better understand the importance of this protein in this type of tumors.

Our study shows that the expression of ACE-2, TMPRSS2 and furin, both at mRNA and protein levels, are altered in neoplastic thyroid lesions. Membranar ACE-2 seems to be upregulated in benign lesions, while TMPRSS2 cytoplasmic expression was downregulated in thyroid carcinomas, which indicates that

expression of such proteins might have a putative role in the tumorigenic process in the thyroid gland.

Resumo

A enzima conversora de angiotensina 2 (ACE-2), a serina protéase transmembranar 2 (TMPRSS2) e a furina estão descritos como intervenientes chave para o mecanismo de entrada do coronavírus da síndrome respiratória aguda grave 2 (SARS-CoV-2) nas células do hospedeiro.

Vários casos de disfunção tiroideia, como tirotoxicose e tiroidite subaguda, assim como novos casos de diabetes tipo 2 (T2D) têm sido reportados em doentes após a infeção por SARS-CoV-2. Contudo a expressão destas moléculas em lesões da tiroide e em neoplasmas neuroendócrinos do pâncreas (PanNENs) permanece ainda por estabelecer.

O principal objetivo do nosso projeto foi caracterizar o padrão de expressão da ACE-2, TMPRSS2 e furina numa série de tecidos de tiroide adjacentes, neoplasias benignas e malignas da tiroide. Adicionalmente, visamos caracterizar a padrão de expressão destas proteínas numa série de PanNENs.

Na série da tiroide, quantificamos a expressão de mRNA destas moléculas por PCR em tempo real (RT-qPCR) em 181 amostras: 44 carcinomas papilares da tiroide clássicos (cPTCs), 13 variantes foliculares de carcinoma papilar da tiroide (FVPTCs), 10 carcinomas foliculares da tiroide (FTCs), 2 carcinomas oncocíticos da tiroide (OTCs), 74 adenomas foliculares (FTAs), 2 bócios e 36 tecidos adjacentes. Em seguida, todas as amostras de tecido correspondentes, fixadas em formol e embebidas em parafina (FFPE) foram incluídas (n=68) para avaliar a expressão proteica e localização subcelular através de imuno-histoquímica (IHQ). Os tecidos FFPE incluíram 26 cPTCs, 11 FVPTCs, 4 FTCs, 2 OTCs, 17 FTAs, 4 bócios e 4 amostras de tiroide adjacente. Em lâminas de FFPE que apresentavam representatividade de tiroide normal adjacente à lesão (n=50), a expressão também foi registada.

A nível de mRNA, observamos que a maior expressão de ACE-2 foi no tecido tiroideu adjacente; todas as lesões tiroideias (bócio, adenomas e carcinomas) mostraram uma redução significativa de expressão de mRNA de ACE-2 comparadas com os tecidos tiroideus adjacentes. Relativamente ao TMPRSS2, não foram encontradas diferenças significativas entre a expressão de mRNA em tecidos adjacentes e lesões tiroideias. Para a furina foi observado um aumento significativo nos níveis de mRNA de adenomas e carcinomas comparados com o tecido adjacente. Diferenças significativas foram também observadas em relação a FTCs, que mostraram uma menor expressão de furina comparativamente com adenomas,

cPTCs e FVPTCs. Adicionalmente, os bócios apresentaram um decréscimo significativo na expressão de mRNA da furina comparativamente com os adenomas.

Relativamente à expressão proteica, o padrão de expressão observado para o ACE-2 foi membranar com uma extensão escassa. Positividade estava limitada a células de pequenos vasos. A expressão proteica de ACE-2 nos tecidos adjacentes e carcinomas papilares (isto é, cPTCs e FVPTCs) estava significativamente reduzida comparativamente com as lesões benignas (isto é, bócio e adenomas foliculares). Em contraste a expressão proteica de ACE-2 observada em cPTCs, FVPTCs e FTCs apresentam um aumento significativo.

O padrão de marcação nas células foliculares para TMPRSS2 e furina foi maioritariamente observado no citoplasma e ocasionalmente no núcleo. A expressão imune citoplasmática e nuclear foi avaliada separadamente. Relativamente à expressão citoplasmática de TMPRSS2, as lesões malignas mostraram um decréscimo significativo na expressão de TMPRSS2 quando comparadas com os tecidos adjacentes. Uma diminuição significativa foi também observada entre FVPTCs e FTCs comparativamente com a expressão dos tecidos adjacentes. Adicionalmente, diferenças significativas foram observadas entre lesões benignas e carcinomas foliculares (isto é, FTCs e OTCs). Não foram encontradas diferenças estatísticas na expressão citoplasmática da furina. Relativamente à marcação nuclear, não foram encontradas diferenças relevantes para o TMPRSS2; enquanto para a furina, tanto os tecidos tiroideus adjacentes como os carcinomas da tiroide apresentaram decréscimo significativo de expressão em comparação com os adenomas. Diferenças significativas foram também verificadas entre adenomas e cPTCs.

Adicionalmente, algumas associações com as características clinicopatológicas foram observadas: tumores mais pequenos e presença de infiltrado linfocítico em lesões benignas estavam associadas a uma maior expressão de mRNA de ACE-2; pacientes mais velhos apresentaram maiores níveis de mRNA de TMPRSS2 e pacientes do sexo masculino com carcinoma papilar da tiroide estavam associados a uma maior expressão de mRNA de furina. Relativamente à expressão proteica, ausência de infiltrado linfocítico e presença de cápsula em tumores papilares, apresentou uma associação com uma maior expressão de ACE-2.

Não foram encontradas diferenças significativas na análise de correlação entre os scores e expressão de mRNA dos três transcritos.

Na série de PanNENs, foi realizada IHC para a ACE-2 em 104 casos: 74 PanNETs G1, 25 PanNETs G2, 3 PanNETs G3, e 2 PanNECs G3. O padrão de
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expressão de ACE-2 observado nestes tumores foi similar ao observado em tiroide (positividade em células de pequenos vasos). Contudo, estudos adicionais são necessários para melhor perceber a importância desta proteína nestes tumores.

O nosso estudo mostrou que a expressão de ACE-2, TMPRSS2 e furina, tanto a nível de mRNA como de proteína, estão alteradas em lesões neoplásicas da tiroide. A expressão membranar de ACE-2 parece estar aumentada em lesões benignas, ao passo que a expressão citoplasmática de TMPRSS2 parece estar reduzida em carcinomas da tiroide, o que indica que a expressão de tais proteínas poderá ter potencial efeito no processo tumorigénico na glândula da tiroide.

1. Introduction

1.1. Thyroid gland

1.1.1. Function and physiology

Thyroid gland is a highly vascularized butterfly-shaped endocrine organ located in the lower, anterior portion of the neck and composed by two lobes (right and left) connected by a structure called isthmus (**Figure 1**) (Armstrong et al., 2022; Khan et al., 2020).

The lobes are essentially composed of follicles, which are the structural and functional units of this gland. Follicles are spherical structures delimited by follicular cells and their interior is made up of a gelatinous substance designated as colloid (Khan et al., 2020). This structure is responsible for the production and storage of thyroid hormones, triiodothyronine (T3) and thyroxine (T4) (Khan et al., 2020). Surrounding the thyroid follicles, there is connective tissue, stroma, lymphatic, and blood vessels as well as the second population of hormone-producing cells, the C cells (Armstrong et al., 2022; Khan et al., 2020; Nilsson & Fagman, 2017). C cells, also designated parafollicular cells, are responsible for the production of calcitonin (Khan & Farhana, 2022), which is a hypocalcemic hormone involved in the maintenance of calcium homeostasis (Armstrong et al., 2022; Khan et al., 2020; Nilsson & Fagman, 2017).

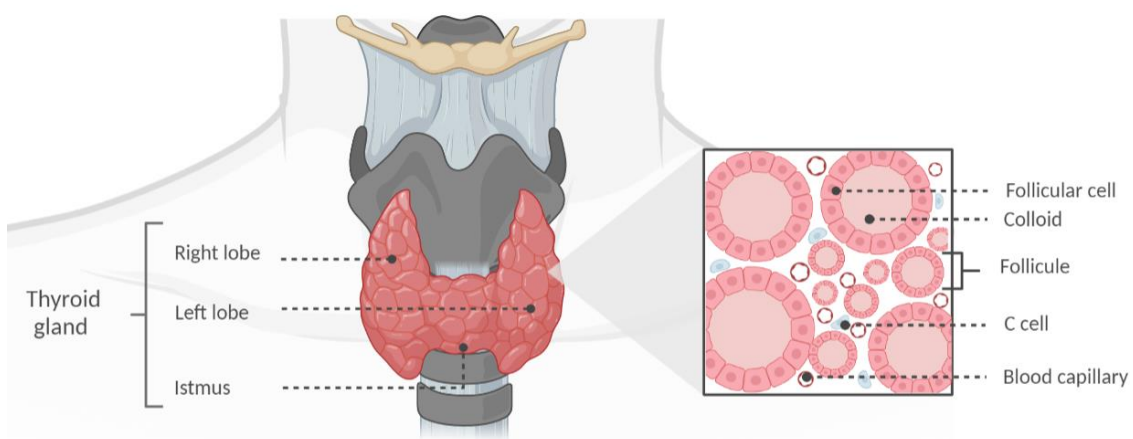


Figure 1 – Schematic representation of thyroid gland physiology

Thyroid gland is an highly vascularized organ located in the inferior portion of the neck, it essentially subdivided in lobes (right and left) connected by the isthmus. The structural and functional units of the gland are the follicles delimited by follicular cells and their interior is filled up with colloid. Thyroid also

has a second population of hormone-producing cells: parafollicular cells or c cells. Image created with BioRender.com.

T3 and T4 hormones are capable of inducing effects on virtually every nucleated cell in the human body, playing important roles in fetal growth and development, regulation of basal metabolic rate, thermogenesis, resting respiratory rate, nervous system and can also exert an important role in the regulation of other endocrine organ's function (Armstrong et al., 2022; Nilsson & Fagman, 2017).

The vast network of capillaries surrounding each one of the follicles ensures that there is a systemic delivery of the produced hormones to the target tissues (Khan et al., 2020; Nilsson & Fagman, 2017).

The overall maintenance of normal thyroid function depends on a negative feedback control system over the hypothalamic-pituitary-thyroid axis (HPT axis) which is an endocrine loop between circulating levels of thyroid hormones, the hypothalamus and the pituitary gland. Therefore, low circulating concentrations of thyroid hormones (T4 and T3) stimulate the biosynthesis and secretion of thyrotropin-releasing hormone (TRH) from the hypothalamus which in turn will promote the production of the thyroid stimulating hormone (TSH) by the anterior pituitary gland that releases TSH into circulation (Feldt-Rasmussen et al., 2021; Gavrilă & Hollenberg, 2019). TSH then binds to its receptor, thyroid stimulating hormone receptor (TSHR), on the thyroid follicular epithelium, which leads to the activation of G proteins, stimulating a series of chain reactions resulting in increased levels of cyclic adenosine monophosphate (cAMP) inducing iodine uptake, cellular differentiation, and subsequent release of thyroid hormones into the systemic circulation (Kumar et al., 2015; Pirahanchi et al., 2022; Porcellini et al., 2003). These hormones bind reversibly to circulating plasma proteins, which have a buffering effect, ensuring the concentration of these hormones within narrow limits and at the same time allowing the hormones to be readily available to the tissues (Fekete & Lechan, 2014).

In peripheral organs, the T4 hormone is then deionized into T3 (active form), and the latter is able to bind to the thyroid hormone nuclear receptors, which leads to the assembly of a multiprotein hormone complex on thyroid hormone response elements (TREs) in target genes, thereby increasing their transcription (Feldt-Rasmussen et al., 2021; Kumar et al., 2015).

In the presence of high levels of these hormones (T3/T4), the negative feedback mechanism is activated, promoting a decrease in TSH secretion and therefore in T3/T4 release (Fekete & Lechan, 2014; Pirahanchi et al., 2022).

Thyroid illnesses are mainly characterized by abnormalities in the organ's function (abnormal hormone production) or morphology (goiter and neoplastic lesions) (Melish, 1990).

Hyperthyroidism is a high hypermetabolic state caused by increased thyroid hormone synthesis and secretion and/or elevated circulating levels of T3 and T4 (thyrotoxicosis) (De Leo et al., 2016; Kumar et al., 2015).

On the other hand, hypothyroidism refers to common conditions caused by structural or functional disorders that interfere with hormone production resulting in thyroid hormone deficiency (Chaker et al., 2017; Kumar et al., 2015).

These disorders are frequently associated with a pathological activation of the immune system against the thyroid. Autoimmune thyroid disorders (AITD) comprise both Grave's disease (GD) and Hashimoto's thyroiditis (HT), which share as common feature lymphocytic infiltration of thyroid parenchyma. The clinical hallmarks that represent GD and HT are respectively, thyrotoxicosis and hypothyroidism (Antonelli et al., 2015; Ferrari et al., 2020).

Goiter is a general term used to describe the enlargement of the thyroid and can be caused by impaired synthesis of thyroid hormone, which is more often the result of dietary iodine deficiency (Kumar et al., 2015). This impairment leads to a compensatory rise in the serum TSH level, which in turn causes hypertrophy and hyperplasia of thyroid follicular cells and, ultimately gross enlargement of the thyroid gland. Goiter can be associated with euthyroidism, hypothyroidism, or hyperthyroidism (Can & Rehman, 2022; Kumar et al., 2015). According to the 2022 World Health Organization (WHO) classification overview of thyroid neoplasms, the best term achieved to designate this entity was thyroid follicular nodular disease (Baloch et al., 2022).

1.2. Thyroid tumors

1.2.1. Epidemiology, etiology, and prognostic factors

Thyroid cancer is the most frequent neoplasm found in the endocrine system (Nguyen et al., 2015; Raue & Frank-Raue, 2016). This malignancy is characterized by having a wide variety of histotypes, with a very heterogeneous frequency among them (Pizzato et al., 2022). Tumors can range from indolent forms, which are the

most common, to highly aggressive forms (Lee et al., 2022). This malignancy shows a female predominance at a ratio of 3:1 (Pizzato et al., 2022; Rahbari et al., 2010).

The 2020 global analysis conducted by the International Agency for Research on Cancer (IARC), shows that the incidence of thyroid cancer has increased over the years, and in contrast, mortality rates appear to have remained stable or even decreased. In this analysis, a large geographical variability in the distribution of thyroid cancer incidence was observed. These data showed that incidence was five times higher in more developed countries (Pizzato et al., 2022). By the year 2030 it is expected a continued increase in the incidence rate and a stable mortality rate, in both sexes (Rahib et al., 2014).

In Portugal, according to Global Cancer Observatory (Globocan) 2020, thyroid cancer ranked 10th in the incidence of new cases for both sexes and was the 4th most frequent in women, while mortality remained low, occupying the 25th position in the mortality ranking (Globocan, 2020).

Several investigators state that the current epidemiological panorama of thyroid cancer is strongly suggestive of an overdiagnosis effect due to increased access to diagnostic imaging methods and fine-needle aspiration biopsies. However, exposure to certain environmental factors cannot be discarded (Kitahara & Sosa, 2020; Pizzato et al., 2022; Raposo et al., 2017).

Due to the high dependence of the thyroid to obtain iodine from the environment, it is more susceptible to genotoxic effects, such as exposure to iodine ionizing radiation, resulting in DNA damage (Calcaterra et al., 2022). Conversely, due to exposure to an iodine deficient environment and consequent TSH stimulation, progressive thyroid follicular cell proliferation can occur, ultimately leading to carcinogenesis (Gharib, 2018).

However, several other risk factors to thyroid carcinoma have been identified such as family history of thyroid cancer, gender, obesity and alcohol consumption (Seib & Sosa, 2019). More recently, environmental exposure to various endocrine disrupting chemicals (EDCs), such as flame retardants, polychlorinated biphenyls (PCBs), phthalates and certain pesticides has been pointed to be potentially associated with the increase in the risk of thyroid cancer (Alsen et al., 2021; Seib & Sosa, 2019).

The great majority of thyroid cancer patients has an excellent prognosis with 5-year relative survival rates above 90% according to American Cancer Society (ACS) and Surveillance, Epidemiology and End results (SEER) program (ACS & SEER, 2022). Nevertheless, several factors such as age, sex, histology, tumor size,

presence of lymph node metastases and molecular alterations may influence the prognosis of this malignancy and perhaps explain why some cases are highly aggressive and metastatic (Palme et al., 2004).

1.2.2. Histotypes

The thyroid parenchyma consists essentially of two types of cells: follicular and parafollicular cells (Arauchi et al., 2017). Depending on the cell of origin, thyroid neoplasms can have very different histological characteristics and clinical outcomes (Figure 2).

The follicular cells can give origin to benign neoplasms, the follicular thyroid adenomas (FTA) (McHenry & Phitayakorn, 2011) or malignant neoplasms such as the well differentiated thyroid carcinomas (WDTC), poorly differentiated thyroid carcinomas (PDTC) and undifferentiated or anaplastic thyroid carcinomas (ATC) (Pstrąg et al., 2018). Within the group of WDTC are included papillary thyroid carcinomas (PTC), follicular thyroid carcinomas (FTC) and oncocytic thyroid carcinomas (OTC) (Pstrąg et al., 2018).

Parafollicular cells give rise to medullary thyroid carcinoma (MTC) (Shah, 2015).

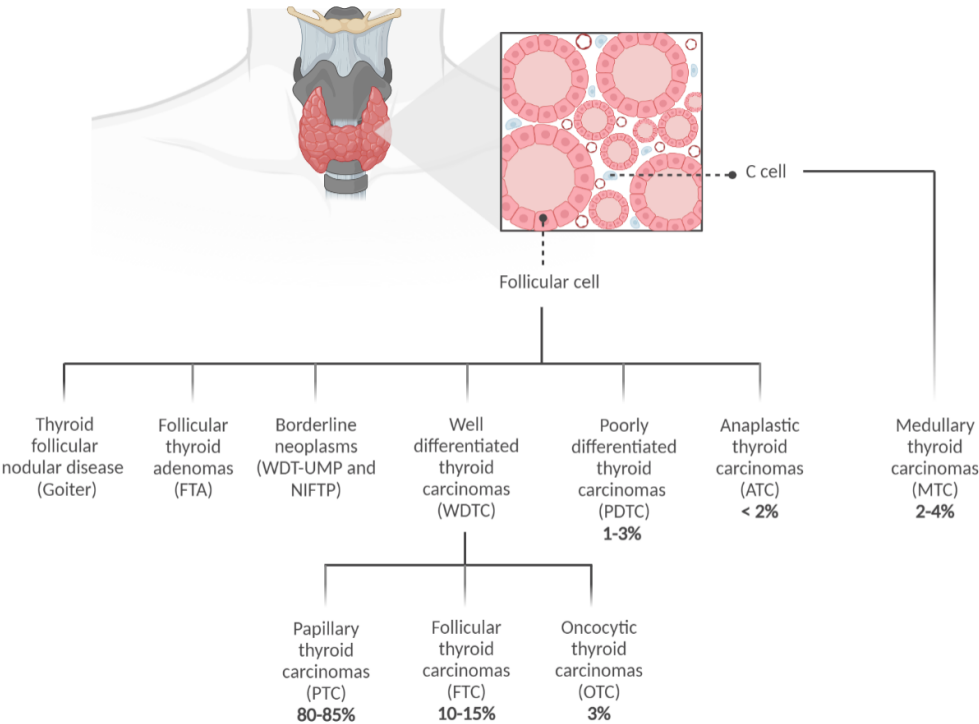


Figure 2 – Histology of thyroid tumors

Thyroid tumors can have different histological features and clinical outcomes according to the cells of origin. Tumors with follicular cell origins are broadly divided into two groups: benign tumors and

malignant tumors. Benign tumors include follicular thyroid nodular disease or also termed goiter, follicular thyroid adenomas (FTA) and borderline neoplasms such as well-differentiated thyroid tumor of uncertain malignant potential (WDT-UMP) and non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP). Within malignant tumors, well-differentiated thyroid carcinomas (WDTc) represent the vast majority of all thyroid carcinomas, which include papillary thyroid carcinomas (PTC) (80-85%), follicular thyroid carcinomas (FTC) (10-15%) and oncocytic thyroid carcinomas (OTC) (3%); a smaller portion of these tumors belong to a group of less differentiated tumors, such as poorly differentiated thyroid carcinomas (PDTC) (1-3%) and anaplastic or undifferentiated thyroid carcinomas (ATC) (<2%). Medullary thyroid carcinomas (MTC) are derived from parafollicular cells and represent about 2-4% of all thyroid carcinomas. Image created with BioRender.com.

1.2.3. Benign neoplasms

1.2.3.1. Follicular thyroid adenomas (FTA)

FTAs are frequent benign neoplasms of the thyroid gland (McHenry & Phitayakorn, 2011; Norris & Farci, 2022). They are typically present as a solitary, homogeneous nodule surrounded by a thin fibrous capsule without features of capsular, vascular, or neighboring thyroid tissues invasion (McHenry & Phitayakorn, 2011; Norris & Farci, 2022). Histologically, the nodules present a follicular architecture that can be described as either micro or macro follicular growth patterns, and the thyrocytes present a (*quasi*) normal cytological appearance (McHenry & Phitayakorn, 2011; Norris & Farci, 2022).

Their size is extremely variable, ranging from few millimeters to dimensions up to 10-15 centimeters (Norris & Farci, 2022). Most patients are euthyroid and asymptomatic and the majority of the nodules are discovered incidentally by ultrasound or palpation (McHenry & Phitayakorn, 2011; Norris & Farci, 2022). Iodine deficiency is one of the risk factors that has been implicated in many studies of follicular lesions (McHenry & Phitayakorn, 2011; Norris & Farci, 2022). Rarely, the adenomas are composed of over 75% of cells with oncocytic features (Barnabei et al., 2009). These cells are distinguished by presenting an abundant eosinophilic cytoplasm, with accumulation of mitochondria, and small regular nuclei (Asa & Mete, 2021; Kumar et al., 2015). The incidence of oncocytic follicular thyroid adenoma (OTA) is uncertain but shows a significantly high prevalence in female when compared to males, in a proportion of 8:1 (Ghossein, 2011).

1.2.4. Borderline and/or low-risk neoplasms

1.2.4.1. Well differentiated thyroid tumor of uncertain malignant potential (WDT-UMP)

WDT-UMP is by definition an encapsulated or well circumscribed tumor composed of well differentiated follicular cells that may have PTC nuclear features and questionable capsular or vascular invasion (Baloch et al., 2022).

This lesion is included in the category of borderline malignancies, as it is not readily diagnosed as benign or malignant. Nevertheless, it is associated with an excellent prognosis (Yassin Fel, 2015).

1.2.4.2. Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP)

NIFTP defines encapsulated thyroid tumors with follicular growth pattern and nuclear features of PTC (Dhanushika et al., 2020). Generally presents an indolent behavior and bears an excellent prognosis (Dhanushika et al., 2020; Geramizadeh & Maleki, 2019; Rosario & Mourao, 2019). This entity was primarily defined to address a subset of low-risk thyroid neoplasms, prevent overtreatment, and diminish the number of surgeries, having in higher consideration the patients quality of life and resource management (Rossi et al., 2019; B. Xu & R. A. Ghossein, 2020).

NIFTP classification is strict, and it must meet specific criteria. According to American Thyroid Association (ATA), the tumor cannot show any evidence of invasion (capsule, blood vessels, lymphatic or thyroid parenchyma); the tumor capsule (when present) must be intact; the tumor cannot have any signs of necrosis or increased mitotic rate; no or minimal papillary appearance (less than 1%) and may not have any signs of other thyroid cancer variants (ATA, 2022).

1.2.5. Malignant neoplasms

1.2.5.1. Papillary thyroid carcinoma (PTC)

PTC is the most frequent histotype, constituting more than 80-85% of all thyroid malignancies (Limaïem, Rehman, et al., 2022; LiVolsi, 2011). These tumors tend to be biologically indolent and have an excellent prognosis with 25-year survival rates of over 95% (LiVolsi, 2011). PTC can arise at any age, but most cases are

diagnosed from the third to fifth decade of life. Nevertheless, it is the most prevalent thyroid tumor found in pediatric patients (LiVolsi, 2011; Sur et al., 2020).

The common etiological factor associated with this type of tumor is radiation exposure, which has been widely documented. In the past century, an abrupt incidence of PTCs has been diagnosed in individuals exposed to large amounts of ionizing radiation, this includes survivors of the atomic bomb attack in World War II and those affected by the Chernobyl nuclear accident; as well as patients treated with low doses of radiation to benign head and neck diseases such as hemangiomas, lymphangiomas, enlarged thymus, enlarged tonsils, and adenoids. (Limaiem, Rehman, et al., 2022; LiVolsi, 2011; Lloyd et al., 2011).

Macroscopically, PTCs may be unifocal or multifocal and their size could be quite variable, with a mean diameter of 2-3 centimeters (Kumar et al., 2015; LiVolsi, 2011). Histologically, these lesions may be well circumscribed or, more often, have poorly defined edges and infiltrative growth patterns into the adjacent thyroid parenchyma. Nuclear features are important diagnostic clues with nuclei showing a clear or empty appearance due to finely dispersed chromatin (orphan Annie-eye nuclei appearance) (Kumar et al., 2015). Cytoplasmic invaginations may give the appearance of intranuclear inclusions or pseudo inclusions. Calcified structures, as psammoma bodies, are commonly present within the papillae cores (Kumar et al., 2015; Lloyd et al., 2011).

PTC can be subdivided into several histological variants, as summarized in **Table 1**, with each one showing a combination of specific growth patterns, cell types, and stromal alterations (Lloyd et al., 2011).

Table 1 – Different variants of PTC.

PTC Variant	
Classic PTC	Solid/Trabecular variant of PTC
Papillary microcarcinoma	Cribriform-morular variant of PTC
Follicular variant of PTC	Columnar cells variant of PTC
Oncocytic variant of PTC	Papillary thyroid carcinoma with fibromatosis /fasciitis-like stroma variant of PTC
Tall cell variant of PTC	
Diffuse sclerosing variant of PTC	Spindle cell variant of PTC
Clear cell variant of PTC	Encapsulated variant of PTC
Hobnail variant of PTC	Warthin-like variant of PTC

In the next topic, only the most frequent variants will be addressed.

a) Classic papillary thyroid carcinoma (cPTC)

cPTC accounts for almost 50% of all papillary tumors (Lang et al., 2006). The diagnosis is made based on the presence of a branching papillary architecture and papillary nuclear features (Orphan Annie eye nuclei) (LiVolsi et al., 2004).

These tumors can be cystic and present calcifications (psammoma bodies). cPTC tends to spread to lymphatics, and a significant proportion of cases present lymph nodes metastases at presentation (LiVolsi et al., 2004).

b) Papillary thyroid microcarcinoma (PTMC)

Papillary microcarcinomas are defined as PTC with less than 1 centimeter of diameter. The detection of these tumors is mostly incidental, occurring when patients undergo imaging exams for other conditions or surgery for benign lesions (Noguchi et al., 2008). These tumors can be encapsulated and associated with an excellent prognosis; or present an infiltrative growth (Limaem, Rehman, et al., 2022; Noguchi et al., 2008). Non-encapsulated PTMC can have more aggressive tumor features, including regional metastasis and disease recurrence similar to cPTC (Arora et al., 2009).

Therefore, in the 5th edition of the WHO classification, it is recommended that PTMC should no longer be considered as a distinct subtype of PTC, since the size *per se*, do not preclude the behavior (Baloch et al., 2022).

c) Follicular variant (FVPTC)

FVPTC is a variant of PTC that is distinguished by having a follicular architecture that resembles follicular neoplasia (follicular adenoma/carcinoma), however the nuclear features are similar to those observed in cPTC (Lloyd et al., 2011). Follicle size can be variable, and these are filled with darker-toned colloid, usually more eosinophilic than adjacent non-neoplastic follicles (Kumar et al., 2015; Lloyd et al., 2011). FVPTC is associated with extrathyroidal extension and lymphatic invasion (Tang et al., 2017; Verma et al., 2020).

Some authors suggest that the clinical behavior of this variant is more aggressive when compared with cPTC (Tang et al., 2017).

d) Oncocytic variant (OVPTC)

This variant is distinguished by a brownish color on gross examination that is relatively similar to what is seen in oncocytic thyroid neoplasms (Lloyd et al., 2011).

The diagnosis is based on the nuclear features of PTC and the presence of an abundant eosinophilic cytoplasm, with accumulation of mitochondria (Lloyd et al., 2011). In terms of architecture, they may present a papillary, follicular, or solid architecture (Sak, 2015).

e) Tall cell variant (TCVPTC)

TCVPTC is the most common aggressive form of thyroid cancer. It is characterized by the presence of cells that become "twice as long (tall) as they are wide" (Coca-Pelaz et al., 2020). According to the overview of the 2022 WHO Classification of Thyroid Neoplasms, TCVPTC is classified as such, when at least 30% of the cells present tall cell features (Baloch et al., 2022).

The tumors have a papillary architecture, and the papillae are elongated and have eosinophilic cytoplasm. In turn, the nuclei are characteristic PTC nuclei but more elongated (LiVolsi, 2010).

This variant is associated with a poor prognosis and a higher death and recurrence when compared to cPTC. This may be attributable to the fact that this variant presents mostly at older ages and with extrathyroidal extension (Morris et al., 2010).

f) Diffuse sclerosing variant (DSVPTC)

DSVPTC is an uncommon variant of PTC and has a higher incidence in pediatrics and in younger ages (Koo et al., 2009; Pillai et al., 2015). It is considered an aggressive variant of PTC. It was initially described as a variant presenting with diffuse involvement of the thyroid gland. Extensive squamous metaplasia, diffuse fibrosis, calcification, psammoma bodies with abundant lymphocytic infiltration are features of this variant. In addition, DSVPTC shows a high prevalence of extrathyroidal extension and cervical lymph node metastases (Pillai et al., 2015; Vuong et al., 2017).

g) Clear cell variant (CCVPTC)

CCVPTC is a rare variant of PTC. The clear-cell appearance results from aberrant accumulation of glycogen in the cytoplasm of tumor cells, however, the nuclear features of PTC are maintained (Juhlin & Hoog, 2019). The architecture is often papillary, but some tumors may show a follicular or insular organization (Sak, 2015).

The prognosis for CCVPTC is considered comparable to that of cPTC (Algahtani et al., 2019).

h) Hobnail variant (HVPTC)

HVPTC is a rare and aggressive subtype of PTC. It exhibits a micro-papillary architecture, loss of cell polarization, a high nuclear/cytoplasmic ratio and an apical nuclear position resembling a hobnail appearance (Lubitz et al., 2014; Poma et al., 2022).

This type of variant is associated with a poor outcome and most patients develop loco-regional disease and distant metastases (Donaldson et al., 2021).

i) Solid variant (SVPTC)

SVPTC is a rare, poorly characterized variant of PTC. This variant is predominantly described in children and has a strong association with exposure to ionizing radiation. It shows a solid/trabecular growth pattern, wherein the tumor cells form hypercellular nodules that retain the cytological features of PTC (Ohashi, 2020; Yang et al., 2021). It is associated with poor prognostic features, such as extrathyroidal extension, lymph node and distant metastases (Coca-Pelaz et al., 2020).

1.2.5.2. Follicular thyroid carcinoma (FTC)

Within thyroid cancer, follicular carcinoma ranks as the second most common carcinoma (D'Avanzo et al., 2004). It represents 10-15% of all thyroid cancers (Ashorobi & Lopez, 2022). This subtype of tumor is more frequent in iodine deficient-areas (Schmid & Farid, 2006). It can be described as "a malignant epithelial tumor showing follicular cell differentiation and lacking the diagnostic nuclear features of papillary thyroid carcinoma" (Sobrinho-Simoes et al., 2011).

FTCs usually appear as isolated tumors, encapsulated or with minimal capsular invasion, or, more rarely, may be highly invasive and present extensive infiltration of the capsule and/or vascular invasion. Microscopically, these tumors present small/medium-sized follicles. Solid or rarely trabecular growth patterns can also be present (Schmid & Farid, 2006; Sobrinho-Simoes et al., 2011).

However, the prognosis also depends on the degree of invasion of the tumor. Minimally invasive tumors have a much better prognosis than widely invasive carcinomas (Sobrinho-Simoes et al., 2011).

1.2.5.3. Oncocytic thyroid carcinoma (OTC)

OTC constitutes about 3% of all thyroid carcinomas and shows a more aggressive behavior. The cut-off established to consider this type of malignancy is the presence of at least 75% of oxyphilic cells (Ahmadi et al., 2016; Kushchayeva et al., 2004). The distinct feature of this variant from its benign counterpart (OTA) is the presence of capsular/vascular invasion (Goffredo et al., 2013; McFadden & Sadow, 2021).

These tumors tend to be associated with vascular invasion and distant metastases, making this variant more aggressive than the other well-differentiated thyroid tumors (Baloch et al., 2022; Goffredo et al., 2013).

1.2.5.4. Poorly differentiated thyroid carcinoma (PDTC)

PDTC is an uncommon subtype of thyroid carcinoma, accounting for 1-3% of all thyroid tumors (B. Xu & R. Ghossein, 2020). The biological behavior is located between differentiated thyroid carcinomas (PTC and FTC) and anaplastic thyroid carcinomas (ATC) (Bellini et al., 2021). The diagnosis of this neoplasm is quite difficult and the current WHO criteria is based on the consensus established at Turin in 2006 (B. Xu & R. Ghossein, 2020).

The Turin proposal establishes that the diagnosis of PDTC must be made according to three criteria: presence of a solid/trabecular/insular growth pattern; absence of PTC nuclear features; at least presence of one these 3 features: mitotic index ≥ 3 high power fields (HPFs), necrosis or convoluted nuclei.

PDTCs are more frequent in older patients, and they also have adverse clinical pathological features such as advanced locoregional disease and distant metastases (Ibrahimpasic et al., 2019).

1.2.5.5. Anaplastic thyroid carcinoma (ATC)

ATC is an extremely rare, undifferentiated, and deadly neoplasm (Bible et al., 2021). It constitutes less than 2% of all cases of thyroid carcinoma and accounts for 20-50% of all deaths for this malignancy (Chintakuntlawar et al., 2019).

Microscopically, these lesions can have quite variable presentations but can be characterized by the presence of spindle shaped cells with a high mitotic index, necrotic cells, an infiltrative growth pattern and vascular invasion (Limaïem, Kashyap, et al., 2022).

Patients with this type of tumor are usually older, in the seventh decade of life. Locoregional and distant metastases are frequent at the time of presentation

(Alobuia et al., 2020; Limaïem, Kashyap, et al., 2022). Most of these tumors are resistant to standard therapies, making the management of the disease very challenging (Chintakuntlawar et al., 2019).

1.2.5.6. Medullary thyroid carcinoma (MTC)

MTC is a rare neuroendocrine tumor that originates from the thyroid parafollicular cells (Lee et al., 2017). It accounts for 2-4% of all thyroid tumors (Ceolin et al., 2019).

This neoplasm in 75% of the cases occurs sporadically and in 25% of the cases is associated with the familial syndrome multiple endocrine neoplasia type 2 (MEN2). The first tends to appear at older ages (fifth or sixth decade of life) and the latter at younger ages (second and third decade of life) due to its hereditary nature (Kim & Kim, 2021).

Usually, patients present an isolated asymptomatic nodule. This lesion is characterized by nests of rounded cells or with ovoid structure with fibrovascular stroma (Master & Burns, 2022). Patient outcome is usually related to tumor stage, patient's age, tumor size and presence of local or distant metastases at the time of presentation. Nonetheless, the majority of the patients in early-stage disease have a good prognosis (Master & Burns, 2022).

1.3. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

The coronavirus disease (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was considered a spreading pandemic in March of 2020 by World health Organization (WHO) (WHO, 2022a). SARS-CoV-2 is a member of enveloped positive sense RNA virus belonging to the family of β -coronaviruses (Scappaticcio et al., 2021).

This virus exploits host proteins in order to mediate cellular infection (**Figure 3**). The main viral receptor is angiotensin converting enzyme 2 (ACE-2), but host cell entry is dependent on target cellular proteases such as transmembrane serine protease 2 (TMPRSS2) and furin that are responsible for processing the SARS-CoV-2 spike protein, ensuring an efficient infection (Jackson et al., 2022; Shang et al., 2020).

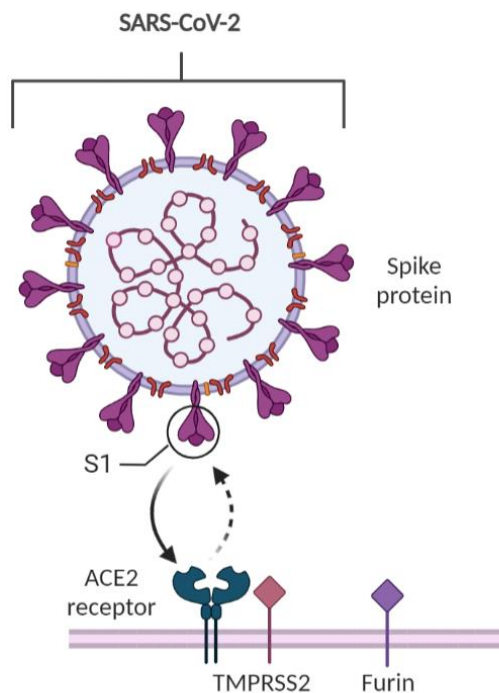


Figure 3 – Interaction of SARS-CoV-2 with ACE-2, TMPRSS2 and furin.

SARS-CoV-2 uses host proteins in order to mediate cellular infection. The virus uses ACE-2 as the main receptor, and the proteases TMPRSS2 and furin are responsible for the proper cleavage/ processing of spike protein. Image created with BioRender.com

1.4. Angiotensin Converting Enzyme 2 (ACE-2)

ACE-2 is a zinc metallo-carboxypeptidase discovered in the late 2000's by two independent research groups as an homolog of angiotensin converting enzyme (ACE) essential in the regulation of renin-angiotensin-aldosterone system (RAAS) (Donoghue et al., 2000; Tipnis et al., 2000).

With an identity of 40% and a similarity of 61% with ACE (G. Li et al., 2020), it was suggested that these two genes arose from a common ancestor (Donoghue et al., 2000).

The *ACE-2* gene is located on chromosome Xp22.2 and generates 6 transcripts through alternative splicing (Scialo et al., 2020). The longest isoform (mACE-2) encodes a protein of 805 amino acids, which is normally located in the plasma membrane with the N-terminal portion of the protein directed towards the extracellular space (Scialo et al., 2020). This portion contains the active catalytic site of the protein, the zinc binding motif sequence HEXXH [(Histidine-Glutamine-X-X-Histidine)], where X represents any amino acid (aa) residue, that is highly conserved in peptidases family (Kuba et al., 2010; Lubbe et al., 2020). The C-terminal end is

localized in the transmembrane domain and has a high homology with collectrin (48%), a renal protein (**Figure 4A**) (Singer & Camargo, 2011).

ACE-2 can also be found in a soluble form, into circulation, at low concentrations (Scialo et al., 2020) after being subjected to a post-translational modification by disintegrins and metalloproteinases domain-containing proteins 10 and 17 (ADAM10 and ADAM17). This isoform only retained the ectodomain (active catalytic site) and loses the transmembrane domain from mACE-2 (Lambert et al., 2005). Nevertheless, the biological relevance of this isoform is still unclear (Kuba et al., 2010).

More recently a short new isoform of ACE-2 has been found, termed as δ ACE-2. This variant has lost the sequence capable of encoding the soluble active part of the protein (Blume et al., 2021; Perez et al., 2022; Williams et al., 2021). However, the real function of this isoform is not yet fully understood (Perez et al., 2022).

ACE-2 expression can be both transcriptionally and post-transcriptionally regulated, and the levels of the protein fluctuate in a dynamic manner under physiological and pathological conditions (Kuba et al., 2010; Lambert et al., 2005; Scialo et al., 2020).

1.4.1. Role of ACE-2 in renin angiotensin aldosterone system (RAAS)

RAAS consists in a sequence of reactions whose main purpose is the maintenance of blood pressure homeostasis and salt balance, which can interfere with the function of many organs, such as the heart, blood vessels and kidneys (Ni et al., 2020; Santos et al., 2018).

This system starts in response to low blood pressure when juxtaglomerular cells of the kidney release renin for the afferent arterioles. Renin is responsible for the conversion of angiotensinogen (synthesized in the liver) into a decapeptide, angiotensin I (Ang I), which in turn is cleaved in angiotensin II (Ang II), an active octapeptide, by ACE (Bian & Li, 2021; De Gasparo et al., 1994; Kaschina et al., 2017; Kumar et al., 2015).

In order to achieve its function, AngII must connect with angiotensin II type 1 receptor (AT1R) or type 2 receptor (AT2R) in the target tissues. Both of the receptors belong to the superfamily of G proteins (Laghlam et al., 2021). ACE/Ang II/AT1R axis leads to the raising of blood pressure through induction of short-term vasoconstriction, stimulation of aldosterone secretion, by the adrenal gland, and increasing tubular sodium (Na^+) reabsorption of water in kidney, which leads to the

increase of blood volume (Bian & Li, 2021; Kumar et al., 2015; Laghnam et al., 2021). AT2R is responsible for counteract the aforementioned effects mediated by AT1R(Bian & Li, 2021; Kumar et al., 2015; Laghnam et al., 2021).

Accumulation of Ang II it's also known for exerting other effects like cell proliferation, inflammatory responses, blood coagulation and extracellular matrix remodeling, oxidative stress, inflammation, atrophy, and fibrosis(Ni et al., 2020; Scialo et al., 2020).

Activation of the ACE/Ang II/AT1R axis, is seen in hypertension, cardiac hypertrophy, heart failure, and other cardiovascular diseases(Kai & Kai, 2020).

Despite great homology with ACE, ACE-2 exerts a counter-regulatory effect on this mechanism. This protein is able to cleave Ang I into the nonapeptide angiotensin 1-9 (Ang 1-9) and Ang II into the heptapeptide angiotensin 1-7 (Ang 1-7). In fact, some studies *in vitro* indicate that the catalytic effectiveness of ACE-2 for Ang II is 400-fold greater than for Ang I, suggesting that the primary role of ACE-2 is the conversion of Ang II into Ang 1-7(Burrell et al., 2004; Laghnam et al., 2021; Vickers et al., 2002).

These two peptides are capable of mediate antagonistic effects of Ang II. Ang 1-7 binds to a G-protein coupled receptor (GPCR) , the mitochondrial assembly receptor (MasR), which leads to vasodilation, antihypertensive, and antifibrosis effects, whereas Ang 1-9 can activate AT2R to trigger natriuresis and nitric oxide (NO) production, mediating vasodilatory effects and decreasing blood pressure (Bian & Li, 2021; Laghnam et al., 2021).

ACE-2/Ang 1-7/MasR axis opposes the ACE/Ang II/AT1R axis effect and has been shown to reverse organ damage in renal and cardiovascular diseases (Bian & Li, 2021; Laghnam et al., 2021).

1.4.2. ACE-2 expression in human tissues

ACE-2 is expressed in a large variety of normal tissues. ACE-2 mRNA and protein expression are enriched in gastrointestinal tract (small intestine, duodenum and colon), kidney, testis and gallbladder (Dai et al., 2020; Hikmet et al., 2020; M.-Y. Li et al., 2020; Salamanna et al., 2020).

When comparing the mRNA expression of ACE-2 in tumors and its corresponding normal tissue, it was described to be increased in colon adenocarcinoma, papillary renal cell carcinoma, pancreatic adenocarcinoma, rectal adenocarcinoma, stomach adenocarcinoma and lung adenocarcinoma, while testicular germ cell tumors, thyroid carcinoma and chromophobe renal cell carcinoma

present decreased ACE-2 expression (Chai et al., 2020; Dai et al., 2020; Ren et al., 2020). Information about ACE-2 protein expression in cancer, is scarce. It's mostly described as expressed in renal and colorectal cancers (Dai et al., 2020; HPA, 2022a; Ren et al., 2020; Uhlén et al., 2015).

ACE-2 may have different roles in different situations and its prognostic value in cancer may be variable according to various characteristics such as gender, race, age, and tumor stage (Feng et al., 2020).

Through bioinformatics analysis, ACE-2 mRNA expression was shown to be related with better prognosis in renal papillary cell carcinoma, lung adenocarcinoma, serous cystadenocarcinoma of the ovary, hepatocellular carcinoma of the liver. On the other hand, it has a negative impact on prognosis in carcinoma of the esophagus, squamous cell carcinoma of the lung, testicular germ cell tumor and lower grade glioma of the brain. These authors also described that tumors with a better prognosis had a positive correlation with ACE-2 expression and immune infiltrates (Feng et al., 2020).

Some studies support that ACE-2 could play a protective role through inhibiting tumor cell proliferation, invasion, migration, angiogenesis, and local inflammation through ACE-2/Ang 1-7/MasR receptor axis in various types of cancers (de Paula Gonzaga et al., 2020; Xu et al., 2017).

Experimental findings shows that ACE-2 is capable of inhibit angiogenesis through suppressing vascular endothelial growth factor A (VEGFa) – vascular endothelial growth receptor 2 (VEGFR2) – extracellular signal-regulated kinase (ERK) pathway in breast cancer (Chai et al., 2020; Zhang et al., 2019) and reduce cell invasion and migration in and non-small cell lung cancer (Chai et al., 2020; Xu et al., 2017).

Although these facts may indicate that ACE-2 has a potential protective factor for cancer progression, the mechanisms to suppress or promote cancer requires further investigation (Xu et al., 2017).

1.5. Transmembrane Serine Protease 2 (TMPRSS2)

TMPRSS2, a type II transmembrane serine protease constituted of 492 amino acids is encoded by the *TMPRSS2* gene located at chromosome 21q22.3 (Thunders & Delahunt, 2020; Wettstein et al., 2022). This protein belongs to the type II transmembrane serine proteases (TTSP), more precisely to the subfamily Hepsin/TMPRSS (Hedstrom, 2002; Paoloni-Giacobino et al., 1997). Structurally (**Figure 4B**), TMPRSS2 contains four domains: A N-terminal portion localized in the

intracellular region, a type II transmembrane domain, a stem region that comprises both low density lipoprotein receptor class A (LDLRA) and scavenger receptor cysteine-rich (SRCR) domains, and a C-terminal serine protease domain that comprises the catalytic triad (Histidine-Aspartate-Serine or HDS) and are located in the extracellular region (Wettstein et al., 2022).

Other TMPRSS2 isoform can arise from alternative splicing of TMPRSS2 mRNA, that differs from the previously described in the N-terminal cytoplasmic tail with an addition of 37 amino acids (Zmora et al., 2015). However, there is no evidence of any proteolytic activities or different physiological roles between isoforms and, for this reason, most studies have focused only on the 492 amino acid isoforms (Wettstein et al., 2022).

Both TMPRSS2 isoforms, like other TTSP members, are glycoproteins expressed as a single chain proenzymes or zymogens (inactive form) that are subjected to autoproteolytic cleavage at the interface of the SRCR and SP domains. This process allows a change in protein conformation required for the transition to their active state. After post-translational modification, the vast majority of mature proteases remain attached to the cell membrane, although a portion is released into the extracellular space (Sarker et al., 2021; Shen et al., 2017; Thunders & Delahunt, 2020; Wettstein et al., 2022). TMPRSS2 has been associated with several processes such as tissue remodeling, inflammatory responses and tumor cell invasion. (Thunders & Delahunt, 2020).

TMPRSS2 mRNA and protein expression is highly expressed in gastrointestinal tissues (small intestine, stomach and colon), lung, salivary gland, esophagus, prostate, liver and thyroid (Baughn et al., 2020; Gkogkou et al., 2020; HPA, 2022b; Piva et al., 2021; Singh et al., 2021; Zhou et al., 2020).

Dysregulated TTSP activity it's a common characteristic in cancers leading to tumor cell proliferation, invasion, and metastasis (Fraser et al., 2022).

TMPRSS2 expression in cancers is very heterogeneous in the literature (Liu et al., 2022; Xiao et al., 2022). According to The Human Protein Atlas (HPA), TMPRSS2 mRNA expression is increased in prostate, urothelial, colorectal, stomach, lung cancer and pancreatic cancer. Prostate cancers, renal, urothelial, lung, colorectal and pancreatic cancers presented weak to moderate protein expression while other cancer tissues such as glioma, thyroid, lung, and colorectal cancers were negative (HPA, 2022b; Karlsson et al., 2021; Uhlén et al., 2015).

TMPRSS2 is also known for being involved in cascades that are relevant for the normal function of the prostate (Lucas et al., 2014).

Some evidences reported that in both neoplastic and hyperplastic prostate, TMPRSS2 expression was significantly higher compared to normal epithelium (Chen et al., 2010; Lucas et al., 2008).

1.6. Furin

Furin is a transmembrane type I, calcium strictly dependent serine endoprotease (Dahms et al., 2016; Molloy et al., 1992). This protein is ubiquitously expressed and belongs to a family of subtilisin/kexin like proprotein convertase (PCSK). This protease family is responsible for the cleavage and conversion of immature pro-proteins into their biologically active forms (Turpeinen et al., 2013). *Furin* gene maps to the chromosome 15q26.1 and encodes a protein of 794 amino acids (Braun & Sauter, 2019; Osman et al., 2022; Parvaz & Jalali, 2021).

At structural level (**Figure 4C**), furin is a single polypeptide constituted by an N-terminal signal peptide (SP), an inhibitory pro-domain, a catalytic endopeptidase domain, a P domain, a cysteine-rich domain (CRD) and a C-terminal cytoplasmic domain (Osman et al., 2022). The pro-domain acts like a chaperone that is necessary for proper guiding, correct folding, activation, and transport of furin. It also serves as a potent auto-inhibitor. Like TMPRSS2, the catalytic domain of furin is composed of catalytic triad (Asparagine-Histidine-Serine or AHS) that is highly conserved within PCSK family and regulated by P domain, through pH modulation and calcium dependent autoproteolytic processing (Thomas, 2002).

After being translated, furin is incorporated in the secretory pathway. The inactive protein is translocated to the membrane of endoplasmic reticulum (ER) membrane via C-terminal domain and then passes to the trans-golgi network (TGN). During this transition the inhibitory pro-domain is removed in two autoproteolytic processes: the first takes place in the ER and the second in the TGN, thus making the enzyme functionally active (Braun & Sauter, 2019; Gawlik et al., 2009; Parvaz & Jalali, 2021).

Furin is responsible for the activation of several proteins essential for proper physiological functioning (Coppola et al., 2008). Some studies, refer that this protease has more than 400 predicted substrates such as growth factors, signaling peptides, hormones, extracellular matrix proteins, transmembrane receptors, serum proteins, coagulation factors and signaling peptides (Coppola et al., 2008; Gawlik et al., 2009; Molloy et al., 1999; Osman et al., 2022; Tian et al., 2011).

Furin is exploited by several viral and bacterial pathogens, enhancing their virulence and spread. It is also involved in pathological conditions such as,

neurological disorders (Alzheimer's disease), arthritis, atherosclerosis, and tumorigenesis (Braun & Sauter, 2019; Coppola et al., 2008).

Furin knock-out mouse models die between day 10.5 and 11.5 validating a vital role and non-redundant functions of this protein in early development stages (Creemers & Khatib, 2008; Roebroek et al., 2004).

Through databases analysis, furin mRNA expression in healthy tissues was found elevated in salivary gland, placenta, liver, pancreas, bone marrow, lung and thyroid tissues (Li et al., 2021; Zhou & Gao, 2021). At protein level, the highest levels of expression were described in cerebral cortex, cerebellum, hippocampus, salivary gland, pancreas, kidney, and placenta (Li et al., 2021).

Furin has been implicated in essential physiological processes but in pathological processes as well. Its overexpression, at mRNA level, has been associated with several human tumors such as ovary cancer, breast, head and neck tumors, brain cancer, non-small cell lung carcinoma, rhabdomyosarcoma, urothelial bladder carcinoma, esophageal carcinoma, head and neck squamous cell carcinoma, lung adenocarcinoma, stomach adenocarcinoma and endometrial carcinoma of the uterine body (Coppola et al., 2008; Jaaks & Bernasconi, 2017; Zhou & Gao, 2021), whereas in clear cell renal cell carcinoma and renal papillary carcinoma the mRNA expression was described as relatively low (Zhou & Gao, 2021). At the protein level, thyroid cancer was the carcinoma that showed the highest expression of this protein, however, moderate expression was observed in lung, liver, prostate, and urothelial cancers (Li et al., 2021).

Some functional studies modulating furin activity show the importance of the protein in the process of many cancer-related substrates and indicate that high furin activity promotes malignant phenotype of cancer cell (Bassi et al., 2003; Coppola et al., 2008; Jaaks & Bernasconi, 2017). An *in vivo* assay, demonstrated that tumors obtained after subcutaneous tissue inoculation of furin transfected cells of head and neck squamous cell carcinoma, were larger and developed earlier when compared to the counterpart controls (Bassi et al., 2003). Interestingly, the inhibition of furin activity was associated with a decrease in substrate activations, proliferation rate and invasive potential of cancer cells, indicating that this enzyme could be an interesting target for cancer therapy (Bassi et al., 2003; Jaaks & Bernasconi, 2017).

Not surprisingly, increased expression of this protein is correlated with aggressiveness and was suggested as prognostic marker for advance or high-grade disease (Braun & Sauter, 2019; Jaaks & Bernasconi, 2017).

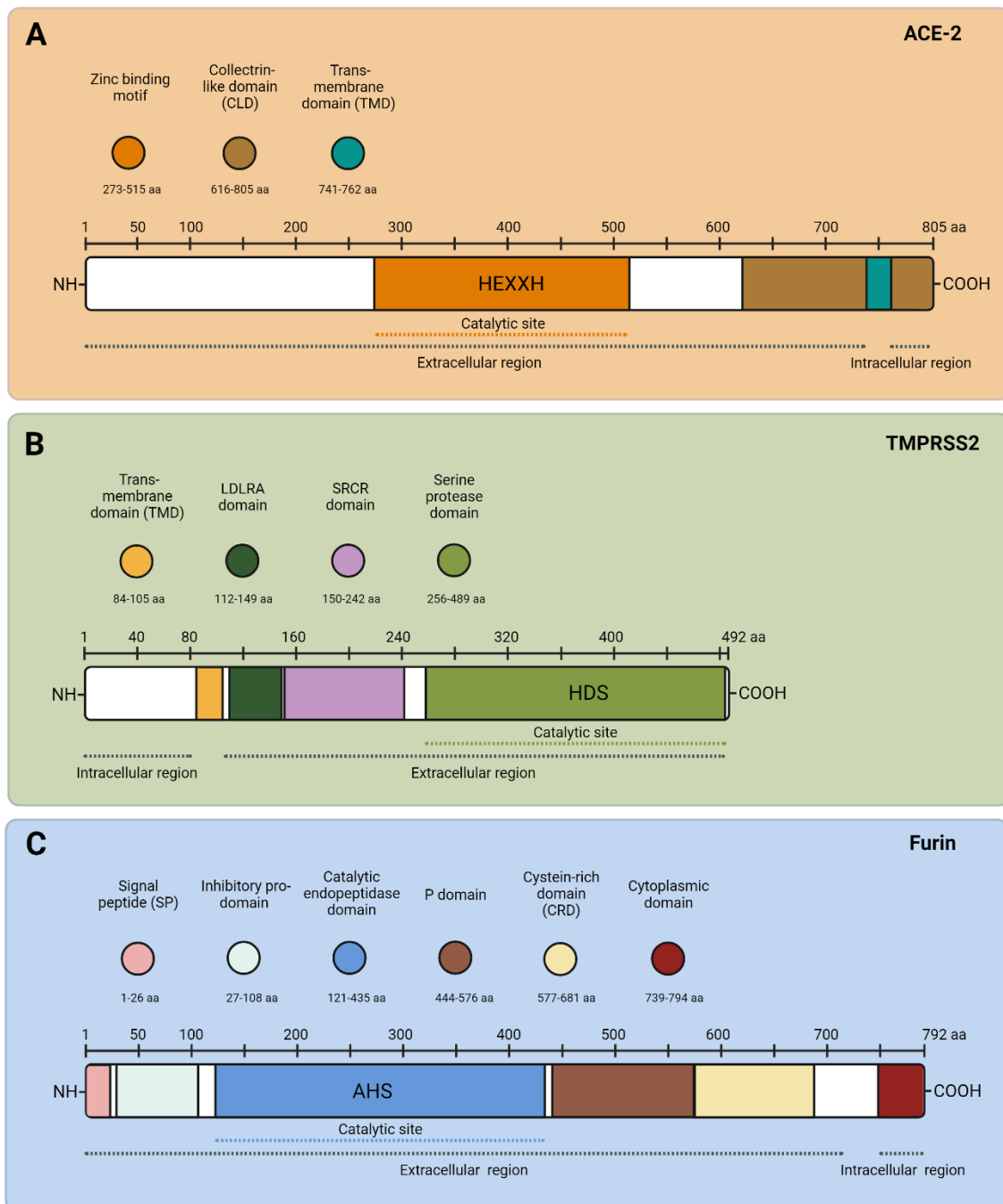


Figure 4 – Schematic representation of ACE-2, TMPRSS2, and furin proteins.

Protein domains are represented with distinct colors and subcellular regions are delimited with dashed lines. The corresponding aa coordinates are presented. All proteins have N and C-terminal domains. **(A)** ACE-2 is composed of a catalytic site containing a zinc-binding motif sequence (HEXXH) and a collectrin-like domain (CLD); **(B)** TMPRSS2 comprises four domains: a type II transmembrane domain, low density lipoprotein receptor class A (LDLRA) domain, scavenger receptor cysteine-rich domain (SRCR) domain and a C-terminal serine protease (SP) domain that comprises the catalytic triad Histidine-Aspartate-Serine (HDS) **(C)** furin is composed of six domains: an N-terminal signal peptide (SP) domain, an inhibitory pro-domain, a catalytic endopeptidase domain that contains a catalytic triad

Asparagine-Histidine-Serine (AHS), a P domain, a cysteine-rich domain (CRD), and a cytoplasmic domain. Image created with BioRender.com

During the pandemic period, several reports of thyroid dysfunction, such as thyroiditis and thyrotoxicosis (Murugan & Alzahrani, 2021; Prete et al., 2021), as well as the development of new onset of T2D in patients after SARS-CoV-2 infection began to emerge (El-Huneidi et al., 2021; Khunti et al., 2021; Metwally et al., 2021). Such evidence suggested that both thyroid and endocrine pancreas might be targets of SARS-CoV2 infection.

However, so far, the expression of molecules involved in the entry mechanism of SARS-CoV-2 in host cells is not well established either in thyroid or in pancreas. More knowledge concerning the expression of ACE-2, TMPRSS2 and furin, both at mRNA and protein levels, in endocrine tissues (normal, benign, and malignant) is an unmet need that we propose to address in the present thesis. Furthermore the putative role of these proteins in endocrine cancers is also poorly understood and deserves to be investigated.

2. Aims

- **Aim I**

- Evaluate the mRNA expression of ACE-2, TMPRSS2 and furin, in a series composed of normal thyroid tissue, benign and malignant thyroid neoplasms.
- Evaluate the protein expression of ACE-2, TMPRSS2 and furin, in a series composed of normal thyroid tissue, benign and malignant thyroid neoplasms.
- Evaluate if the expression of ACE-2, TMPRSS2 and furin (at mRNA and/or protein level) correlate with the clinicopathological features of thyroid neoplasms.

- **Aim II**

- Characterize ACE-2 protein expression in a series composed of normal pancreas and PanNENs.

The results and discussion of Aim II are presented in **Appendix II**.

3. Material and Methods

3.1. Biological samples

The thyroid samples used in this study belong to a series composed of adjacent thyroid tissue (AT), benign and malignant thyroid neoplasms collected over the past 20 years by the group of Cancer Signaling and Metabolism at Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal. A total of 181 frozen tissues were included in the study, comprising adjacent thyroid tissue (n=36), goiters (n=2), follicular adenomas (FTAs) (n=74), classic papillary thyroid carcinomas (cPTCs) (n=44), follicular variant of papillary thyroid carcinoma (FVPTCs) (n=13), follicular thyroid carcinoma (FTCs) (n=10), and oncocytic thyroid carcinoma (OTCs) (n=2). Additionally, we have selected (when available) the corresponding formalin-fixed paraffin embedded (FFPE) tissues (n=68), respectively, adjacent thyroid tissues (n=4), goiters (n=4), follicular adenomas (n=17), cPTCs (n=26), FVPTCs (n=11), FTCs (n=4), and OTCs (n=2). In FFPE tissues presenting both adjacent thyroid tissue and tumor (n=50), the analysis of adjacent tissue was performed separately. All these selected samples had RNA previously extracted and, in some samples, converted to cDNA for subsequent molecular characterization. The selected samples were collected on the period between 1989 and 2012.

3.2. Reverse transcription

To convert the RNA into cDNA in some of the selected samples, RevertAid RT, Reverse Transcription Kit, (REF: K1691, Thermo Scientific, USA) was used, following the manufacturer's instructions. All the reagents necessary and instructions for the execution of the technique were present in the kit.

In order to avoid contamination by RNases and preserve the purity and integrity of the RNA, all equipment and work areas were decontaminated with RNase Away (REF: 7001, Thermo Scientific, USA).

Before cDNA synthesis and for RT-qPCR applications, the RNA template needs to be free of genomic DNA, therefore DNase treatment was performed.

For cDNA synthesis, 1 µg of RNA was used from each sample, and then Random Hexamer primers were joined (1 µL) along with nuclease-free water until reached the final volume of 12 µL (**Table 2**).

Table 2 – Composition random primers mix.

Random primers mix	
RNA	1 µg
Random Hexamer primer	1 µL
Nuclease-free water	To 12 µL
Total	12 µL

After this step, a reverse transcriptase reaction mix composed of Reaction Buffer, RiboLock RNase inhibitor, dNTP mixture, and RevertAid RT was added to the isolated RNA (**Table 3**). The final volume obtained after the combination of both mixtures was 20 µL.

Table 3 – Composition of reverse transcriptase reaction mix.

Reverse transcriptase reaction mix	
5 µL Reaction Buffer	4 µL
RiboLock RNase Inhibitor	1 µL
10 mM dNTP Mix	2 µL
RevertAid RT (200 U/µL)	1 µL
Total	8 µL

To avoid contamination between reagents and genomic DNA, negative controls of the technique were made: control without the RNA sample for each gene of interest; and all the components of the mixture but without reverse transcriptase.

3.3. Quantitative real time polymerase chain reaction (RT-qPCR)

Real-time quantitative PCR (RT-qPCR) was performed to evaluate the mRNA expression of ACE-2, TMPRSS2 and furin in our thyroid series. As an endogenous control of the technique, TBP (Human TATA-box binding protein) qPCR assay (REF: 222242490, Integrated DNA Technologies (IDT), USA) was used.

The PCR reaction mixture was composed of Taqman ® Universal PCR Master Mix (REF: 4324018, Applied Biosystems, USA), PCR probes [ACE-2 qPCR assay (REF: 228806911), TMPRSS2 (REF: 28806907), furin (REF: 228806915) purchased from IDT, USA] and purified water to a final volume of 9 µL (**Table 4A, 4B and 4C**).

The assays were optimized to amplify two genes in the same reaction (multiplex) and tested two-by-two with different fluorescence ranges. The ones that proved to be most effective in the same reaction were the ACE-2 and TMPRSS2 assays.

Table 4 – Composition of PCR reaction mix of **(A)** ACE-2 and TMPRSS2, **(B)** furin and **(C)**TBP.

A RT-qPCR reaction mix 1		B RT-qPCR reaction mix 2		C RT-qPCR reaction mix 3	
Taqman PCRMaster mix	5 µL	Taqman PCRMaster mix	5 µL	Taqman PCRMaster mix	5 µL
ACE-2 qPCR assay	0,5 µL	Furin qPCR assay	0,5 µL	TBP qPCR assay	0,5 µL
TMPRSS2 qPCR assay	0,5 µL	H ₂ O	3,5 µL	H ₂ O	3,5 µL
H ₂ O	3,0 µL	cDNA	1 µL	cDNA	1 µL
cDNA	1 µL	Total	10 µL	Total	10 µL
Total	10 µL				

Amplification was performed using the QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) under the following conditions: an initial step at 95°C for 10 min, followed by 50 cycles of 15s at 95°C and 1 min at 60°C (**Figure 5**).

Triplicates and non-template controls (NTC) were made for all samples. Amplification was considered up to the threshold cycle (Ct) 37, for all the assays.

The ΔC_t was calculated by subtracting the mean of Cts obtained for each of the genes of interest to the mean Cts of the housekeeping gene. Quantification of gene expression was obtained using the $2^{-\Delta C_t}$ method.

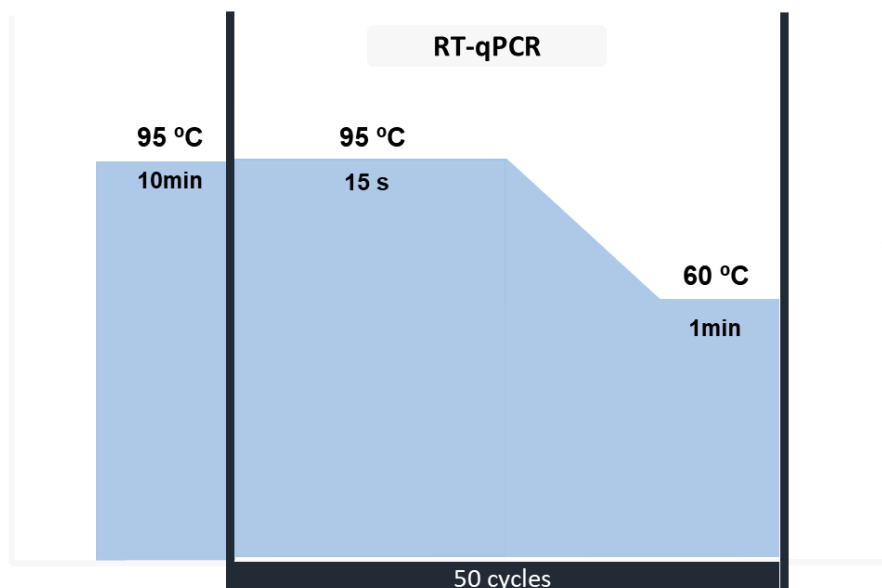


Figure 5 – RT-qPCR reaction conditions.

3.4. Hematoxylin-Eosin Staining (H&E)

For hematoxylin-eosin staining, 4 µm thick sections of FFPE tissues for each case of thyroid series were used. First, the slides were deparaffined with two consecutive rinses in xylol (Enzymatic, Portugal) for 10 minutes each. The samples were then hydrated in decreased ethanol concentrations (Enzymatic, Portugal): 10 minutes in ethanol 100%, 5 minutes in ethanol 100%, 5 minutes in ethanol 95% and 5-minutes ethanol 70%. The slides were rinsed under running water for 5 minutes.

After this step, the samples were stained with Gill's Hematoxylin (REF: CP813, DiaPath, Italy) for 4 minutes and 30 seconds. In order to remove the excess of this reagent, the slides were rinsed in water for 5 minutes after which they were placed in ethanol 70% for 5 minutes. Subsequently, tissues were counterstained with alcoholic eosin (REF:71204, Richard Allan Scientific, USA) for 3 minutes.

Samples were dehydrated through consecutive rinses in increasing ethanol concentrations: 5 minutes in ethanol 95%, followed by two rinses in ethanol 100% for 5 minutes each. For conservation and preservation of the tissues, two more consecutive rinses in xylol for 10 min were required. Lastly, the slides were finalized using mounting medium (REF: 4112, Richard Allan Scientific, USA) and observed under microscope.

3.5. Immunohistochemistry (IHC)

In order to evaluate the amount and subcellular localization of the studied proteins (ACE-2, TMPRSS2, and furin) in the thyroid and PanNENs series, 4 µm

thick sections of FFEP samples were used to perform immunohistochemistry. To start, all the tissues were deparaffinized by two consecutive passages in xylol for 10 min each. Hydration was performed through rinses in decrease concentrations of ethanol in the respective sequence: ethanol 100% (10 minutes), ethanol 100% (5 minutes), ethanol 96% (5 minutes) and ethanol 70% (5 min). Then slides were placed in running water for 5 minutes.

Heat-induced antigen retrieval was performed in a steamer at 90°C for 45 minutes in ethylenediaminetetraacetic acid (EDTA) buffer [Epitope Retrieval Solution (1x Concentrate) pH9 (REF: RE7119, Leica, Germany)]. After this step, the slides were left to cool at room temperature (RT) for 20 minutes. Then, samples were rinsed in 10% phosphate-buffered saline (PBS) and 0.02% tween washing buffer (referred as PBS-T 0.02%) for 5 minutes, two consecutive times. Subsequently, the tissues were circumscribed with hydrophobic pen (REF: H-400, ImmeEdge Pen, Vector Laboratories, USA). UltraVision Quanto Detection System HRP (REF: TL-125-QHL, EpreDia, USA) kit was used during the whole technique. The volume of reagent used per slide was c.100 µL.

UltraVision Hydrogen peroxide block (REF: TA-125-H202Q, EpreDia, USA) was used to block the endogenous peroxidase for 10 min at room temperature. After that, two rinses with PBS-T 0.02% were done for 5 min each. Then, an additional blockage (U-block, Ventana Medical Systems, USA) was used for 10 minutes, followed by two more rinses with washing buffer for 5 min each.

The tissue block was made using UltraVision Protein Block (REF: TA-125-PBQ, EpreDia, USA) for 10 minutes.

In the next step, the tissues were incubated with the primary antibody, overnight at 4°C. The antibodies used for each protein and the respective concentrations were:

- Anti-ACE-2 Monoclonal Antibody (CL4035) (REF: MA5-31395, Invitrogen, USA) at concentration of 1:2500;
- Recombinant anti-TMPRSS2 monoclonal antibody (EPR3862) (REF: ab109131, Abcam, UK) at concentration of 1:1000;
- Anti-Furin Polyclonal Antibody (REF: PA5-96680, Invitrogen, USA) at concentration of 1:250;

Antibody Diluent OP Quanto (REF: TA-125-ADQ, Thermo Scientific, USA) was used to achieve the desired concentration of each antibody.

After overnight incubation, three consecutive rinses were done with PBS-T 0.02% for 5 min each.

The following step consisted in the addition of Primary Antibody Amplifier Quanto (REF: TL-125-QPB, EpreDia, USA) followed by HRP Polymer Quanto (REF: TL-125-QPH, EpreDia, USA) for 10 minutes each. In between these steps, slides were washed with PBS-T 0.02% for two consecutive times, for 5 minutes each.

To reveal the staining of the tissues, DAB Quanto (REF: TA-125-QHDX, EpreDia, USA) was used for 3 minutes. Then slides were washed in running water (5 minutes).and counterstained with Gill's Hematoxylin (REF: CP813, DiaPath, USA) for 1 min. The excess amount of reagent was removed by placing the slides for 5 minutes under running water.

The slides were settled in 0.02% (W/V) ammonia water (Emsure, Merck, Germany) for 30 seconds with some agitation and then placed under running water for 1 minute.

To dehydrate the tissues, the slides were passed through increasing concentrations of ethanol: ethanol 96% (5 minutes) and 2 times in ethanol 100% (for 5 minutes each) and two consecutive rinses in xylol for 10 min each. The slides were assembled using mounting medium and then observed under microscope to evaluate the expression pattern. The human kidney was used as a positive control of the technique since it is described as one of the organs with the highest expression of these proteins(Dong et al., 2020; Zhou et al., 2020). The same tissue was used for the negative control, only the primary antibody was omitted in order to assess non-specific binding of the secondary antibody.

3.6. Immunohistochemical evaluation

The immunohistochemical expression of the three proteins in thyroid series was blindly evaluated by a pathologist and classifications were based on the 4th edition of the WHO endocrine tumors classifications from 2017(IARC & WHO, 2017). Each tissue was read in its entirety and a score was assigned according to the location, intensity of staining and percentage of cells positive for staining.

The vast majority of cases evaluated contained tumor and adjacent normal thyroid tissue (AT) on the same slide, and therefore scores were given for each of the tissues separately.

The results were evaluated semi-quantitatively by the immunoreactive score (IRS) method (Fedchenko & Reifenrath, 2014) (**Table 5**), which consists in the multiplication of the staining intensity score (**Figure 6A to 6L**), that was subdivided into absent (0), weak (1), moderate (2) and strong (3); by the percentage of positive

cells, which was subdivided into < 10% (0) 10% - 25% (1), 26 - 50%(2), 51 - 75%(3) and > 75% (4). The results obtained through the IRS score range from 0 to 12.

Table 5 – Immunohistochemical classification.

Staining intensity	0	Absent
	1	Weak
	2	Moderate
	3	Strong
Percentage of positive cells	0	< 10%
	1	10% - 25%
	2	26% - 50%
	3	51% - 75%
	4	> 75%
IRS score	Staining intensity x Percentage of positive cells = [0-12]	

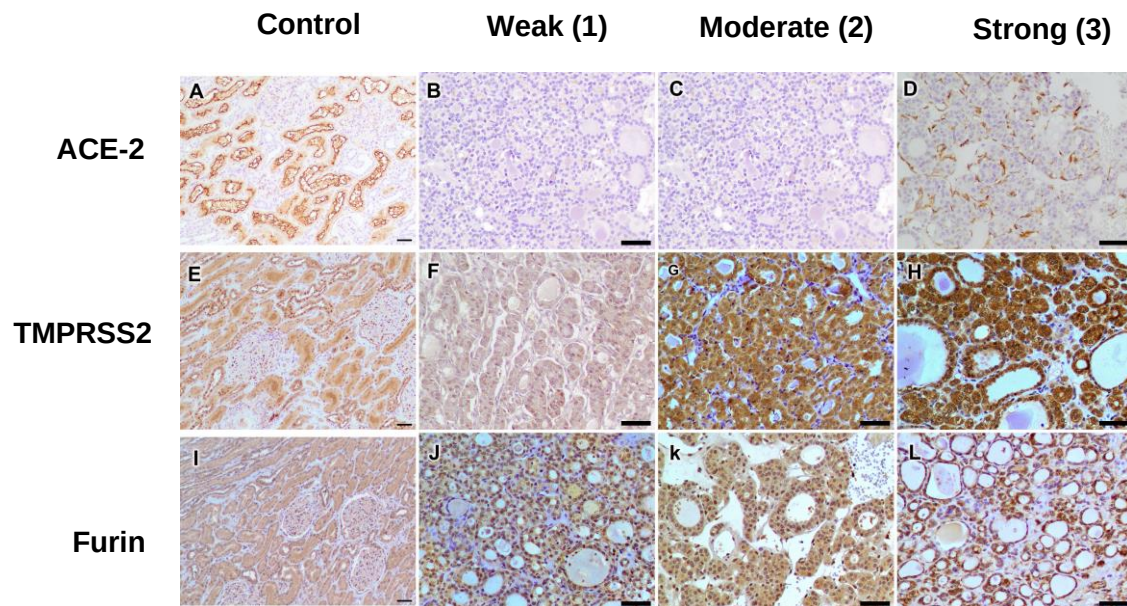


Figure 6 - Representation of the positive control and the different staining intensities of ACE-2, TMPRSS2 and furin in thyroid lesions.

Schematic representation of the staining observed for the positive control (human kidney) and the different staining intensities from weak to strong, for ACE-2 (A-D, respectively), TMPRSS2 (E-H, respectively) and furin (I-L, respectively) in thyroid lesions. Images of positive control are at 100x magnification and of thyroid lesions are at 200x magnification. Scale bar shows 50 µm.

For each protein a cut-off was established according to median score from adjacent thyroid tissue and the staining pattern (**Table 6**).

For ACE-2 only membrane staining was assessed, and a cut-off was established between < 3 and ≥ 3 .

TMPRSS2 and furin showed two locations for the staining: cytoplasmic and nuclear, that were individually classified. Concerning cytoplasmic staining, a cut-off in TMPRSS2 immune score was set between < 12 and 12 . In furin, the cut-off considered was less < 8 and ≥ 8 . Regarding nuclear staining, the same cutoff was established for both TMPRSS2 and furin, < 1 was considered negative and ≥ 1 that was considered positive.

Table 6 – Cut-offs established for immune reactive staining scores of ACE-2, TMPRSS2, and furin.

ACE-2	IRS membranar staining	< 3
		≥ 3
TMPRSS2	IRS cytoplasmic staining	< 12
		12
	IRS nuclear staining	< 1 (Negative)
		≥ 1 (Positive)
Furin	IRS cytoplasmic staining	< 8
		≥ 8
	IRS nuclear staining	< 1 (Negative)
		≥ 1 (Positive)

For the clinicopathological characteristics of the patients the following criteria were considered (**Table 7**): patients age was divided according to the cut-off established by the 8th edition of American Joint Committee on Cancer (AJCC) (Tuttle et al., 2017) into < 55 years and ≥ 55 years; patients' sex was categorized in females and males; the number of lesions was separated into 1 lesion and > 1 lesion per patient; tumor size was divided into ≤ 3 cm and > 3 cm according to the tumors median size present in the series; tumor capsule, capsule invasion, vascular invasion, lymph node metastasis, extrathyroidal extension and lymphocytic infiltration were separated into dichotomous variables (i.e., absence or presence), according to the presence or absence of such characteristics.

Table 7 – Criteria established for patients clinicopathological features

Patient characteristics	
Age	< 55
	≥ 55
Sex	Female
	Male
Number lesions per patient	1
	> 1
Tumor size	≤ 3 cm
	> 3 cm
Tumor capsule	Absence
	Presence
Capsule invasion	Absence
	Presence
Vascular invasion	Absence
	Presence
Lymph node metastasis	Absence
	Presence
Extrathyroidal extension	Absence
	Presence
Lymphocytic infiltration	Absence
	Presence

3.7. Statistical Analysis

Statistical analysis was performed solely on thyroid series. GraphPad Prism 8 software version 8.0.2 (GraphPad, California, USA) and IBM SPSS Statistics 27 (IBM, New York, USA) were used for graphic drawing and statistical analyzes. To determine if the samples follow a normal distribution, Anderson-Darling, D'Agostino & Pearson and Shapiro-Wilk tests were done. In order to compare mRNA expression and protein expression between all group pairs, Mann-Whitney t-test was performed. When the compared groups have a similarly shaped distribution, the Mann-Whitney test compares the medians of the different groups. When the distributions have different shapes, this test ranks all values from lowest to highest, and then compares the mean ranks (Hart, 2001). Therefore, in TMPRSS2 and furin protein expression, regardless median and interquartile range (IQR), the range values, obtained from the difference between the minimum and maximum scores were taken into account. Statistically significant differences were considered when p -value <0.05 . A Spearman's correlation test was used for the correlation between the protein and RNA expression. For statistical evaluation associations between clinicopathological data and immune reactivity score of each protein, firstly a continuous analysis (Mann-Whitney test) was performed, followed by a chi-square test on 2x2 tables; whenever more than 1 cell (25.0%) expected to count less than 5, the Fisher's Exact test was considered.

4. Results

4.1. Thyroid series description

The population of this study was composed by 159 patients presenting thyroid lesions.

Each patient had RNA extracted from frozen tumor and, in a subset, its respective histology (FFPE tissue). The clinical and pathological data of the patients are summarized in **Table 8**. Approximately 80% of our thyroid series was composed of females (n=127). Patients median age was 42 ± 21 years (median $\pm$ IQR), ranging from 11 to 82 years. Most patients presented a single lesion (n=81), and the median tumor size was 3.00 ± 2.50 cm (median $\pm$ IQR). Nearly 70% of tumors (n=92) were encapsuled; capsule invasion occurred in 12 cases (13.2%), and vascular invasion was present in 27 cases (20.1%). Lymph node metastasis was verified in 16 cases (48.5%), extrathyroidal extension (ETE) was observed in 21 cases (21.6%), and lymphocytic infiltrate was present in 48 cases (36.6%).

Table 8 – Clinicopathological characteristics of the thyroid series patients.

Patient characteristics	Total (n=159)
Age	(n=150)
Mean $\pm$ SD	42.9 $\pm$ 15.3
Median $\pm$ IQR	42.0 $\pm$ 21.0
Min – Max	11 - 82
Sex	(n=153)
Female	127 (83.0%)
Male	26 (17.0%)
Clinicopathological features	
Number of lesions	(n=129)
1	81 (62.8%)
> 1	48 (37.2%)
Tumor size (cm)	(n=120)
Mean $\pm$ SD	3.2 $\pm$ 1.6
Median $\pm$ IQR	3.0 $\pm$ 2.5
Min – Max	0.5 - 8.0
Tumor capsule	(n=134)
Absence	42 (31.3%)
Presence	92 (68.7%)
Capsule invasion	(n=91)
Absence	79 (49.7%)
Presence	12 (13.2%)
Vascular invasion	(n=134)
Absence	107 (79.9%)
Presence	27 (20.1%)
Lymph node metastases	(n=33)
Absence	17 (51.5%)
Presence	16 (48.5%)
Extrathyroidal extension	(n=97)
Absence	76 (78.4%)
Presence	21 (21.6%)
Lymphocytic infiltration	(n=131)
Absence	83 (63.4%)
Presence	48 (36.6%)

Abbreviations: SD (Standard deviation), IQR (Interquartile range), Min (Minimum) and Max (Maximum).

4.2. mRNA expression in adjacent thyroid tissue, benign and malignant tumors.

The results obtained from mRNA expression were stratified and analyzed in three different ways for each molecule.

1. Firstly, adjacent thyroid tissues , goiters, follicular thyroid adenomas (FTAs), and thyroid carcinomas were analyzed and compared as separated groups;
2. Then goiters and FTAs were analyzed together as a benign lesions group, and thyroid carcinomas were analyzed separately divided according to the main histological group: papillary carcinomas (PCs), that include cPTC and FVPTC, and thyroid carcinomas with follicular architecture (FCs), that include FTC and OTC;
3. Finally, all the available histotypes were analyzed and compared individually.

Mann-Whitney test was used to search for statistical differences between all group pairs.

The median values and IQR of mRNA expression for each one of the groups and for each one of the transcripts are summarized in **Supplementary table 1 (Appendix I)**.

4.2.1 ACE-2 mRNA expression

The highest ACE-2 mRNA expression was verified in adjacent thyroid tissues (0.372 ± 0.377 , median $\pm$ IQR), followed by thyroid carcinomas (0.176 ± 0.170 , median $\pm$ IQR), adenomas (0.153 ± 0.207 , median $\pm$ IQR) and goiters (0.071 ± 0.024 , median $\pm$ IQR) (**Figure 7A**). All thyroid lesions (goiter, adenomas, and carcinomas) showed significantly reduced mRNA expression compared to the adjacent thyroid tissues.

ACE-2 mRNA expression in thyroid carcinomas was higher in FCs (0.235 ± 0.239 , median $\pm$ IQR) than in PCs (0.171 ± 0.161 , median $\pm$ IQR), whereas the benign lesions presented the lowest expression (0.141 ± 0.207 , median $\pm$ IQR) (**Figure 7B**). As for the previous stratification, a statistically significant decrease in ACE-2 mRNA expression was observed between adjacent thyroid tissues and each of the comparison groups.

Concerning the histotype analysis, the highest mRNA expression levels were verified in OTCs (0.348 ± 0.000 , median $\pm$ IQR), followed by FVPTCs (0.143 ± 0.192 , median $\pm$ IQR), FTCs (0.177 ± 0.265 , median $\pm$ IQR), cPTCs (0.171 ± 0.161 , median

$\pm$ IQR), adenomas (0.153 ± 0.207 , median $\pm$ IQR), and goiters (0.071 ± 0.024 , median $\pm$ IQR) (**Figure 7C**). All comparisons were statistically significant, except for OTCs, but only one case was included with that diagnosis.

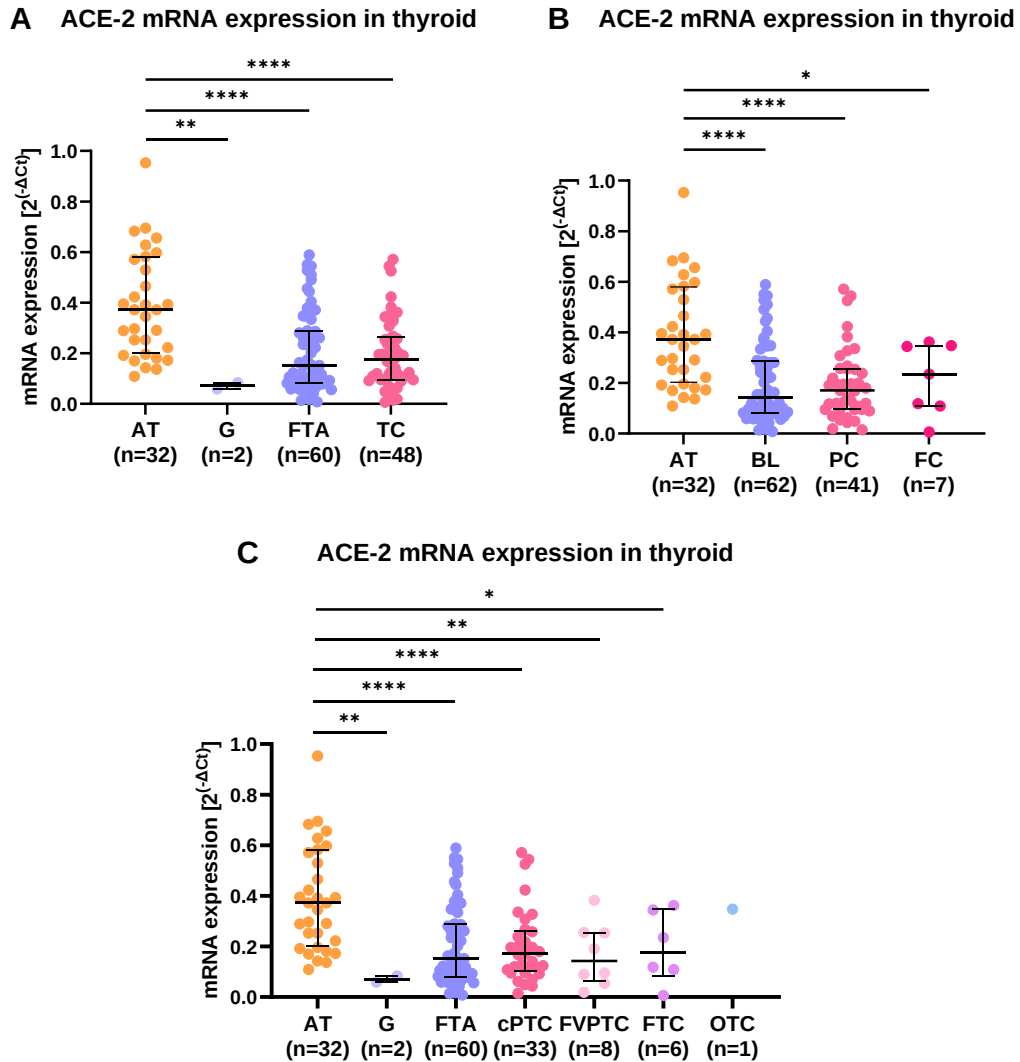


Figure 7 – ACE-2 mRNA expression.

ACE-2mRNA expression levels were stratified differently: in **(A)** stratification was made between groups of AT, G, FTA, and TC; in **(B)** AT, BL, PC, and FC; and **(C)** AT, G, FTA, cPTC, FVPTC, FTC and OTC. Results are shown as median $\pm$ IQR. * p -value ≤ 0.05 , ** p -value ≤ 0.01 , and **** p -value ≤ 0.0001 . **Abbreviations:** AT (adjacent thyroid tissues), G (goiters), FTA (follicular thyroid adenomas), TC (thyroid carcinomas), BL (benign lesions), PC (papillary carcinomas), FC (follicular carcinomas), cPTC (classic papillary thyroid carcinomas), FVPTC (follicular variant of papillary thyroid carcinomas), FTC (follicular thyroid carcinomas) and OTC (oncocytic thyroid carcinomas).

4.2.2. TMPRSS2 mRNA expression

The mRNA expression levels of TMPRSS2 between the group pairs was similar. The highest expression was seen in adjacent thyroid tissues (0.662 ± 0.717 , median $\pm$ IQR) followed by thyroid carcinomas (0.580 ± 1.040 , median $\pm$ IQR), adenomas (0.575 ± 0.926 , median $\pm$ IQR), and goiters (0.488 ± 0.753 , median $\pm$ IQR) (**Figure 8A**).

Within thyroid carcinomas, FCs exhibited more mRNA expression (0.686 ± 1.020 , median $\pm$ IQR) than PCs (0.555 ± 1.046 , median $\pm$ IQR) (**Figure 8B**).

Among the malignant histotypes, FVPTCs (1.099 ± 0.912 , median $\pm$ IQR) and FTCs (0.915 ± 1.113 , median $\pm$ IQR) were the ones with the highest expression (**Figure 8C**).

No statistically significant differences were observed.

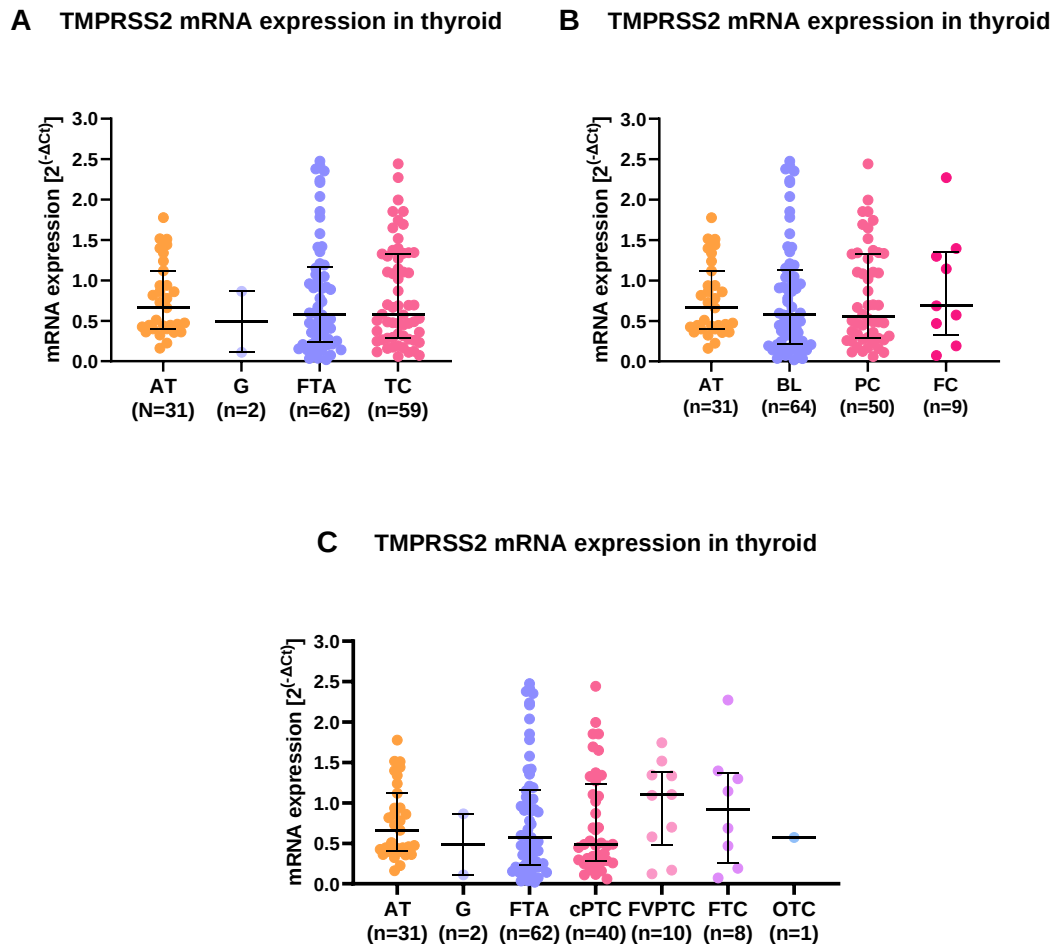


Figure 8 – TMPRSS2 mRNA expression.

TMPRSS2 mRNA expression levels were stratified differently: in **(A)** stratification was made between groups of AT, G, FTA, and TC; in **(B)** AT, BL, PC, and FC; and **(C)** AT, G, FTA, cPTC, FVPTC, FTC and

OTC. Results are shown as median $\pm$ IQR. **Abbreviations:** AT (adjacent thyroid tissues), G (goiters), FTA (follicular thyroid adenomas), TC (thyroid carcinomas), BL (benign lesions), PC (papillary carcinomas), FC (follicular carcinomas), cPTC (classic papillary thyroid carcinomas), FVPTC (follicular variant of papillary thyroid carcinomas), FTC (follicular thyroid carcinomas) and OTC (oncocytic thyroid carcinomas).

4.2.3. Furin mRNA expression

The highest furin mRNA expression levels were observed in adenomas (0.067 ± 0.255 , median $\pm$ IQR), followed by carcinomas (0.047 ± 0.182 , median $\pm$ IQR), adjacent thyroid tissues (0.020 ± 0.013 , median $\pm$ IQR), and goiters (0.015 ± 0.013 , median $\pm$ IQR) (**Figure 9A**). Compared with furin expression levels in adjacent thyroid tissues, a statistically significant increase in benign (0.064 ± 0.254 , median $\pm$ IQR) and malignant thyroid tumors (0.047 ± 0.182 , median $\pm$ IQR) was observed (**Figure 9A and 9B**). Within benign lesions, goiters presented a significant reduction in furin mRNA expression compared to adenomas.

Among thyroid carcinomas, FCs were the group with the lowest furin mRNA expression (0.011 ± 0.030 , median $\pm$ IQR) (**Figure 9B**). A significant increase was verified in expression in benign lesions and PCs (0.067 ± 0.192 , median $\pm$ IQR) compared to the adjacent thyroid tissues. A significant decrease in the expression of FCs when compared to furin expression of benign lesions and PCs was observed.

Regarding the histotypes analysis, cPTC was the papillary histotype with the highest expression (0.067 ± 0.213 , median $\pm$ IQR), whereas FTC was the follicular histotype with the lowest expression levels (0.011 ± 0.006 , median $\pm$ IQR) (**Figure 9C**). Apart from OTC and goiter (0.109 ± 0.000 , median $\pm$ IQR) ($n=1$), all comparisons with adjacent thyroid tissues expression were statistically significant. Significant differences were also observed in relation to FTC, which presented lower expression than adenomas, cPTCs, and FVPTCs (0.046 ± 0.133 , median $\pm$ IQR).

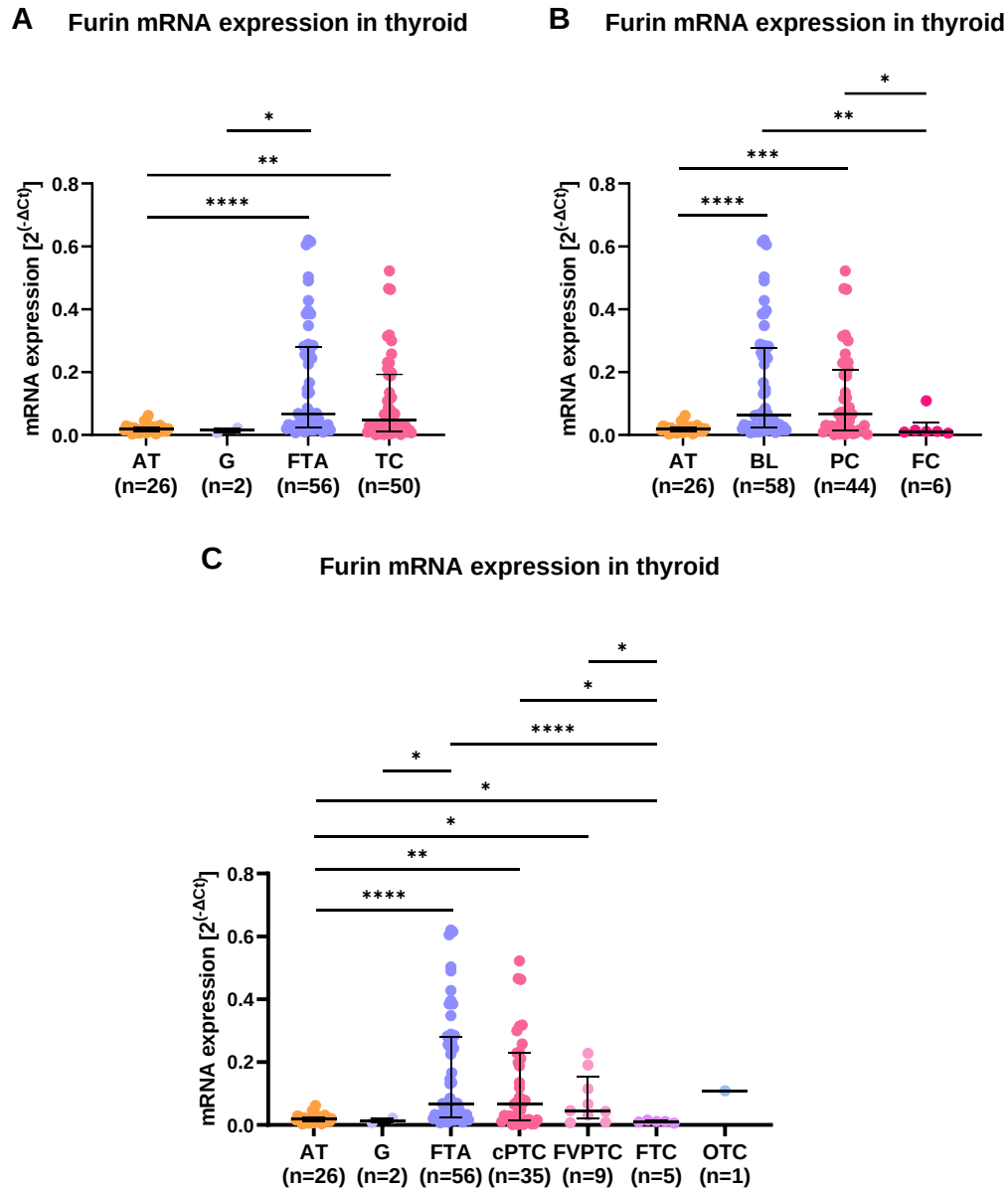


Figure 9 – Furin mRNA expression.

Furin mRNA expression levels were stratified differently: in **(A)** stratification was made between groups of AT, G, FTA, and TC; in **(B)** AT, BL, PC, and FC; and **(C)** AT, G, FTA, cPTC, FVPTC, FTC and OTC. Results are shown as median $\pm$ IQR. * p -value ≤ 0.05 , ** p -value ≤ 0.01 , *** p -value ≤ 0.001 , and **** p -value ≤ 0.0001 . **Abbreviations:** AT (adjacent thyroid tissues), G (goiters), FTA (follicular thyroid adenomas), TC (thyroid carcinomas), BL (benign lesions), PC (papillary carcinomas), FC (follicular carcinomas), cPTC (classic papillary thyroid carcinomas), FVPTC (follicular variant of papillary thyroid carcinomas), FTC (follicular thyroid carcinomas) and OTC (oncocytic thyroid carcinomas).

4.3. Association of mRNA expression and clinicopathologic features

The association between the expression of ACE-2, TMPRSS2, and furin and the clinicopathological characteristics of the patients was evaluated. Mann-Whitney test was performed between transcripts expression in benign lesions, papillary and follicular carcinomas, and patients clinicopathological features (dichotomic variables) as described in **Table 7**.

4.3.1. ACE-2

Regarding the ACE-2 mRNA expression, an association was observed with lesions size and presence of lymphocytic infiltration in benign lesions (adenomas and goiter). Lesions with smaller dimensions (≤ 3 cm) showed significantly higher ACE-2 expression compared to lesions with a larger size (>3 cm) (**Figure 10A**). Lesions presenting lymphocytic infiltration showed significantly increased ACE-2 expression in comparison to lesions without it (**Figure 10B**).

No significant associations were observed in papillary and follicular carcinomas between ACE-2 expression and the patients clinicopathological features.

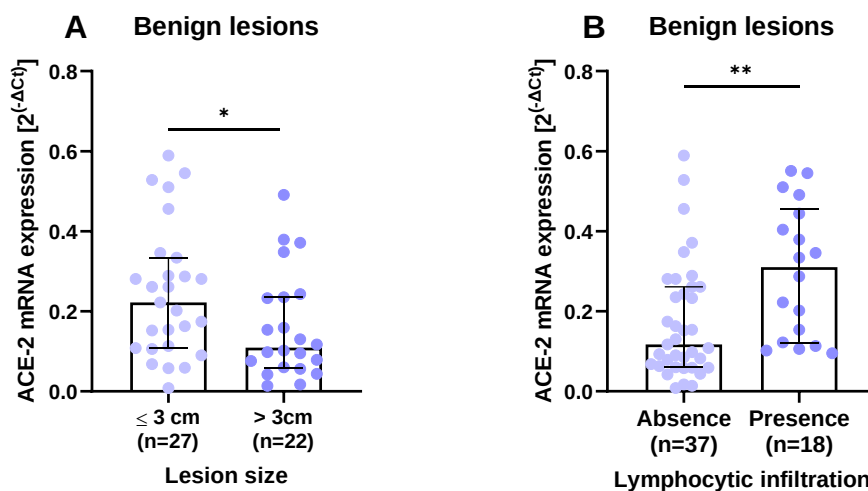


Figure 10 – ACE-2 mRNA expression association with lesion size and presence/absence of lymphocytic infiltration in benign lesions.

(A) ACE-2 expression in benign lesions with lesion size ≤ 3 and > 3 cm; **(B)** ACE-2 expression in benign lesions with the presence and absence of lymphocytic infiltration. Results are shown as median $\pm$ IQR. * p -value ≤ 0.05 and ** p -value ≤ 0.01 .

4.3.2. TMPRSS2

Concerning TMPRSS2 mRNA expression, an association with the age of patients in papillary carcinomas could be established. Older patients (≥ 55 years) showed a significant increase in TMPRSS2 expression compared to younger patients (< 55 years) (**Figure 11**). No significant associations were observed between patients' clinicopathological features in benign lesions and follicular carcinomas.

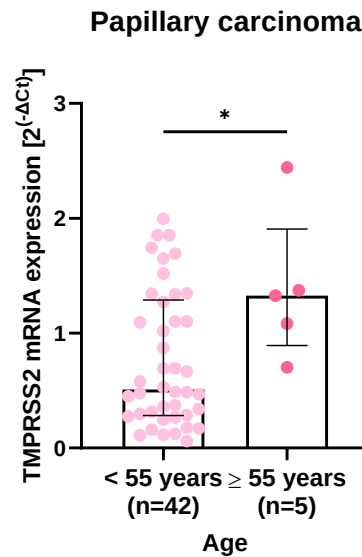


Figure 11 – TMPRSS2 mRNA expression association with age of the patients with papillary carcinoma.

TMPRSS2 expression in papillary tumors in patients with < 55 years and ≥ 55 years. Results are shown as median $\pm$ IQR. * p -value ≤ 0.05 .

4.3.3. Furin

Furin demonstrated an association with patients' sex in papillary carcinomas. Males presented a statistically significant increase in furin expression compared to females (**Figure 12**). No other significant associations were observed in benign lesion and follicular carcinomas.

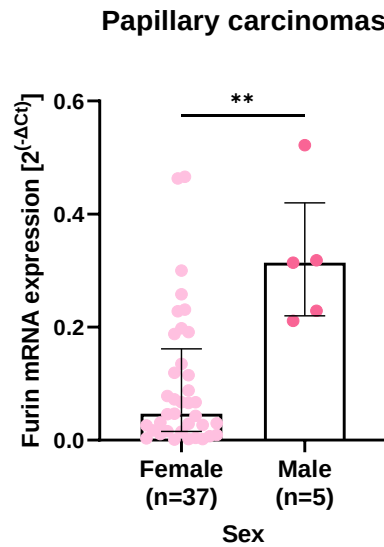


Figure 12 – Furin mRNA expression association with sex of the patients with papillary carcinomas.

Furin expression in papillary tumors in females and males. Results are shown as median $\pm$ IQR. ***p*-value ≤ 0.01 .

4.4. Protein expression in thyroid in adjacent thyroid tissue, benign and malignant tumors

Protein expression was quantified using an immunoreactive score (IRS), as described in the methodology chapter. The results obtained from the protein expression analysis were stratified in the same way as for the mRNA expression.

All median values, IQR and the range values, for each group are summarized in **Supplementary table 1 (Appendix I)**.

4.4.1. ACE-2 protein expression

The staining pattern observed for ACE-2 in thyroid lesions was predominantly membranous and mainly positive in vascular cells (**Figure 13A and 13B**). No ACE-2 staining was observed in the follicular cells.

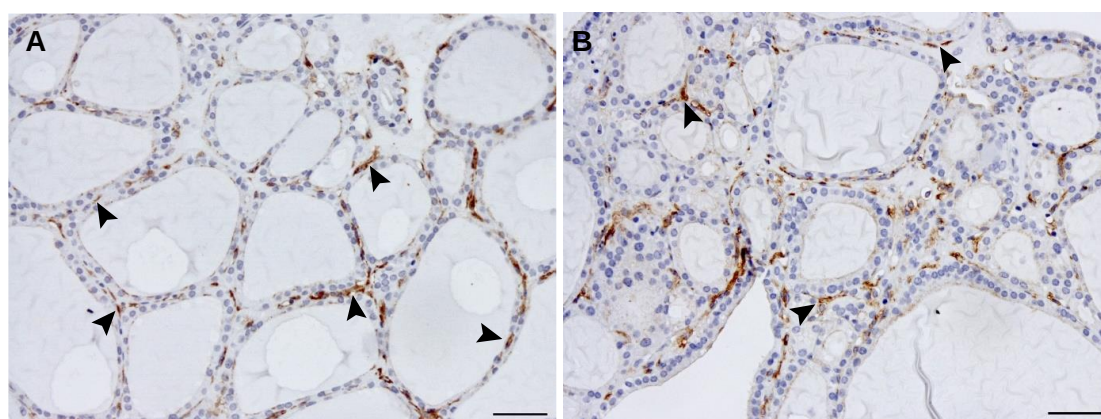


Figure 13 — Immunohistochemical staining pattern of ACE-2.

Representative images of ACE-2 staining in **(A)** adjacent thyroid tissue and **(B)** follicular variant of papillary thyroid carcinoma. The staining is mainly membranous, located in vascular cells (**arrow heads**). Images are at 200x magnification and scale bar shows 50 μ m.

Goiters (5.000 ± 6.000 , median IRS $\pm$ IQR) and adenomas (4.000 ± 6.500 , median IRS $\pm$ IQR) were the tissues with the highest ACE-2 immune expression, followed by the adjacent thyroid tissues (2.000 ± 2.750 , median IRS $\pm$ IQR) and thyroid carcinomas (2.000 ± 2.000 , median IRS $\pm$ IQR) that presented the same level of expression (**Figure 14A**). The ACE-2 IRS score in adjacent thyroid tissues and thyroid carcinomas was significantly lower than in adenomas.

Within thyroid carcinomas, FCs exhibited the highest ACE-2 score (3.000 ± 3.750 , median IRS $\pm$ IQR) (**Figure 14B**). ACE-2 expression in adjacent thyroid tissues and PCs (1.500 ± 2.000 , median IRS $\pm$ IQR) was significantly reduced compared to benign lesions (4.000 ± 6.000 , median IRS $\pm$ IQR).

Among the malignant tumors, cPTCs presented the lowest ACE-2 protein levels (0.000 ± 2.000 , median IRS $\pm$ IQR) (**Figure 14C**). In contrast to adjacent thyroid tissues, adenomas exhibited a significant increase, whereas cPTCs showed a significant decrease. Benign lesions (goiter and adenomas), FVPTCs (3.500 ± 4.000 , median IRS $\pm$ IQR) and FTCs (3.000 ± 2.750 , median IRS $\pm$ IQR) exhibited a significant increase in ACE-2 protein expression compared to cPTCs.

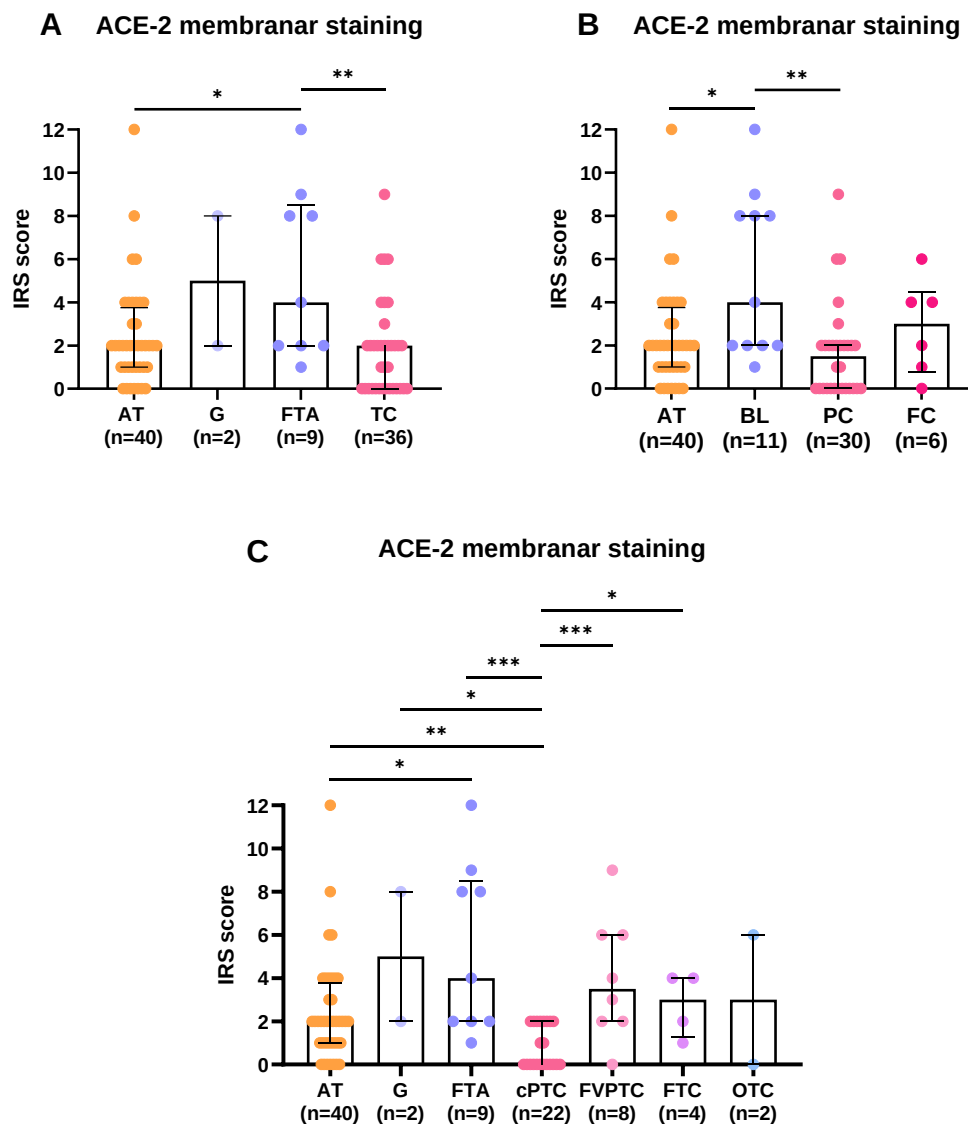


Figure 14 – ACE-2 membranar expression.

ACE-2 IRS scores were stratified differently: in **(A)** stratification was made between groups of AT, G, FTA, and TC; in **(B)** AT, BL, PC, and FC; and **(C)** AT, G, FTA, cPTC, FVPTC, FTC and OTC. Results are shown as median $\pm$ IQR. *p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001 . **Abbreviations:** AT (adjacent thyroid tissues), G (goiters), FTA (follicular thyroid adenomas), and TC (thyroid carcinomas), BL (benign lesions), PC (papillary carcinomas), and FC (follicular carcinomas), cPTC

(classic papillary thyroid carcinomas), FVPTC (follicular variant of papillary thyroid carcinomas), FTC (follicular thyroid carcinomas) and OTC (oncocytic thyroid carcinomas).

4.4.2. TMPRSS2 protein expression

The staining pattern observed for TMPRSS2 was predominantly cytoplasmic and occasionally nuclear located in follicular cells (**Figure 15A and 15B**). Cytoplasmic and nuclear staining were analyzed separately.

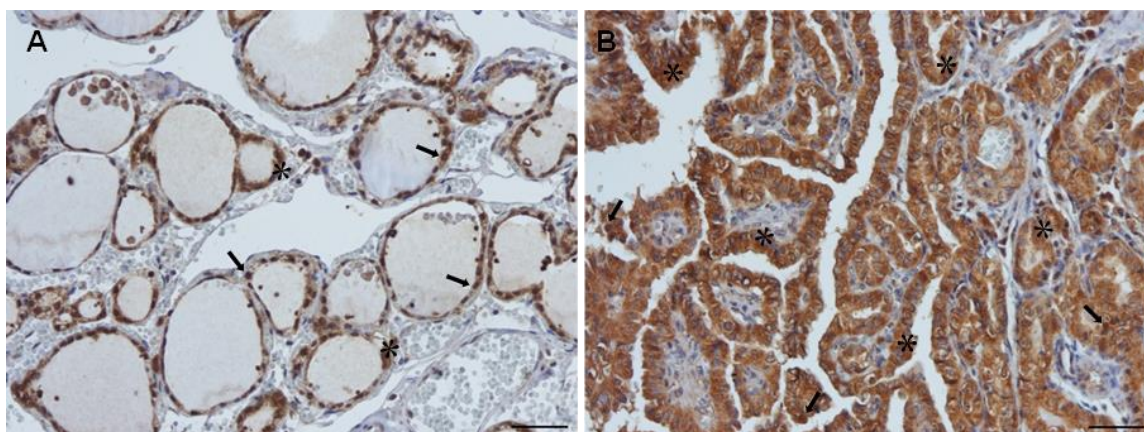


Figure 15 – Immunohistochemical staining pattern of TMPRSS2.

Representative images of TMPRSS2 staining in **(A)** adjacent thyroid tissue and **(B)** classic papillary thyroid carcinoma. The staining is mainly cytoplasmic (**asterisks**), and occasionally nuclear (**arrows**), located to follicular cells. Images are at 200x magnification and scale bar shows 50 μ m.

a) Cytoplasmic Staining

The highest TMPRSS2 IRS score at cytoplasmic location occurred in the thyroid adjacent tissues (12.000 ± 4.000 , 8.000; median IRS $\pm$ IQR, range value), followed by goiters (10.000 ± 4.000 , 4.000; median IRS $\pm$ IQR, range value), thyroid carcinomas (8.000 ± 4.000 , 9.000; median IRS $\pm$ IQR, range value), and adenomas (8.000 ± 4.000 , 8.000; median IRS $\pm$ IQR, range value) that presented de same IRS score median values (**Figure 16A**).

In thyroid carcinomas (PCs and FCs) a significant decrease in TMPRSS2 score comparatively to the adjacent thyroid tissues was observed (**Figure 16 A and 16B**).

The median expression scores observed was equal in benign lesions (8.000 ± 4.000 , 8.000; median IRS $\pm$ IQR, range value) and PCs (8.000 ± 4.000 , 9.000; median IRS $\pm$ IQR, range value). FCs (8.000 ± 1.250 , 5.000; median IRS $\pm$ IQR, range value) presented a significant decrease in TMPRSS2 cytoplasmic expression compared benign lesions (**Figure 16B**).

All malignant histotypes presented a similar level of expression, nevertheless in FVPTC (8.000 ± 0.000 , 4.000; median IRS $\pm$ IQR, range value), and FTC (8.000 ± 3.750 , 5.000; median IRS $\pm$ IQR, range value), a significant decrease was observed comparatively to thyroid adjacent tissue (**Figure 16C**).

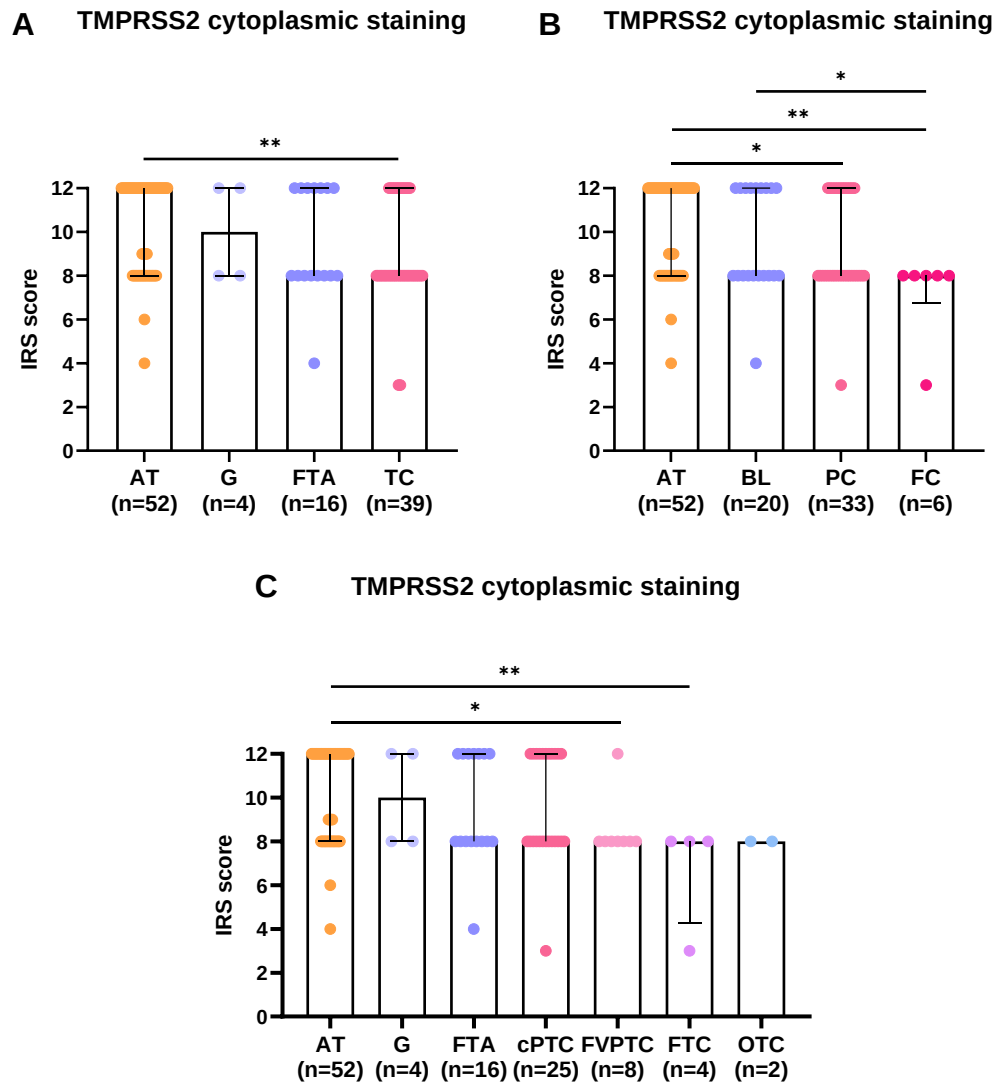


Figure 16 – TMRSS2 cytoplasmic expression.

TMRSS2 cytoplasmic IRS scores were stratified differently: in **(A)** stratification was made between groups of AT, G, FTA, and TC; in **(B)** AT, BL, PC, and FC; and **(C)** AT, G, FTA, cPPTC, FVPTC, FTC and OTC. Results are shown as median $\pm$ IQR. *p-value ≤ 0.05 and **p-value ≤ 0.01 . **Abbreviations:** AT (adjacent thyroid tissues), G (goiters), FTA (follicular thyroid adenomas), and TC (thyroid carcinomas), BL (benign lesions), PC (papillary carcinomas), and FC (follicular carcinomas), cPPTC (classic papillary thyroid carcinomas), FVPTC (follicular variant of papillary thyroid carcinomas), FTC (follicular thyroid carcinomas) and OTC (oncocytic thyroid carcinomas).

b) Nuclear staining

TMPRSS2 nuclear expression was detected at the same level in all analyzed groups. Adenomas (0.000 ± 3.500 , 8.000 ; median IRS $\pm$ IQR, range value) presented a highest levels of TMPRSS2 nuclear immune score, followed by thyroid carcinomas (0.000 ± 3.000 , 9.000 ; median IRS $\pm$ IQR, range value), goiters (0.000 ± 3.000 , 4.000 ; median IRS $\pm$ IQR, range value), and adjacent tissue (0.000 ± 2.000 , 8.000 ; median IRS $\pm$ IQR, range value) (**Figure 17A**). FCs (0.000 ± 5.000 , 5.000 ; median IRS $\pm$ IQR, range value) presented the higher furin expression score compared to PCs (0.000 ± 2.500 , 9.000 ; median IRS $\pm$ IQR, range value) (**Figure 17B**).

Among malignant histotypes, FVPTC (1.000 ± 5.250 , 4.000 ; median IRS $\pm$ IQR, range value) presented the highest TMPRSS2 nuclear score (**Figure 17C**). However, no relevant differences were found.

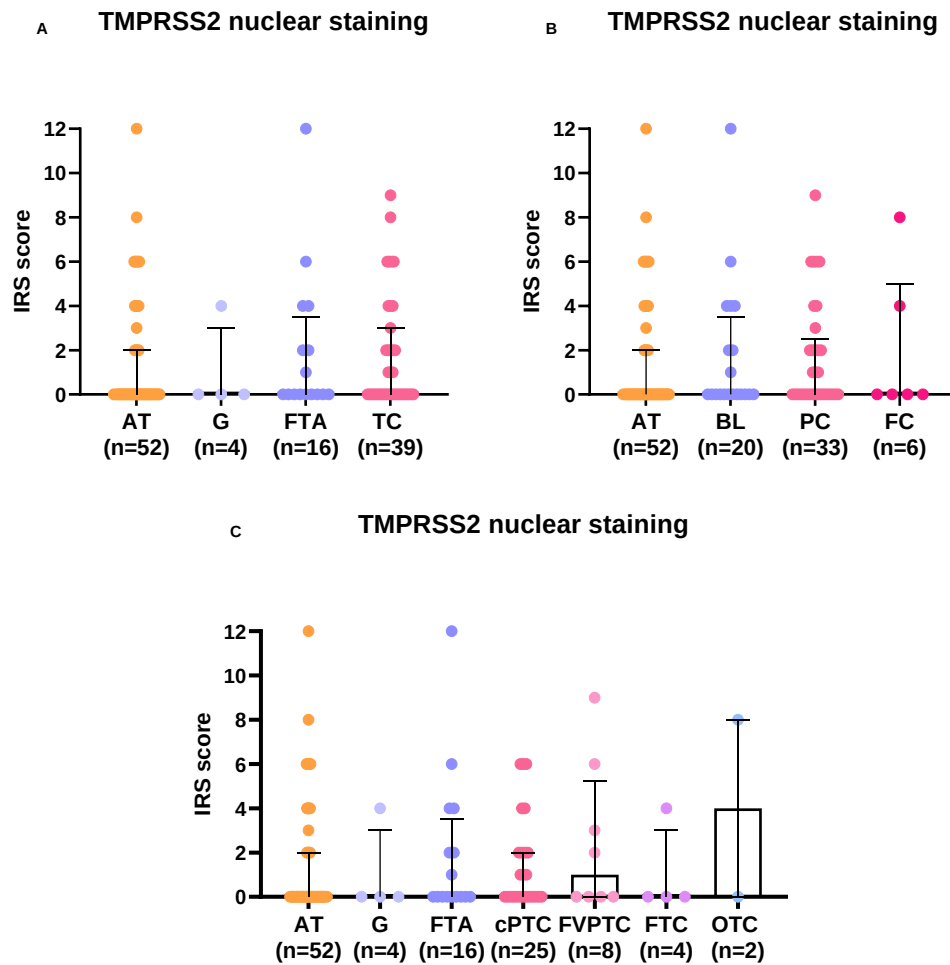


Figure 17 – TMRSS2 nuclear expression.

TMRSS2 nuclear IRS scores were stratified differently: in **(A)** stratification was made between groups of AT, G, FTA, and TC; in **(B)** AT, BL, PC, and FC ; and **(C)** AT, G, FTA, cPTC, FVPTC, FTC and OTC. Results are shown as median $\pm$ IQR. **Abbreviations:** AT (adjacent thyroid tissues), G (goiters), FTA (follicular thyroid adenomas), and TC (thyroid carcinomas), BL (benign lesions), PC (papillary carcinomas), and FC (follicular carcinomas), cPTC (classic papillary thyroid carcinomas), FVPTC (follicular variant of papillary thyroid carcinomas), FTC (follicular thyroid carcinomas) and OTC (oncocytic thyroid carcinomas).

4.4.3. Furin protein expression

Similarly, to TMPRSS2 profile, the furin staining patterns in the thyroid gland were predominantly cytoplasmic and occasionally nuclear, specifically located in follicular cells (**Figure 18A, 18B and 18C**).

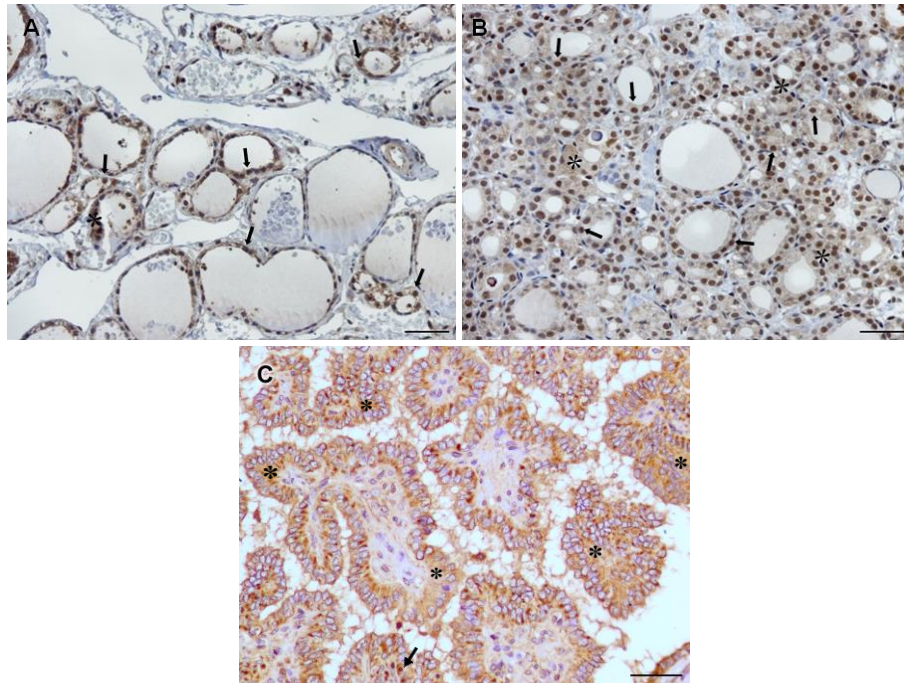


Figure 18 – Immunohistochemical staining pattern of furin.

Representative images of furin staining in (A) adjacent thyroid tissue (B) follicular thyroid adenoma and (C) classic papillary thyroid carcinoma. The staining is both cytoplasmic (asterisks) and nuclear (arrows), located to follicular cells. Images are at 200x magnification and scale bar shows 50 μ m.

a) Cytoplasmic Staining

Furin IHC expression median levels were equal in normal adjacent tissues (8.000 ± 8.000 , 12.000; median IRS $\pm$ IQR, range value), adenomas (8.000 ± 4.000 , 9.000; median IRS $\pm$ IQR, range value), and carcinomas (8.000 ± 5.000 , 8.000; median IRS $\pm$ IQR, range value); goiters presented a slightly lower expression (7.500 ± 9.000 , 9.000; median IRS $\pm$ IQR, range value) (**Figure 19A**). When combining goiters and adenomas in the same group of benign lesions (8.000 ± 8.000 , 9.000; median IRS $\pm$ IQR, range value), no differences between the median expression levels with adjacent tissues and malignant tumors (papillary and follicular carcinomas) were observed (**Figure 19B**).

All histotypes presented similar furin immune score with the exception of OTCs that had the highest expression (8.500 ± 1.000 , 1.000; median IRS $\pm$ IQR, range value), and goiters that presented the lowest (**Figure 19C**).

No statistically differences were found.

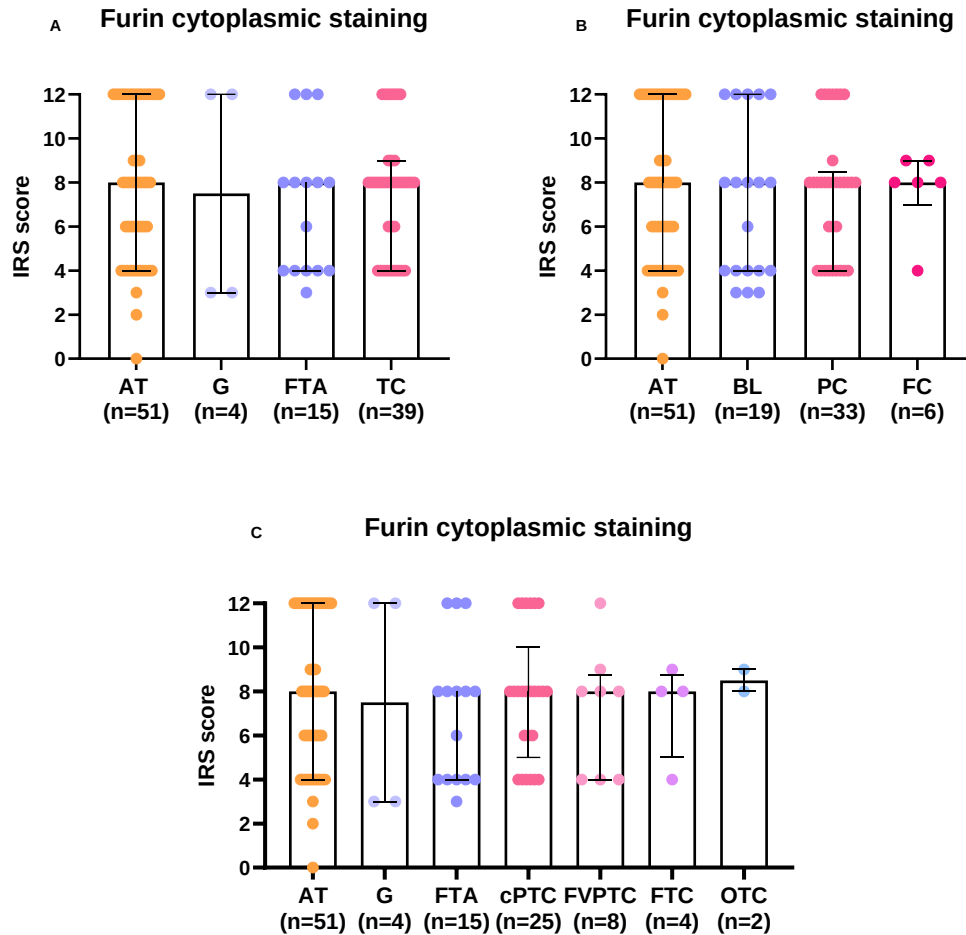


Figure 19 – Furin cytoplasmic expression.

Furin cytoplasmic IRS scores were stratified differently: in **(A)** stratification was made between groups of AT, G, FTA, and TC; in **(B)** AT, BL, PC, and FC; and **(C)** AT, G, FTA, cPTC, FVPTC, FTC and OTC. Results are shown as median $\pm$ IQR. **Abbreviations:** AT (adjacent thyroid tissues), G (goiters), FTA (follicular thyroid adenomas), and TC (thyroid carcinomas), BL (benign lesions), PC (papillary carcinomas), and FC (follicular carcinomas), cPTC (classic papillary thyroid carcinomas), FVPTC (follicular variant of papillary thyroid carcinomas), FTC (follicular thyroid carcinomas) and OTC (oncocytic thyroid carcinomas).

b) Nuclear Staining

Nuclear furin expression was similar in all the analyzed groups (0.000, median IRS), however statistical tests retrieved significance out of some comparisons. In contrast to adenomas (0.000 ± 4.000, 8.000; median IRS ± IQR, range value), adjacent thyroid tissues (0.000 ± 0.000, 8.000; median IRS ± IQR, range value) and thyroid carcinomas (0.000 ± 0.000, 2.000; median IRS ± IQR, range value) presented a significant decrease in furin expression (**Figure 20A**).

Within thyroid carcinomas, a significant decrease was verified in PCs (0.000 ± 0.000, 2.000; median IRS ± IQR, range value) when compared to benign lesions (0.000 ± 2.000, 8.000; median IRS ± IQR, range value) (**Figure 20B**).

Concerning all histotypes, cPTCs (0.000 ± 0.000, 2.000; median IRS ± IQR, range value) exhibited a significant decrease in furin nuclear expression comparatively to adenomas (**Figure 20C**).

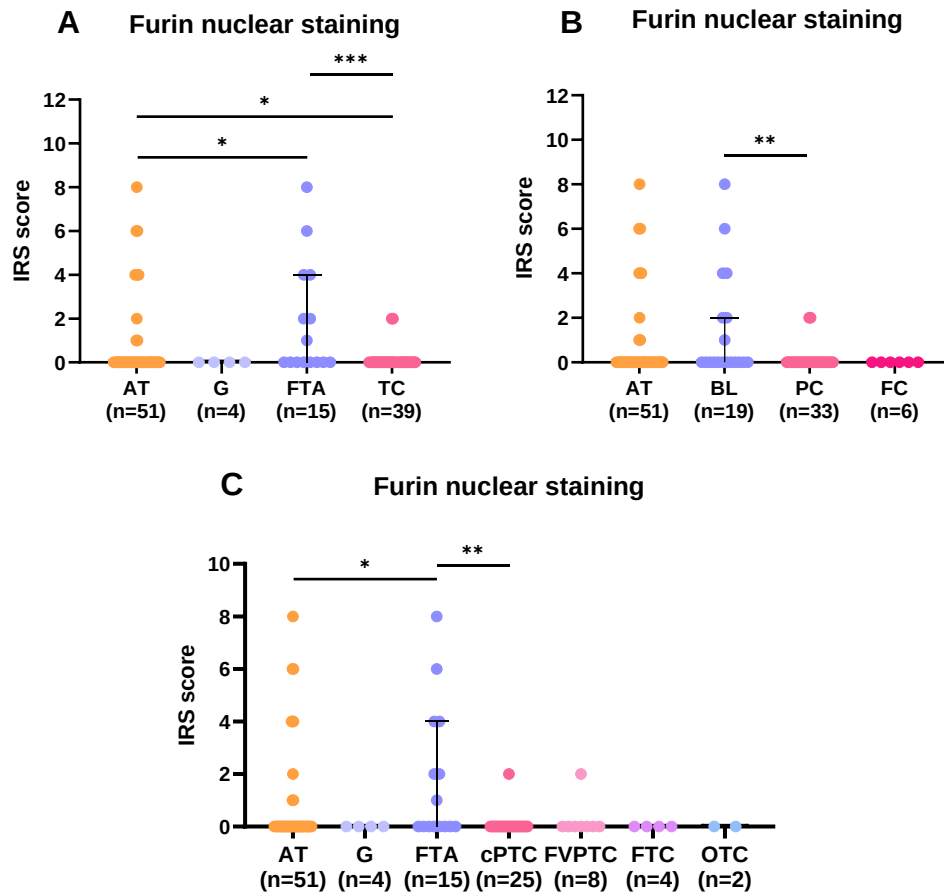


Figure 20 – Furin nuclear expression.

Furin nuclear IRS scores were stratified differently: in **(A)** stratification was made between groups of AT, G, FTA and TC; in **(B)** AT, BL, PC, and FC; and **(C)** AT, G, FTA, cPTC, FVPTC, FTC and OTC. Results are shown as median $\pm$ IQR. *p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001 . **Abbreviations:** AT (adjacent thyroid tissues), G (goiters), FTA (follicular thyroid adenomas), and TC (thyroid carcinomas), BL (benign lesions), PC (papillary carcinomas), and FC (follicular carcinomas), cPTC (classic papillary thyroid carcinomas), FVPTC (follicular variant of papillary thyroid carcinomas), FTC (follicular thyroid carcinomas) and OTC (oncocytic thyroid carcinomas).

4.5. Correlation between RNA and protein expression

Correlation analysis between mRNA expression by RT-qPCR of the three transcripts and the respective protein expression by IHC was performed, retrieving no statistical differences (data not shown).

4.6. Association of protein expression and clinicopathologic features

The associations between the expression of each of the proteins under study (ACE-2, TMPRSS2, and furin) and the patients' clinicopathological characteristics was evaluated. Firstly, a continuous analysis was performed (Mann-Whitney test) in benign lesions, papillary and follicular carcinomas; next, the protein expression was categorized, as described previously in the material and methods chapter (**Table 6 and 7**). The chi-square test was performed between dichotomous variables.

Concerning the ACE-2 protein expression, in a continuous analysis, we observed in papillary carcinomas, that the absence of lymphocytic infiltrate was associated with a higher immune expression of ACE-2 compared to those with presence (**Figure 21**).

The chi-square test revealed statistically significant differences with the absence/presence of tumor capsule in papillary carcinomas. Encapsulated tumors were associated with a higher ACE-2 IRS score (**Supplementary table 2**)

Regarding cytoplasmic and nuclear protein expression of TMPRSS2 and furin, no associations with clinicopathological features of patients were found (**Supplementary table 3 and 4**).

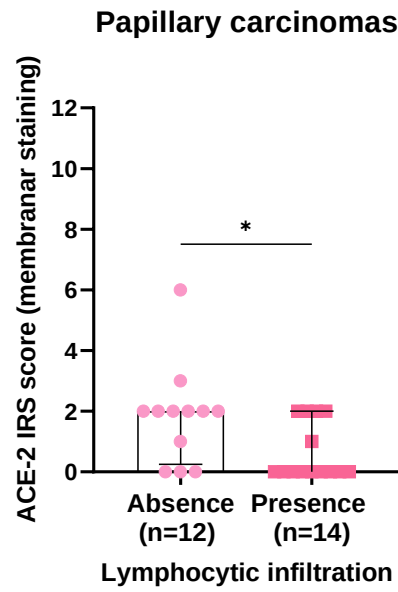


Figure 21 – Association of ACE-2 protein expression with the absence/presence of lymphocytic infiltration in papillary carcinomas patients.

The absence of lymphocytic infiltration in papillary carcinomas patients was associated with a higher immune ACE-2 expression. Results are shown as median $\pm$ IQR. * p -value ≤ 0.05 .

5. Discussion

Thyroid carcinoma is the most common neoplasia in the endocrine system with an increasing incidence (Nguyen et al., 2015; Raue & Frank-Raue, 2016).

During the COVID-19 pandemic, several cases of subacute thyroiditis and thyrotoxicosis were reported in patients after SARS-CoV-2 infection (Murugan & Alzahrani, 2021; Prete et al., 2021), suggesting that this virus invades and affects thyroid cells. However, the implications in thyroid cancer require further investigation. Recently, it was reported that the incidence of COVID-19 was lower in thyroid cancer patients than in normal population (Murugan & Alzahrani, 2021; Prete et al., 2021).

This event highlighted the need for proper characterization of the expression of key host determinants for SARS-CoV-2 viral infection, namely ACE-2, TMPRSS2, and furin, which remains to be established in normal thyroid and in thyroid tumors (Murugan & Alzahrani, 2021). Our aim with this study was to primarily characterize these molecules in a series of thyroid lesions composed of benign and malignant neoplasms. We wanted to verify if these proteins can have a potential role on thyroid carcinogenesis. The expression of ACE-2, TMPRSS2, and furin was analyzed at mRNA and at protein levels; the adjacent thyroid tissues of the lesions were also evaluated and used as a baseline.

Our first approach was to characterize the mRNA expression of ACE-2, TMPRSS2, and furin. Regarding the ACE-2 mRNA expression levels, our results showed that benign and malignant thyroid lesions presented significantly less expression than adjacent thyroid tissue, however we did not observe relevant differences between these two latter groups. Up to date, several studies reported the high ACE-2 mRNA expression levels in normal thyroid tissues and in normal thyroid cell lines (Hikmet et al., 2020; Hoffmann et al., 2020; Park et al., 2022; Rotondi et al., 2021; Scappaticcio et al., 2021). Still, there are few studies that characterized the ACE-2 mRNA expression in thyroid lesions. Some studies are in agreement with our results, showing a significant reduction in ACE-2 mRNA expression between thyroid cancer versus normal control (Bao et al., 2020; Chai et al., 2020). On the other hand, in a study using a thyroid series composed only of benign and malignant thyroid lesions, the authors noted a decrease in ACE-2 mRNA expression in benign lesions (goiters and adenomas) versus the expression in malignant lesions (PTC and FTC) (Narayan et al., 2020).

In our study, all thyroid tissues showed elevated mRNA levels TMPRSS2 expression, compared to housekeeping gene expression, however we did not

observe relevant differences between expression in adjacent tissues and thyroid lesions. Although the information available in the literature regarding the expression of this transcript is limited, some studies reported the relatively high mRNA expression levels of TMPRSS2 in normal thyroid and in a follicular thyroid cell line (Lazartigues et al., 2020; Park et al., 2022; Scappaticcio et al., 2021; Zhou et al., 2020). Besides thyroid tumors, TMPRSS2 has also been shown to be dysregulated in several other tumors such as prostate and lung adenocarcinomas (Chen et al., 2010; Lucas et al., 2014; Xiao et al., 2022). So far, only *in silico* analyses have been performed, pointing that the expression of TMPRSS2 is downregulated in thyroid carcinomas compared to healthy thyroid tissues (Bao et al., 2020; Meng et al., 2022; Ravaioli et al., 2020; Xiao et al., 2022).

In our results, we observed a significant increase in the mRNA expression of this transcript in benign and malignant lesions compared to adjacent tissue expression. Relevant differences were found between benign lesions (goiter vs adenomas) and malignant lesions (papillary vs follicular carcinomas). Additionally, we observed that benign lesions showed a significant higher expression than follicular carcinomas. Within thyroid carcinomas, we observed that increased furin mRNA expression seemed to be associated with the papillary pattern. Some studies reported that thyroid gland has been reported as one of the organs with the highest expression of furin (Zhou & Gao, 2021; Zhou et al., 2020). The furin mRNA expression is altered in various types of cancer such as ovary cancer, breast, head and neck, brain, non-small cell lung carcinoma, and rhabdomyosarcoma (Coppola et al., 2008; Jaaks & Bernasconi, 2017). Nevertheless, the literature still lacks studies on furin mRNA expression in both normal and thyroid tumors.

In this work we also identified significant associations between the expression of each of the transcripts (ACE-2, TMPRSS2 and furin) and the clinicopathological characteristics of the patients.

Concerning ACE-2 mRNA expression, smaller lesions (≤ 3 cm) and the presence of lymphocytic infiltration were associated with higher ACE-2 expression in patients with benign lesions. The same associations, regarding lesions size, have been reported in thyroid tumors (Narayan et al., 2020), however in our series we did not verify statistically significant associations between ACE-2 mRNA expression and the size of the malignant lesions.

Relatively to lymphocytic infiltration, it was reported that the mean levels of ACE-2 mRNA can be positively modulated by cytokines, in cell cultures of primary normal human thyroids after treatment with IFN- γ and, to a lesser extent, with TNF- α (Coperchini et al., 2021). IFN- γ and TNF- α are respectively produced and secreted

by activated lymphocytes (Castro et al., 2018) and monocytic cells (Zhu et al., 2020). These cytokines are also known to be involved in thyroid autoimmune pathogenesis and capable of affecting the differentiation, growth and, function of thyroid cells (Miyazaki et al., 1999)

For the TMPRSS2 mRNA expression, our study showed that older patients with papillary carcinomas demonstrated an association with a higher expression of TMPRSS2. In thyroid cancer, age is considered an important prognostic factor, especially in papillary tumors, being older patients associated with worse prognosis, disease-specific survival, and disease-free survival (Cho et al., 2012; Jonklaas et al., 2012; Kauffmann et al., 2018). To our knowledge, there is no information on the association between age and the mRNA expression of TMPRSS2 in normal thyroid tissues or in thyroid lesions. However, this trend has been observed in the olfactory epithelium (David et al., 2022) and lung tissues from mice (Schuler et al., 2020), and oral epithelia from humans (Peng et al., 2021). In contrast, a study that analyzed the total mRNA expression of TMPRSS2 in the lung reported no significant age differences (Asselta et al., 2020). Nevertheless, Ravaioli and collaborators, in a study analyzing data from public repositories have reported higher TMPRSS2 expression in tumors from older patients (Ravaioli et al., 2020).

Finally, regarding furin mRNA expression, our results showed a significant association with the patient sex in papillary tumors, since males presented a higher furin expression compared to females. To our knowledge there's still a lack of information in the literature concerning this association in thyroid tumors or in normal thyroid tissue. Nevertheless, in a study performed in bronchial brushing samples reported that furin levels were higher in males (Kermani et al., 2021). However, in lung adenocarcinomas, the association seems to be contradictory, since furin mRNA levels were significantly higher females (Luo et al., 2022).

After mRNA expression characterization, we attempted to establish the cellular sub-localization of the ACE-2, TMPRSS2 and furin, using IHC, and to evaluate their expression using an IRS score (Fedchenko & Reifenrath, 2014).

The main staining pattern observed for ACE-2 was membranous, positive mainly in endothelial cells of small vessels. This staining pattern was observed equally in both adjacent thyroid tissue and in the lesions. The benign lesion groups (i.e., goiter and adenomas) had the highest score compared to normal tissue and thyroid carcinomas. Papillary thyroid carcinomas (i.e., cPTC and FVPTC) were the ones that showed the lowest expression of this protein. We also observed significant differences between the expression of ACE-2 in normal tissue and adenomas and papillary carcinomas. These results are consistent with what is described in the

literature. Several evidences had already demonstrated that ACE-2 was mainly expressed in cells of small vessels (Bargehr et al., 2021; Guney & Akar, 2021; Zulli et al., 2008). A study reported that ACE-2 staining in thyroid was essentially located to small subsets of cells, such as endothelial cells and pericytes (Hikmet et al., 2020).

Park *et al.* verified that ACE-2 was mainly positive at microvessels or lymphatics between thyroid follicles of humans and rats, but no staining was detected in follicular cells. The same authors observed that ACE-2 was exclusively expressed in the thyroid pericytes from rats, using a pericyte marker neuron-gial antigen 2 (NG2) (Park et al., 2022). A study developed in our research group by Macedo *et al.*, also analyzed the expression of ACE-2 in normal thyroid tissues from patients who died due to SARS-CoV-2 infection. Concordantly with our results, ACE-2 displayed a basal staining that seems to colocalize with endothelial cells (Macedo et al., 2022).

With regards to the expression of this protein in thyroid lesions, the information is controversial and there are few works on the matter.

A systematic review of tissue microarrays performed by Meiners *et al.* showed that the highest positive ACE-2 levels in capillaries occurred in tumors with (neuro)endocrine activity such as thyroid tumors (Meiners et al., 2021). They also showed that adenomas of the thyroid gland had, in blood vessels, a higher ACE-2 positive fraction compared to that seen in papillary tumors (Meiners et al., 2021).

In contrast, other study reported that ACE-2 was mainly located in the cytoplasm of follicular cells and sporadically also in their nuclei (Narayan et al., 2020). The authors evaluated the expression of ACE-2 and detected a high immunoreactivity in FTCs and PTCs compared to the other tissues (goiters and adenomas) (Narayan et al., 2020). In the study performed by Ricardo *et al.*, the expression of this protein was assessed in thyroid carcinomas, specifically in papillary tumors, and no ACE-2 expression was observed restricted to thyroid follicular cells (Ricardo et al., 2022). Those divergent results can be related with methodological differences, namely the use of different antibodies. From the available literature the most consistent data support our findings, *i.e.*, the restricted expression of ACE-2 in the endothelial cells of small vessels.

For TMPRSS2 protein expression, two main staining patterns were found: cytoplasmic and nuclear. Regarding the TMPRSS2 cytoplasmic score, we observed a significant lower TMPRSS2 expression in carcinomas than in adjacent tissue expression. A statistically significant difference between benign lesions and follicular carcinomas was also found. Regarding nuclear staining, no significant differences were observed between the groups.

There are few studies on TMPRSS2 protein expression in the literature in normal thyroid tissue, and we are not aware of any study in thyroid carcinomas. Park *et al.* demonstrated in normal thyroid tissues that TMPRSS2 was expressed in the colloid within follicles, but not in follicular cells or microvessels (Park *et al.*, 2022). In a previous work of our group, Macedo *et al.* reported that the expression of TMPRSS2 observed in normal thyroid samples from COVID-19 deceased patients, was quite high and the staining was mostly cytoplasmic and occasionally nuclear (Macedo *et al.*, 2022).

In previous studies in other cancers nuclear expression of TMPRSS2 was not reported, so further studies are needed in order to clarify the specificity of the nuclear staining, or, alternatively, if we are facing any technical issue or an unspecific cross reaction.

We have also analyzed furin protein expression, where we detected that this proteins was also expressed in follicular cells and showed essentially a cytoplasmic and focal nuclear staining pattern. In cytoplasmic labeling no significant differences could be observed between the groups (adjacent tissue, benign and malignant lesions). Regarding the nuclear expression of furin, a significant increase in adenomas was verified, in contrast to the expression observed in adjacent tissue and carcinomas. Additionally, a significant decrease was noted between papillary carcinomas and adenomas.

From the few studies available, furin expression was generally reported as low in normal thyroid tissue furin expression with cytoplasmic staining (Macedo *et al.*, 2022). The only study evaluating furin expression in thyroid carcinomas, more specifically in papillary thyroid carcinomas, demonstrated that furin staining was observed in all analyzed tissues limited to the cytoplasm in a granular pattern (Ricardo *et al.*, 2022).

Despite furin cytoplasmic expression, HPA reported that several cancers presented furin nuclear staining (HPA, 2022c; Uhlén *et al.*, 2015).

The next step was to correlate the mRNA levels and protein expression in adjacent thyroid and thyroid lesions. We observed that no significant correlation was found between mRNA expression and protein expression of the evaluated molecules. In general, mRNA expression is known to have a positive correlation with protein levels, but the strength of the correlation varies greatly according to the gene/protein under study (Guo *et al.*, 2008). Nonconformities between mRNA and protein have been already reported in several studies (Wang, 2008; Wegler *et al.*, 2020). In this way, the level of mRNA expression is only informative, but not predictive of protein expression, and therefore it is essential to compare these two parameters. Still, the

more prominent, and decisive role is expected to be played by protein expression in the cell's functioning (Park et al., 2022).

Finally, we attempted to evaluate if ACE-2, TMPRSS2 and furin protein expression associate with clinicopathological features.

Relatively to ACE-2 protein expression, we found significant associations between ACE-2 expression and the clinicopathological characteristics of papillary carcinoma patients. The absence of lymphocytic infiltration and the presence of tumor capsule were associated with a higher ACE-2 protein score.

There's a lack of information in the literature regarding the association of ACE-2 protein expression and the features of thyroid tumors. To our knowledge, only one study reported a positive association of ACE-2 positive small blood vessels and a low tumor stage in papillary thyroid carcinomas (Meiners et al., 2021). No associations were found regarding cytoplasmic and nuclear protein expression of TMPRSS2 and furin and clinicopathological features of the patients.

Nevertheless, our study has two major limitations: the first was the number of the cases composing our study population in thyroid series, and the multiple histotypes included, that resulted in small effective numbers for some groups; increasing the number of cases would increase statistical significance. The second limitation was the number of cases with histology/RNA available. Only 40% of the cases used to study the mRNA expression of each of the transcripts had FFPE tissues available for protein expression evaluation; this may have hampered the establishment of correlation between mRNA and protein expression.

In the beginning of our study we also aimed to characterize the expression of ACE-2, TMPRSS2 and furin molecules in PanNENs, since the information on this matter is scarce. Due to time limitations this aim was not totally addressed, but the results obtained are presented in **Preliminary PanNENs series work (Appendix II)**.

Nevertheless, our results consist in a first approach to the characterization of these transcripts/proteins in endocrine lesions and may be a promising contribution to understand the role of ACE-2, TMPRSS2 and furin molecules in endocrine tumorigenesis.

6. Conclusion and future perspectives

In this study we were able to describe for the first time the expression of ACE-2, TMPRSS2 and furin in adjacent thyroid tissue and thyroid lesions, both at mRNA and protein level.

For ACE-2, at mRNA level, all thyroid lesions (goiter, adenomas, and carcinomas) showed significantly reduced mRNA expression compared to the adjacent thyroid tissue. Benign lesions with smaller dimensions showed significantly higher ACE-2 mRNA expression compared to lesions with a larger size. Moreover, benign lesions presenting lymphocytic infiltration showed significantly increased ACE-2 mRNA expression in comparison to lesions without it. At immunohistochemical evaluation, cPTC were the group with the lowest expression of ACE-2. Higher ACE-2 protein expression in papillary carcinomas associates with absence of lymphocytic infiltrate and with encapsulated tumors.

The mRNA expression levels of TMPRSS2 in all thyroid lesions was similar, and no significant differences were verified. Nevertheless, TMPRSS2 mRNA expression, showed association with the age of patients with papillary carcinomas. Older patients (≥ 55 years) showed a significant higher expression in TMPRSS2 mRNA compared to younger patients (< 55 years). At immunohistochemical evaluation a significant decrease in cytoplasmatic TMPRSS2 score was observed in thyroid carcinomas (papillary and follicular) when compared to the adjacent thyroid tissue. No associations with clinicopathological features of patients were found regarding cytoplasmic and/or nuclear TMPRSS2 protein expression.

The mRNA expression levels of furin in benign and malignant thyroid tumors were significantly higher than in the adjacent thyroid tissue. cPTC was the malignant histotype with the highest expression of furin mRNA expression. Male patients with papillary carcinomas presented a statistically significant increase in furin mRNA expression compared to females. Furin cytoplasmic immunohistochemical expression was similar in adjacent thyroid tissues, benign lesions, and carcinomas. No associations with clinicopathological features of patients were found regarding cytoplasmic and/or nuclear furin protein expression.

Our study shows that the expression of proteins involved in SARS-CoV-2 entry are deregulated in neoplastic thyroid lesions. These findings indicate that expression of such proteins may play a putative role in the tumorigenic process in the thyroid gland;

In the future, it would be interesting to enlarge the series in the different tumor types and further characterize the mRNA/protein levels of ACE-2, TMPRSS2

and furin. Also we intend to characterize those molecules by RT-qPCR and western-blot in different thyroid carcinoma cell lines.

7. References

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8. Appendix

8.1. Appendix I: Thyroid series supplementary data

Supplementary table 1 – Median, IQR and range values from mRNA and protein expression of ACE-2, TMPRSS2 and furin.

The median values and IQR observed for both mRNA and protein expression for each group: adjacent thyroid tissues, benign lesions (goiters and adenomas), thyroid carcinomas, PC (cPTC and FVPTC) and FC (FTC and OTC). Range values, obtained between the difference of the minimum and maximum immune scores were also presented for TMPRSS2 and furin protein expression. The color code varies from blue to red according to the median values, with dark blue representing lower values and dark red representing higher values.

	ACE-2		TMPRSS2			Furin		
	RNA	Protein	RNA	protein cytoplasmic	protein nuclear	RNA	protein cytoplasmic	protein nuclear
	(median ± IQR)	(median ± IQR)	(median ± IQR)	(median ± IQR, range value)	(median ± IQR, range value)	(median ± IQR)	(median ± IQR, range value)	(median ± IQR, range value)
Adjacent thyroid tissue	0.372 ± 0.377	2.000 ± 2.750	0.662 ± 0.717	12.000 ± 4.000, 8.000	0.000 ± 2.000, 8.000	0.020 ± 0.013	8.000 ± 8.000, 12.000	0.000 ± 0.000, 8.000
Benign	0.141 ± 0.207	4.000 ± 6.000	0.575 ± 0.913	8.000 ± 4.000, 8.000	0.000 ± 3.500, 8.000	0.064 ± 0.2543	8.000 ± 8.000, 9.000	0.000 ± 2.000, 8.000
Goiter	0.071 ± 0.024	5.000 ± 6.000	0.488 ± 0.753	10.000 ± 4.000, 4.000	0.000 ± 3.000, 4.000	0.015 ± 0.013	7.500 ± 9.000, 9.000	0.000 ± 0.000, 0.000
Adenoma	0.153 ± 0.207	4.000 ± 6.500	0.575 ± 0.926	8.000 ± 4.000, 8.000	0 ± 3.500, 8.000	0.067 ± 0.255	8.000 ± 4.000, 9.000	0.000 ± 4.000, 8.000
Carcinoma	0.176 ± 0.170	2.000 ± 2.000	0.580 ± 1.040	8.000 ± 4.000, 9.000	0.000 ± 3.000, 9.000	0.047 ± 0.182	8.000 ± 5.000, 8.000	0.000 ± 0.000, 2.000
PC	0.171 ± 0.162	1.500 ± 2.000	0.555 ± 1.046	8.000 ± 4.000, 9.000	0.000 ± 2.500, 9.000	0.067 ± 0.192	8.000 ± 4.500, 8.000	0.000 ± 0.000, 2.000
cPTC	0.171 ± 0.161	0.000 ± 2.000	0.487 ± 0.952	8.000 ± 4.000, 9.000	0.000 ± 2.000, 9.000	0.067 ± 0.213	8.000 ± 5.000, 8.000	0.000 ± 0.000, 2.000
FVPTC	0.143 ± 0.192	3.500 ± 4.000	1.099 ± 0.912	8.000 ± 0.000, 4.000	1.000 ± 5.250, 4.000	0.046 ± 0.133	8.000 ± 4.75, 8.000	0.000 ± 0.000, 2.000
FC	0.235 ± 0.239	3.000 ± 3.750	0.686 ± 1.020	8.000 ± 1.250, 5.000	0.000 ± 5.000, 5.000	0.011 ± 0.030	8.000 ± 2.000, 5.000	0.000 ± 0.000, 0.000
FTC	0.177 ± 0.265	3.000 ± 2.750	0.915 ± 1.113	8.000 ± 3.750, 5.000	0.000 ± 3.000, 5.000	0.011 ± 0.006	8.000 ± 3.750, 5.000	0.000 ± 0.000, 0.000
OTC	0.348 ± 0.00	3.000 ± 6.000	0.572 ± 0.000	8.000 ± 0.000, 0.000	4.000 ± 8.000, 0.000	0.109 ± 0.000	8.500 ± 1.000, 1.000	0 ± 0.000, 0.000

Abbreviations: IQR (interquartile range), PC (papillary carcinomas), cPTC (classical thyroid carcinomas), FVPTC (follicular variant thyroid carcinomas), FC (follicular carcinomas), FTC (follicular thyroid carcinoma) and OTC (oncocytic thyroid carcinoma).

Supplementary table 2 – Associations between ACE-2 protein expression and clinicopathological data.

Crosstabulation between ACE-2 protein expression and the clinicopathological characteristics of the patients (age, gender, number of lesions, tumor capsule, capsule invasion, lymph node metastasis, extrathyroidal extension and lymphocytic infiltration). Chi-Square test was performed in 2x2 tables, and in cases when > 1 cells (25,0%) have expected count less than 5, the Fisher's Exact was considered. * Statistical significances are yellow marked.

Patients characteristics	ACE-2							
	Adjacent tissue (n=40)		Benign lesions (n=11)		Papillary lesions (n=30)		Follicular lesions (n=6)	
	< 3	≥ 3	< 3	≥ 3	< 3	≥ 3	< 3	≥ 3
Age	(n=38)		(n=10)		(n=29)		(n=6)	
< 55 years	23 (82.1%)	8 (80.0%)	4 (100.0%)	5 (83.3%)	20 (83.3%)	4 (80.0%)	2 (66.7%)	3 (100.0%)
≥ 55 years	5 (17.9%)	2 (20.0%)	0 (0.0%)	1 (16.7%)	4 (16.7%)	1 (20.0%)	1 (33.3%)	0 (0.0%)
p-value	1.000		1.000		1.000		1.000	
Gender	(n=38)		(n=10)		(n=29)		(n=6)	
Female	23 (82.1%)	9 (90.0%)	3 (75.0%)	6 (100.0%)	21 (87.5%)	3 (60.0%)	2 (66.7%)	2 (66.7%)
Male	5 (17.9%)	1 (10.0%)	1 (25.0%)	0 (0.0%)	3 (12.5%)	2 (40.0%)	1 (33.3%)	1 (33.3%)
p-value	1.000		0.400		0.195		1.000	
Number lesions p/ patient	-		(n=10)		(n=25)		(n=5)	
1			3 (75.0%)	3 (66.7%)	12 (52.2%)	0 (0.0%)	1 (33.3%)	0 (0.0%)
> 1			1 (25.0%)	2 (33.3%)	11 (47.8%)	2 (100.0%)	2 (66.7%)	2 (100.0%)
p-value	-		1.000		0.480		1.000	
Tumor size	-		(n=7)		(n=20)		(n=4)	
≤ 3 cm			2 (100.0%)	3 (60.0%)	9 (52.9%)	3 (100.0%)	2 (100.0%)	2 (100.0%)
> 3cm			0 (0.0%)	2 (40.0%)	8 (47.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
p-value	-		1.000		0.242		-	
Tumor capsule	-		(n=9)		(n=25)		(n=6)	
Absent			1 (25.0%)	0 (0.0%)	21 (91.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Present			3 (75.0%)	5 (100.0%)	2 (8.7%)	2 (100.0%)	3 (100.0%)	3 (100.0%)
p-value	-		0.444		0.020*		-	
Capsule invasion	-		(n=9)		(n=3)		(n=5)	
Absent			4 (100.0%)	5 (100.0%)	2 (100.0%)	1 (100.0%)	1 (33.3%)	0 (0.0%)
Present			0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (66.7%)	2 (100.0%)
p-value	-		-		-		1.000	
Vascular invasion	-		(n=9)		(n=23)		(n=6)	
Absent			4 (100.0%)	5 (100.0%)	10 (47.6%)	1 (50.0%)	2 (66.7%)	2 (66.7%)
Present			0 (0.0%)	0 (0.0%)	11 (52.4%)	1 (50.0%)	1 (33.3%)	1 (33.3%)
p-value	-		-		1.000		1.000	
Lymph node metastases	-		(n=1)		(n=10)		(n=4)	
Absent			1 (100.0%)	0 (0.0%)	6 (60.0%)	0 (0.0%)	2 (100.0%)	2 (100.0%)
Present			0 (0.0%)	0 (0.0%)	4 (40.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
p-value	-		-		-		-	
Extrathyroidal extension	-		(n=3)		(n=25)		(n=5)	
Absent			2 (100.0%)	1 (100.0%)	14 (60.9%)	2 (100.0%)	2 (100.0%)	3 (100.0%)
Present			0 (0.0%)	0 (0.0%)	9 (39.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
p-value	-		-		0.520		-	
Lymphocytic infiltration	-		(n=9)		(n=26)		(n=6)	
Absent			2 (66.7%)	2 (33.3%)	10 (41.7%)	2 (100.0%)	3 (100.0%)	2 (66.7%)
Present			1 (33.3%)	4 (66.7%)	14 (58.3%)	0 (0.0%)	0 (0.0%)	1 (33.3%)
p-value	-		0.524		0.203		1.000	

Supplementary table 3 – Associations between TMPRSS2 protein expression and clinicopathological data

Crosstabulation between TMPRSS2 protein expression and the clinicopathological characteristics of the patients (age, gender, number of lesions, tumor capsule, capsule invasion, lymph node metastasis, extrathyroidal extension and lymphocytic infiltration). Chi-Square test was performed in 2x2 tables, and in cases when > 1 cells (25,0%) have expected count less than 5, the Fisher's Exact was considered.

Patients Characteristics	TMPRSS2															
	Adjacent tissue (n=52)				Benign lesions (n=20)				Papillary carcinomas (n=33)				Follicular carcinomas (n=6)			
	Cytoplasmic staining		Nuclear staining		Cytoplasmic staining		Nuclear staining		Cytoplasmic staining		Nuclear staining		Cytoplasmic staining		Nuclear staining	
	< 12	≥ 12	< 1	≥ 1	< 12	≥ 12	< 1	≥ 1	< 12	≥ 12	< 1	≥ 1	< 12	≥ 12	< 1	≥ 1
Age	(n=50)		(n=50)		(n=19)		(n=19)		(n=32)		(n=32)		(n=6)		(n=6)	
< 55 years	17 (77.3%)	26 (92.9%)	32 (91.4%)	11 (73.3%)	9 (90.0%)	9 (100.0%)	11 (100.0%)	7 (87.5%)	16 (80.0%)	11 (91.7%)	13 (81.3%)	14 (87.5%)	5 (83.3%)	0 (0.0%)	3 (75.0%)	2 (100.0%)
≥ 55 years	5(22.7%)	2(7.1%)	3 (8.6%)	4(26.7%)	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (12.5%)	4 (20.0%)	1 (8.3%)	3 (18.8%)	2 (12.5%)	1 (16.7%)	0 (0.0%)	1 (25.0%)	0 (0.0%)
p-value	0.217		0.176		1.000		0.421		0.626		1.000		-		1.000	
Gender	(n=50)		(n=50)		(n=19)		(n=19)		(n=32)		(n=32)		(n=6)		(n=6)	
Female	21 (95.5%)	23 (82.1%)	30 (85.7%)	14 (93.3%)	10 (100.0%)	8 (88.9%)	10 (100.0%)	8 (88.9%)	16 (80.0%)	11 (91.7%)	13 (81.3%)	14 (87.5%)	4 (66.7%)	0 (0.0%)	3 (75.0%)	1 (50.0%)
Male	1 (4.5%)	5 (17.9%)	5 (14.3%)	1 (6.7%)	0 (0.0%)	1 (11.1%)	1 (11.1%)	0 (0.0%)	4 (20.0%)	1 (8.3%)	3 (18.8%)	2 (12.5%)	2 (33.3%)	0 (0.0%)	1 (25.0%)	1 (50.0%)
p-value	0.211		0.654		0.474		1.000		0.626		1.000		0.698		1.000	
Number lesions p/ patient	-		-		(n=20)		(n=20)		(n=28)		(n=28)		(n=5)		(n=5)	
1					9 (81.8%)	7 (77.8%)	10 (83.3%)	6 (75.0%)	8 (50.0%)	6 (50.0%)	8 (50.0%)	6 (50.0%)	1(20.0%)	0 (0.0%)	1(25.0%)	0 (0.0%)
> 1					2 (18.2%)	2 (22.2%)	2 (16.7%)	2 (25.0%)	8 (50.0%)	6 (50.0%)	8 (50.0%)	6 (50.0%)	4 (80.0%)	0 (0.0%)	3 (75.0%)	1 (100.0%)
p-value	-		-		1.000		1.000		1.000		1.000		-		1.000	
Tumor size	-		-		(n=19)		(n=19)		(n=26)		(n=26)		(n=6)		(n=6)	
≤ 3 cm					4 (40.0%)	4 (44.4%)	5 (41.7%)	3 (42.9%)	10 (66.7%)	7 (63.6%)	11 (68.8%)	6 (60.0%)	3 (50.0%)	0 (0.0%)	2 (50.0%)	1 (50.0%)
> 3cm					6 (60.0%)	5 (55.6%)	7 (58.3%)	4 (57.1%)	5 (33.3%)	4 (36.4%)	5 (31.3%)	4 (40.0%)	3 (50.0%)	0 (0.0%)	2 (50.0%)	1 (50.0%)
p-value	-		-		1.000		1.000		1.000		0.692		-		1.000	
Tumor capsule	-		-		(n=19)		(n=19)		(n=28)		(n=28)		(n=6)		(n=6)	
Absent					0 (0.0%)	1 (12.5%)	1 (8.3%)	0 (0.0%)	14 (82.4%)	10 (90.9%)	13 (86.7%)	11 (84.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Present					11 (100.0%)	7 (87.5%)	11 (91.7%)	7 (100.0%)	3 (17.6%)	1 (9.1%)	2 (13.3%)	2 (15.4%)	6 (100.0%)	0 (0.0%)	4 (100.0%)	2 (100.0%)
p-value	-		-		0.421		1.000		1.000		1.000		-		-	
Capsule invasion	-		-		(n=19)		(n=19)		(n=2)		(n=2)		(n=5)		(n=5)	
Absent					11 (100.0%)	8 (100.0%)	12 (100.0%)	7 (100.0%)	1 (100.0%)	1 (100.0%)	1(100.0%)	1(100.0%)	1 (20.0%)	0 (0.0%)	1 (25.0%)	0 (0.0%)
Present					0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (80.0%)	0 (0.0%)	3 (75.0%)	1 (100.0%)
p-value	-		-		-		-		-		-		-		1.000	
Vascular invasion	-		-		(n=19)		(n=19)		(n=26)		(n=26)		(n=6)		(n=6)	
Absent					11 (100.0%)	8 (100.0%)	12 (100.0%)	7 (100.0%)	7 (46.7%)	6 (54.5%)	8 (57.1%)	5 (41.7%)	4 (66.7%)	0 (0.0%)	3 (75.0%)	1 (50.0%)
Present					0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (53.3%)	5 (45.5%)	6 (42.9%)	7 (58.3%)	2 (33.3%)	0 (0.0%)	1 (50.0%)	1 (50.0%)
p-value	-		-		-		-		0.691		0.431		-		1.000	
Lymph node metastases	-		-		(n=1)		(n=1)		(n=13)		(n=13)		(n=4)		(n=4)	
Absent					0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)	5 (55.6%)	2 (50.0%)	4 (50.0%)	3 (60.0%)	4 (100.0%)	0 (0.0%)	3 (100.0%)	1 (100.0%)
Present					0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (44.4%)	2 (50.0%)	4 (50.0%)	2 (40.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
p-value	-		-		-		-		1.000		1.000		-		-	
Extrathyroidal extension	-		-		(n=6)		(n=6)		(n=27)		(n=27)		(n=5)		(n=5)	
Absent					1 (100.0%)	5 (100.0%)	4 (100.0%)	2 (100.0%)	10 (58.8%)	8 (80.0%)	10 (72.4%)	8 (61.5%)	5 (100.0%)	0 (0.0%)	3 (100.0%)	2 (100.0%)
Present					0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	7 (41.2%)	2 (20.0%)	4 (28.6%)	5 (38.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
p-value	-		-		-		-		0.406		0.695		-		-	
Lymphocytic infiltration	-		-		(n=19)		(n=19)		(n=29)		(n=29)		(n=6)		(n=6)	
Absent					7 (70.0%)	4 (44.4%)	8 (66.7%)	3 (42.9%)	9 (52.9%)	3 (25.0%)	6 (37.5%)	6 (46.2%)	5 (83.3%)	0 (0.0%)	4 (100.0%)	1 (50.0%)
Present					3 (30.0%)	5 (55.6%)	4 (33.3%)	4 (57.1%)	8 (47.1%)	9 (75.0%)	10 (62.5%)	7 (53.8%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	1 (50.0%)
p-value	-		-		0.370		0.377		0.251		0.638		-		0.333	

Supplementary table 4 – Associations between furin protein expression and clinicopathological data

Crosstabulation between furin protein expression and the clinicopathological characteristics of the patients (age, gender, number of lesions, tumor capsule, capsule invasion, lymph node metastasis, extrathyroidal extension and lymphocytic infiltration). Chi-Square test was performed in 2x2 tables, and in cases when > 1 cells (25,0%) have expected count less than 5, the Fisher's Exact was considered.

Patients Characteristics	Furin											
	Adjacent tissue (n=51)				Benign lesions (n=19)				Papillary carcinomas (n=33)			
	Cytoplasmic staining		Nuclear staining		Cytoplasmic staining		Nuclear staining		Cytoplasmic staining		Nuclear staining	
	< 8	≥ 8	< 1	≥ 1	< 8	≥ 8	< 1	≥ 1	< 8	≥ 8	< 1	≥ 1
Age	(n=49)		(n=49)		(n=18)		(n=18)		(n=32)		(n=32)	
< 55 years	17 (77.3%)	25 (92.6%)	35 (85.4%)	7 (87.5%)	8 (100.0%)	9 (90.0%)	10 (90.9%)	7 (100.0%)	8 (72.7%)	19 (90.5%)	26 (86.7%)	1 (50.0%)
≥ 55 years	5 (22.7%)	2 (7.4%)	6 (14.6%)	1 (12.5%)	0 (0.0%)	1 (10.0%)	1 (9.1%)	0 (0.0%)	3 (27.3%)	2 (9.5%)	4 (13.3%)	1 (50.0%)
p-value	0.219		1.000		1.000		1.000		0.310		0.292	
Gender	(n=49)		(n=49)		(n=18)		(n=18)		(n=32)		(n=32)	
Female	19 (86.4%)	24 (88.9%)	35 (85.4%)	8 (100%)	8 (100.0%)	9 (90.0%)	10 (90.0%)	7 (100.0%)	9 (81.8%)	18 (85.7%)	26 (86.7%)	1 (50.0%)
Male	3 (13.6%)	3 (11.1%)	6 (14.6%)	0 (0.0%)	0 (0.0%)	1 (10.0%)	1 (9.1%)	0 (0.0%)	2 (18.2%)	3 (14.3%)	4 (13.3%)	1 (50.0%)
p-value	1.000		0.571		1.000		1.000		1.000		0.292	
Number lesions p/ patient	-		-		(n=19)		(n=19)		(n=28)		(n=28)	
1					7 (77.8%)	8 (80.0%)	9 (75.0%)	6 (85.7%)	5 (45.5%)	9 (52.9%)	13 (48.1%)	1 (100.0%)
> 1					2 (22.2%)	2 (20.0%)	3 (25.0%)	1 (14.3%)	6 (54.5%)	8 (47.1%)	14 (51.9%)	0 (0.0%)
p-value	-		-		1.000		1.000		0.699		1.000	
Tumor size	-		-		(n=18)		(n=18)		(n=26)		(n=26)	
≤ 3 cm					6 (66.7%)	2 (22.2%)	3 (27.3%)	5 (71.4%)	6 (60.0%)	11 (68.8%)	17 (68.0%)	0 (0.0%)
> 3cm					3 (33.3%)	7 (77.8%)	8 (72.7%)	2 (28.6%)	4 (40.0%)	5 (31.3%)	8 (32.0%)	1 (100.0%)
p-value	-		-		0.153		0.145		0.692		0.346	
Tumor capsule	-		-		(n=18)		(n=18)		(n=28)		(n=28)	
Absent					1 (11.1%)	0 (0.0%)	0 (0.0%)	1 (14.3%)	8 (80.0%)	16 (88.9%)	23 (85.2%)	1 (100.0%)
Present					8 (88.9%)	9 (100.0%)	11 (100.0%)	6 (85.7%)	2 (20.0%)	2 (11.1%)	4 (14.8%)	0 (0.0%)
p-value	-		-		1.000		0.389		0.601		1.000	
Capsule invasion	-		-		(n=18)		(n=18)		(n=3)		(n=3)	
Absent					1 (100.0%)	2 (100.0%)	11 (100.0%)	7 (100.0%)	1 (100.0%)	2 (100.0%)	3 (100.0%)	0 (0.0%)
Present					0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
p-value	-		-		-		-		-		1.000	
Vascular invasion	-		-		(n=18)		(n=18)		(n=26)		(n=26)	
Absent					9 (100.0%)	9 (100.0%)	11 (100.0%)	7 (100.0%)	5 (55.6%)	8 (47.1%)	13 (52.0%)	0 (0.0%)
Present					0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (44.4%)	9 (52.9%)	12 (48.0%)	1 (100.0%)
p-value	-		-		-		-		1.000		1.000	
Lymph node metastases	-		-		(n=1)		(n=1)		(n=13)		(n=13)	
Absent					1 (100.0%)	0 (0.0%)	0 (0.0%)	1 (100.0%)	3 (75.0%)	4 (44.4%)	7 (58.3%)	0 (0.0%)
Present					0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (25.0%)	5 (55.6%)	5 (41.7%)	1 (100.0%)
p-value	-		-		-		-		0.559		0.462	
Extrathyroidal extension	-		-		(n=6)		(n=6)		(n=27)		(n=27)	
Absent					2 (100.0%)	4 (100.0%)	3 (100.0%)	3 (100.0%)	7 (77.8%)	11 (61.1%)	17 (65.4%)	1 (100.0%)
Present					0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (22.2%)	7 (38.9%)	9 (34.6%)	0 (0.0%)
p-value	-		-		-		-		0.667		1.000	
Lymphocytic infiltration	-		-		(n=18)		(n=18)		(n=29)		(n=29)	
Absent					6 (66.7%)	4 (44.4%)	8 (66.7%)	2 (33.3%)	4 (36.4%)	8 (44.4%)	12 (42.9%)	0 (0.0%)
Present					3 (33.3%)	5 (55.6%)	4 (33.3%)	4 (66.7%)	7 (63.6%)	10 (55.6%)	16 (57.1%)	1 (100.0%)
p-value	-		-		0.637		0.321		0.717		1.000	

8.2. Appendix II: Preliminary PanNEN series work

8.2.1. Pancreas

8.2.1.1. Function and physiology

Pancreas is a glandular organ of the gastrointestinal system located in the abdominal cavity, connected to several organs, such as stomach, spleen, duodenum, and liver. Human pancreas is a compact organ subdivided into distinct parts: head, neck, body, and tail. Pancreas is composed of an exocrine and an endocrine component that influence the function of our entire body (Karpinska & Czauderna, 2022; Leung, 2010).

The exocrine component, which represents more than 95% of the pancreatic mass, is composed of ductal and acinar cells that are responsible for the secretion of digestive enzymes, essential for normal digestion, absorption, and assimilation of nutrients (Chang & Leung, 2014).

The endocrine counterpart, representing near 2% of the total pancreas volume, is organized into clusters of cells called islets of Langerhans, which are scattered throughout the pancreatic parenchyma and are responsible for the release of hormones that exert pivotal actions in regulation and maintenance of glucose homeostasis (In't Veld & Marichal, 2010). The endocrine pancreas is highly vascularized and innervated (Eberhard & Lammert, 2009).

Pancreatic islets are composed of three major cell types, which influence each other's secretion (Leung, 2010):

- α -cells (alpha cells) are responsible for production of glucagon which stimulates the release of glucose from the glycogen stores in the liver, by glycogenolysis resulting in increased blood sugar. These cells constitute about 20-40% of human islet cells (In't Veld & Marichal, 2010; Kumar et al., 2015; Wendt & Eliasson, 2020).
- β -cells (beta cells) secrete insulin, which is the hormone that regulates glucose uptake by the tissues and reduces blood glucose levels. Comprises nearly 50-80% of islet cells in humans (In't Veld & Marichal, 2010; Kumar et al., 2015).
- δ -cells (delta cells) produce somatostatin which negatively regulates the release of both insulin and glucagon. It represents about 1-5% of the islet mass (Wendt & Eliasson, 2020).

8.2.1.2. Glucose homeostasis

The glucose homeostasis is a tightly regulated process. During a fasting state, both plasma glucose levels and insulin levels are low. In this condition, α -cells are stimulated to produce glucagon which will stimulate hepatic gluconeogenesis and glycogenolysis, leading to an increase in glucose levels and therefore preventing hypoglycemic states (Kumar et al., 2015).

In a post-prandial state, β -cells are stimulated to produce insulin in response to an increased glucose load, leading to a decrease of the glucagon levels. Insulin will promote glucose uptake by peripheral tissues (e.g., brain, skeletal muscle), increase glycogen synthesis, and hepatic lipogenesis. Consequently, there is a conversion of glycogen to triglycerides by skeletal muscle and adipose tissue, maintaining the normoglycemia (Hiriart et al., 2014; Kumar et al., 2015).

8.2.1.3. Diabetes

Diabetes belongs to a group of metabolic diseases that share a common feature which is hyperglycemia resulting from defective insulin secretion, insulin action or, more usually, both situations. The vast majority of diabetes cases fall into two broad categories: type 1 or type 2 diabetes (Kumar et al., 2015).

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of pancreatic β cells with consequent absolute insulin deficiency (Lucier & Weinstock, 2022).

Type 2 diabetes (T2D) is a progressive metabolic disorder caused by a combination of peripheral resistance to insulin action or an inappropriate secretory response of pancreatic β -cells resulting in insulin resistance (American Diabetes, 2012; Association, 2011; Goyal & Jialal, 2022). According to WHO, more than 95% of people with diabetes have type 2 diabetes (WHO, 2022b). This type of diabetes are largely associated with increased consumption of unhealthy diets and sedentary lifestyles that leads to obesity or overweight (Khan et al., 2020; J. Liu et al., 2020).

A wide variety of monogenic and secondary causes are responsible for the remaining cases of diabetes (Kumar et al., 2015).

8.2.2. Pancreatic neuroendocrine neoplasms (PanNENs)

The PanNENs are tumors arising from Langerhans islet cells and represent less than 3% of all pancreas neoplasms (Kelgiorgi & Dervenis, 2017; Sonbol et al., 2022; Souche et al., 2020).

These tumors can have a sporadic or hereditary origin. The vast majority of PanNENs arises sporadically, whereas less than 10% of them occur in the context of familial syndromes, being the most representative multiple endocrine neoplasia type 1 (MEN1), Von Hippel Lindau (VHL), Neurofibromatosis type 1 (NF1) or Tuberous Sclerosis Complex (TSC). Patients with those syndromes are typically diagnosed with neoplasia's at earlier ages, with a multifocal presentation (McKenna & Edil, 2014; Soczomski et al., 2021; Souche et al., 2020).

Clinically, PanNENs can be one of two types: functioning (F-PanNENs) or nonfunctioning tumors (NF- PanNENs). F-PanNENs represent about 30% of all PanNENs and are characterized by their capacity of secretion of metabolically active peptides such as insulin, gastrin, somatostatin, glucagon, and vasoactive intestinal peptide (VIP), inducing clinical symptoms. These tumors comprise insulinomas, gastrinomas or more rarely glucagonomas, VIPoma's and somatostatinomas (Bartolini et al., 2018; Chen et al., 2021; Lee et al., 2017; Souche et al., 2020). However, the vast majority of PanNENs are NF- PanNENs, accounting for almost 65-85% of cases (Falconi et al., 2016; Souche et al., 2020). Since these tumors do not produce hormones or produce them at very low levels, patients are usually asymptomatic in the early stages and often diagnosed incidentally, or at higher disease stages (Cloyd & Poultides, 2015; Kelgiorgi & Dervenis, 2017).

According to the 5th edition of the WHO digestive systems tumors classifications, PanNENs are classified either as well differentiated tumors (PanNETs) or, rarely, as poorly differentiated tumors also known as neuroendocrine carcinomas (PanNECs)(IARC & WHO, 2019). On the basis of the proliferative activity of the neoplastic cells, measured by the Ki-67 expression and/or mitotic index, PanNENs can be graded as low (G1), intermediate (G2), or high grade (G3). PanNETs can be G1-G3, but PanNECs are always G3(Choe et al., 2019; Rindi et al., 2022).

8.2.2. Epidemiology, etiology, and prognostic factors

PanNENs are rare neoplasms but represent the second most common pancreatic neoplasm (McKenna & Edil, 2014). A large part of these tumors is asymptomatic, slow-growing and indolent, resulting in late diagnose in some patients, at later disease stages, as explained in the anterior topic (Chen et al., 2021; Cloyd & Poultides, 2015). Nearly 50% of patients have nodal or distant metastases by the time of diagnosis (Souche et al., 2020).

These neoplasms have a slight male predominance (55% male vs 45% female) (McKenna & Edil, 2014) and its peak of incidence occurs between sixth or seventh decade of life (Halfdanarson et al., 2008).

The incidence of PanNENs has been reported to be increasing over the last decades (Kelgiorgi & Dervenis, 2017; McKenna & Edil, 2014). According to an analysis using the SEER database, Sonbol *and colleagues* showed that annual incidence rates between 2000-2016 increased from 0.27 to 1.00 per 1 000 000 people, while mortality decreased. This increase in incidence is also accompanied by a significant improvement in survival of the disease at different stages (Sonbol et al., 2022).

The large increase in the incidence rate is suggestive of increased use and improvement of diagnostic imaging, leading also to an incidental detection of part of these tumors (McKenna & Edil, 2014; Sonbol et al., 2022).

Smoking and alcohol consumption, the presence of a first-degree family history of any cancer, and type 2 diabetes have been described as risk factors associated with the development of sporadic PanNENs (Giraldi et al., 2021; Haugvik et al., 2015).

Several clinical factors, such as age, tumor stage, tumor size, the presence of regional or distant metastases, vascular invasion, and necrosis, may influence the prognosis of PanNENs patients (Brooks et al., 2019; Gao et al., 2018).

Surgery is the only curative approach in PanNENs patients (Li et al., 2022).

8.2.3. PanNENs series description

The samples of the PanNENs series were provided by Centro Hospitalar e Universitário de São João, Porto, Portugal and from Acibadem International Hospital, Istanbul, Turkey. The pancreatic neuroendocrine neoplasm (PanNEN) series was composed of 104 patients. PanNENs were classified, according to WHO 2019 classification (5th edition)(IARC & WHO, 2019), as PanNET G1 (n=74), PanNET G2 (n=25), PanNET G3 (n=3), and PanNEC G3 (n=2). The selected samples belong to a period between 1988 and 2018.

FFPE tissue was available for the totality of this series and the ACE-2 expression was preliminary performed. The clinical and pathological data of each patient are summarized in **Supplementary table 5**. In the present study there is a female sex predominance (60%). The median age of the patients at diagnosis was 54 years (54.00 ± 23.00 median $\pm$ IQR). Most patients presented non-functioning tumors (68.3%). Out of the functioning tumors, the majority were insulinomas.

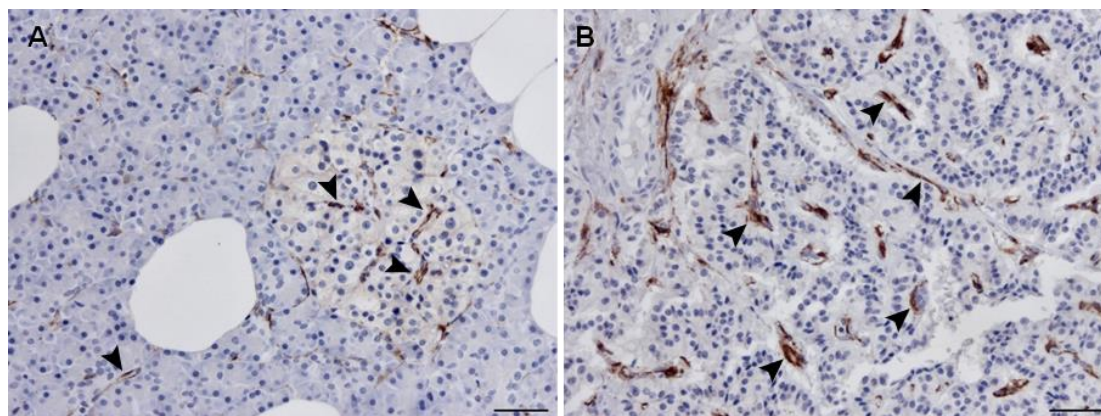
Supplementary table 5 – Clinicopathological characteristics of the PanNENs series patients.

Patient characteristics	Total (n=104)
Age	(n=104)
Mean $\pm$ SD	51.9 $\pm$ 14.9
Median $\pm$ IQR	54.0 $\pm$ 23.0
Min – Max	15 - 81
Sex	(n=104)
Female	56 (53.8%)
Male	48 (46.2%)
Clinically functionality	(n=104)
Non-functioning	71 (68.3%)
Functioning	33 (31.7%)
WHO classification 2019 (5th edition)	(n=104)
PanNET G1	74 (71.2%)
PanNET G2	25 (24.0%)
PanNET G3	3 (2.9%)
PanNEC G3	2 (1.9%)

Abbreviations: SD (standard deviation), IQR (interquartile range), Min (Minimum) and Max (maximum).

8.2.4. ACE-2 expression in PanNENs

The staining observed for ACE-2 in the pancreas was similar to the one observed in thyroid, the staining pattern was essentially in the membrane and positive in the vasculature cells (**Supplementary figure 1A and 1B**).



Supplementary figure 1 – Immunohistochemical staining pattern of ACE-2 in pancreas.

Representative images of ACE-2 staining in **(A)** normal pancreas and **(B)** pancreatic neuroendocrine neoplasm. The staining is mainly membranous, located to vascular cells (**arrow heads**).

8.2.5. Discussion

PanNENs are tumors of the endocrine portion of the pancreas that represent less than 3% of all pancreas neoplasms (Kelgiorgi & Derveniz, 2017; Sonbol et al., 2022; Souche et al., 2020).

Similar to thyroid, during the COVID-19 pandemic, cases of the new onset of T2D have been reported in patients after the SARS-CoV2 infection and several studies suggest that ACE-2 is expressed in endocrine cells of the pancreas, including β cells (El-Huneidi et al., 2021; Khunti et al., 2021; Memon & Abdelalim, 2021; Metwally et al., 2021). However, the expression of this transcript/protein in β cells has been described in few reports and with some contradiction (El-Huneidi et al., 2021; Memon & Abdelalim, 2021).

Since there is few information available in the literature about the expression of ACE-2 in PanNENs, our second aim of this work was to establish the expression of these molecules in these tumors.

ACE-2 expression has important implications for pancreatic β cell function, regulation of insulin secretion, blood flow, and maintenance of glucose homeostasis (Brar et al., 2017; Chodavarapu et al., 2016; Niu et al., 2008). Although most of the studies were performed in rodents, the involvement of ACE-2/Ang 1-7 axis in

pancreatic β cells has been reported to reduce β -cell dedifferentiation and improve islet microcirculation through suppression of inducible nitric oxide synthase (iNOS), an enzyme responsible for the metabolism of reactive oxygen species production/activity (Xuan et al., 2018). The ACE-2/Ang 1-7 axis has also been reported to inhibit pancreatitis through nitric oxide (NO)-dependent signals (Wang et al., 2015). In contrast, ACE-2 deficient mice presented impairment of glucose tolerance and reduced insulin secretion (Shoemaker et al., 2015).

Our series had a slightly predominance of female sex (53.8%), and most tumors were clinically non-functioning (68.3%). The median age at diagnostics was 54 years which is in accordance with the literature (Ro et al., 2013). Most of the tumors that composed our series were PanNET G1 (71.2%).

This was a preliminary evaluation of ACE-2 expression in PanNENs. The staining pattern observed was essentially membranous, located to islet microvasculature. The ACE-2 staining observed was similar between non-neoplastic and neoplastic tissues. Although few evidence on this matter, our results are in agreement with the literature.

At the mRNA level, ACE-2 expression in the pancreas was slightly higher compared to the expression in the lung (F. Liu et al., 2020; Yang et al., 2010). Later, other authors showed that the expression of ACE-2 mRNA in a beta cell lines (EndoC-Bh1) was similar to the expression level of human pancreatic islets (Fignani et al., 2020).

In a study analyzing the expression of this transcript between diabetic (T2D) and non-diabetic patients, ACE-2 expression was significantly increased in hyperglycemic diabetic islets compared to non-diabetic/normoglycemic islet (Taneera et al., 2020). Additionally, Fignani *et al.* reported that ACE-2 expression could be modulated in response to inflammatory stress. The presence of proinflammatory cytokines (IL-1 β -IFN γ and TNF α) increases ACE-2 expression in both β cell lines and primary human pancreatic islets (Fignani et al., 2020).

At the protein level, several evidences have shown that ACE-2 expression is strongly positive in endocrine tissue compared to the exocrine counterpart. It is preferentially expressed in subsets of insulin-producing cells and also expressed in the cells of the pancreatic microvasculature such as pericytes and moderately expressed in rare scattered ductal cells (Fignani et al., 2020; Yang et al., 2020).

The newly described short ACE-2 isoform is also prevalently expressed in human beta cells (Fignani et al., 2020).

In contrast, other authors have reported that most islet cell subsets express no or low levels of ACE-2 protein and that this expression was only present in the islet and exocrine tissue micro vasculature and in a subset of pancreatic ducts (Coate et al., 2020; Kusmartseva et al., 2020).

There's only one study that explored the ACE-2 immunostaining in PanNENs. Meiners *et al.* reported that ACE-2 vessel staining was detected with more intense signal around the islets of Langerhans, than in exocrine pancreas. They also reported, similar to thyroid, that the highest rates of vessel positivity occurred in tumors with endocrine/neuroendocrine activity. PanNETs ranked first in the tumor fraction with ACE-2 positive blood vessels (70.3%) followed by PanNEC that occupied the 9th position (21.4%)(Meiners et al., 2021). These data could potentially indicate that loss/inhibition of ACE-2 expression could be associated with more aggressive tumors or, instead, be influenced by the different genetic signatures of such distinct pancreatic neuroendocrine histotypes.

At this time, we could only conclude that the immune expression of ACE-2 observed in these tumors was similar to that observed in thyroid. However, further studies are needed to better understand the importance of the SARS-CoV-2 host mediators proteins in PanNENs and their potential role in the carcinogenesis process.

8.2.6. Future perspectives

In PanNENs, the data collected in our study was limited, and we could only conclude that the immunoprofile of ACE-2 observed in these tumors is similar to that observed in thyroid (expression restricted to cells in small vessels). We intend now to evaluate the expression in the PanNENs series, using an IRS score as described for thyroid samples, and evaluated putative clinicopathological associations.

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