UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
GRIGORE T. POPA IAȘI

HABILITATION THESIS

THE THYROID BETWEEN MALIGNANCY AND AUTOIMMUNITY: THE PATHOLOGICAL WAY FROM DIAGNOSIS TO RESEARCH

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CONTENTS

ABBREVIATIONS

^{131}I: Iodine-131
^{134}Cs: Caesium 134
^{137}Cs: Caesium 137
AAO-HNS: American Academy of Otolaryngology-Head and Neck Surgery
AChR: acetylcholine receptor
AHNS: American Head and Neck Society
AIT: autoimmune thyroiditis
AITD: autoimmune thyroid disease
AJCC/UICC: American Joint Committee on Cancer/Union for International Cancer Control
ASCO–CAP: American Society of Clinical Oncology/College of American Pathologists
ASDR: age-standardized death rates
ASIR: age-standardized incidence rate
ATA: American Thyroid Association
ATC: anaplastic thyroid carcinoma
BCL2: B-cell lymphoma 2
CC: colon carcinoma
CEA: carcinoembryonic antigen
CgA: chromogranin
CK: cytokeratin
CKD: chronic kidney disease
DTC: differentiated thyroid carcinomas
EAIT: experimental autoimmune thyroiditis
ECM: extracellular matrix
EMT: epithelial-mesenchymal transition
EOMG: early-onset myasthenia gravis
ETA: European Thyroid Association
ETE: extrathyroidal extension
FMTC: familial medullary thyroid carcinoma syndrome
FNAB: fine-needle aspiration biopsy
FPTC: follicular variant of papillary thyroid carcinoma
FTC: follicular thyroid carcinomas
GPX: glutathione peroxidase
Hb: hemoglobin
HE: hematoxylin & eosin stain
HOXB9: homeobox B9
HPT: hyperparathyroidism
HPT-JT: hypeparathyroidism - jaw tumor syndrome
HT: Hashimoto's thyroiditis
HTT: hyalinizing trabecular tumor
ICAM-1: intercellular adhesion molecule-1 expression
ICD-O: international classification of diseases for oncology
IFN-γ: interferon-gamma
IHC: immunohistochemistry
LDH: lactate dehydrogenase
LOMG: late-onset myasthenia gravis
LRP4: low-density lipoprotein receptor-related protein 4
LRRN4: leucine rich repeat neuronal 4
MEN: multiple endocrine neoplasia

MG: myasthenia gravis
MGFA: Myasthenia Gravis Foundation of America
MGG: May-Grünwald–Giemsa stain
MI-RP: minimally-invasive radio-guided parathyroidectomy
MTC: medullary thyroid carcinoma
MuSK: muscle-specific tyrosine kinase
NIFTP: noninvasive follicular thyroid neoplasm with papillary-like nuclear features
NIS: Na+/I– symporter
NLRR: leucine rich repeat neuronal protein family
NOD: non-obese diabetic mice
NSE: neuron-specific enolase
OAMG: ocular-associated myasthenia gravis
PDTC: poorly differentiated thyroid carcinoma
PGP9.5: protein gene product 9.5
PHPT: primary hyperparathyroidism
PN: periostin
PT: parathyroid gland
PTC: papillary thyroid carcinomas
PTH: parathyroid hormone
PTMC: papillary thyroid microcarcinoma
RAI: radioiodine therapy
RB1: retinoblastoma tumor suppressor
RCC: renal cell carcinoma
RET: rearranged during transfection proto-oncogene
ROS: reactive oxygen species
SCC: squamous cell carcinoma
Se: selenium
SEER: National Cancer Institute Surveillance, Epidemiology and End Results
SHPT: secondary hyperparathyroidism
SIRT1: silent mating-type information regulation 2 homologue 1
Syn: synaptophysin
TAMG: thymoma-associated myasthenia gravis
TC: thyroid carcinoma
TERT: telomerase reverse transcriptase
TFH: thymic follicular hyperplasia
Tg: thyroglobulin
THPT: tertiary hyperparathyroidism
TLFH: thymic lympho-follicular hyperplasia
TN: thyroid nodule
TNF-α: tumor necrosis factor-alpha
TNM: tumor-node-metastasis
TPO: thyroid peroxidase
TR: thyoredoxin reductases
TRAIL: TNF-related apoptosis-inducing ligand
TSH: thyroid-stimulating hormone
TSH-R: thyroid-stimulating hormone receptor
TTF1: thyroid transcription factor 1
TUBB3: β-tubulin
UICC: Union for International Cancer Control
VG: van Gieson stain
WHO: World Health Organization

ABSTRACT

A *habilitation thesis* consists of the synthesis of a cumulative material that reflects the author's personal work and in which scientific, professional and academic experience is the support to confer *venia legendi* and open the way to a career as a professor.

This *habilitation thesis* sums up my scientific, professional and academic activities and achievements after the completion of my doctoral education (2006-2022), as well as some of my future projects that have taken shape and will materialize in the years to come. The thesis was structured in accordance with the recommendations of the National Council for Attestation of University Degrees, Diplomas and Certificates (CNATDCU) and with the methodology of the Doctoral School of "Grigore T. Popa" University of Iași.

The habilitation thesis includes three sections.

Section I reviews my main postdoctoral professional, academic and scientific achievements.

Section II is the main part of the thesis, as it includes the most important research findings that define my personal scientific profile. Relying on my professional expertise, as a pathologist, in thyroid pathology, this section provides an overview of my main research directions, materialized in main flow publications that validate my efforts to decipher the substrate of microscopic changes in tumoral and nontumoral thyroid.

Chapter 1, the introduction, focuses on the main landmarks that create the nosological framework of thyroid pathology. General data related to the incidence of thyroid carcinoma worldwide and in Romania, as well as concepts related to thyroid tumour etiopathogenesis are shown, with an emphasis on the two main tumour entities: papillary carcinoma and medullary carcinoma - tumours of major importance due to their occurrence in the general population, to their tumoral and familial aggregation and to their progression. The paper also includes information on nontumoral autoimmune thyroid pathology, as a predisposing factor to the occurrence of thyroid carcinomas or autoimmune pathological aggregations.

The next *four chapters* (*chapters 2 to 5*) dwell on papillary thyroid carcinoma, as the main topic of my research. The studies referred in Chapter 2 demonstrate the increase in the incidence of thyroid carcinoma in the north-eastern part of the country, a certified goitre-prone area, as well as the increase in the sensitivity of techniques for clinical and paraclinical identification of this malignancy. Chapter 3 lays the foundations for the in-depth characterization of a new tumour subclass that of papillary thyroid microcarcinoma, with different prognostic value depending on tumour localization and histological subtype. The new lesion entity has been causing heated debates about the specificity of its surgical and oncological approach. Chapter 4 brings to the fore the refinement of papillary thyroid carcinoma diagnosis, improved by the use of immunohistochemical techniques and by relating them to histological subclasses. The reported studies focus on the expression of a broad range of prognostic molecular markers of papillary thyroid carcinoma (HER2-neu, E-cadherin, β-catenin, MOC-31, tubulin, periostin). These molecules have the potential to identify aggressive histological subtypes, which may guide therapy customization. Chapter 5 connects scientific thyroid pathology research with parathyroid gland pathology, in terms of common therapeutic implications.

The findings of my research materialized in papers published in: *Diagnostics* (IF: 3,706), *Int J Clin Exp Pathol* (IF: 1,396), *Appl Sci* (IF: 2,474), *Rom J Morphol Embryol* (IF:

0,912 and 1,5), *Farmacia* (IF:1,507), *Biomed Res Int* (IF: 2,583), *BMC Surgery* (IF: 1,775) and *Acta Endocrinol-Buch* (IF: 0,411).

Chapter 6 is devoted to medullary carcinoma, a particular form of thyroid carcinoma, with proven familial aggregation and intermediate prognosis between well-differentiated and undifferentiated forms. The general overview on this tumour, which includes etiopathogenic aspects, clinical manifestations, difficulties of cytological and histopathological diagnosis and immunohistochemical confirmation techniques, is complemented by a study that assesses the clinicomorphological profile of a significant group of patients (unpublished data at the time of thesis drafting).

The findings of this research materialized in papers published in: *Rom J Morphol Embryol* (IF: 1,411).

Chapter 7 makes the transition towards the scientific research on nontumoral thyroid lesion pathology, by approaching autoimmune pathology, which associates various lesion entities and aggregations with other types of autoimmune pathologies, which may cause a high number of dysfunctions and multiorgan failure. Thus, starting from a theoretical presentation of autoimmune thyroiditis (etiopathogenesis, immunological mechanisms, clinical and ultrasonographic features, cytology, histopathological aspects, immunohistochemical profile, differential diagnosis), scientific research addresses to a rare lesion association, namely Hashimoto's thyroiditis and myasthenia gravis, as well as the assessment, in an experimental study, of a possible etiopathogenic factor involved in the occurrence of autoimmune lesions, namely selenium - as a must for good health. Planimetric morphology research is pioneering work in the morphometric approach of experimental thyroid pathology.

The findings of this research materialized in papers published in: *Rom J Morphol Embryol* (IF: 0,912 and 1,033), *Environ Eng Manag J* (IF: 1,096) and *Exp Ther Med* (IF: 2.447).

Section III describes my future professional, academic and research projects.

My research will focus mainly on thyroid tumour pathology, and especially to poorly differentiated, anaplastic and medullary thyroid carcinomas, with guarded prognosis, but with possible chances of identifying new molecular markers with prognostic value for tumour progression. I will also get more involved in researching digestive tumour pathology, more precisely the expression of certain proteins associated with tumorigenesis, angiogenesis and epithelio-mesenchymal transition.

Section IV includes a number of 800 references for my studies, many of them not older than five years, which proves the topicality of the topics tackled in this thesis.

REZUMAT

Conceperea unei *teze de habilitare* impune sintetizarea unui material cumulativ care reflectă activitatea personală, și în care experienţa ştiinţifică, profesională şi academică constituie suportul pentru a se conferi *venia legendi* şi a deschide calea către o carieră de profesor universitar.

Conținutul prezentei *teze de habilitare* însumează preocupările și realizările științifice, profesionale și academice după finalizarea studiilor doctorale (2006-2022), dar și o parte din proiectele de viitor care au prins contur și vor avea finalitate în anii ce vor urma. Structurarea acestei teze este realizată în conformitate cu recomandările Consiliului Naţional de Atestare a Titlurilor, Diplomelor și Certificatelor Universitare (CNATDCU) și a metodologiei Școlii Doctorale a Universității "Grigore T. Popa" din Iași.

Teza de abilitare este alcătuită din trei secţiuni.

Secțiunea I trece în revistă principalele realizări profesionale, academice și științifice obţinute postdoctoral.

Secțiunea II constituie centrul de greutate al tezei, prin concentrarea rezultatelor de referință ale activității de cercetare care definește profilul științific personal. Pornind de la expertiza profesională, ca anatomopatolog, în patologia tiroidiană, această secțiune oferă imaginea de ansamblu a direcțiilor abordate în cercetare, finalizate prin publicații în fluxul principal care validează preocupările de descifrare a substratului modificărilor microscopice în tiroida tumorală și non-tumorală.

Capitolul 1, cu caracter introductiv, subliniază principalele repere care creează cadrul nosologic al patologiei tiroidiene. Sunt prezentate date generale legate de incidenţa carcinomului tiroidian la nivel mondial şi national, precum și concepte referitoare la etiopatogenia tumorală tiroidiană, cu accent asupra celor două entități tumorale principale: carcinomul papilar și carcinomul medular– forme tumorale cu importanţă majoră prin prezența în populație, agregare tumorală sau familială şi evoluție. Totodată, textul include informații referitoare la patologia non-tumorală autoimună tiroidiană, ca factor predispozant în apariţia unor carcinoame tiroidiene sau agregări de patologii de tip autoimun.

Următoarele *patru capitole* (*capitolele 2 – 5*) vizează carcinomul papilar tiroidian, ca ax principal al cercetării. În Capitolul 2, studiile realizate demonstrează creşterea incidenţei carcinomului tiroidian în arealul nord-estic al ţării, zonă guşogenă certificată, precum şi creşterea sensibilităţii tehnicilor de identificare clinică şi paraclinică a acestei malignități. Capitolul 3 deschide perspectiva caracterizării aprofundate a unei noi subclase tumorale, cea a microcarcinomului papilar tiroidian, cu valoare prognostică diferită în funcţie de localizare şi subtip histologic tumoral. Noua entitate lezională determină astăzi dezbateri aprinse asupra modalităţii specifice de abordare chirurgicală şi oncologică. Capitolul 4 aduce în prim-plan rafinamentul diagnosticului carcinomului papilar tiroidian, optimizat prin prin aplicarea tehnicilor imunohistochimice şi prin raportarea acestora la subclasele histologice. Studiile raportate sunt axate asupra expresiei unui panel larg de markeri moleculari cu valoare prognostică în stratificarea carcinomului papilar tiroidian (HER2-neu, E-cadherin, β-catenin, MOC-31, tubulin, periostin). Aceste molecule au potențialul de a identifica subtipuri histologice agresive, cu repercutare în particularizarea terapiei. Capitolul 5, interconectează cercetarea ştiinţifică a patologiei tiroidiene cu patologia glandei paratiroide, prin prisma implicaţiilor terapeutice comune.

Contribuțiile personale în urma acestor cercetări au fost publicate în: *Diagnostics* (IF: 3,706), *Int J Clin Exp Pathol* (IF: 1,396), *Appl Sci* (IF: 2,474), *Rom J Morphol Embryol* (IF: 0,912 şi 1,5), *Farmacia* (IF:1,507), *Biomed Res Int* (IF: 2,583), *BMC Surgery* (IF: 1,775) şi *Acta Endocrinol-Buch* (IF: 0,411).

Capitolul 6 este dedicat carcinomului medular, ca formă particulară de carcinom tiroidian, cu agregare familială demonstrată şi prognostic intermediar între formele bine diferenţiate şi cele nediferenţiate. Prezentarea generală a acestei entităţi tumorale, în care sunt dezvoltate aspectele etiopatogenice, manifestările clinice, dificultăţile de diagnostic citologic, histopatologic şi modalităţile confirmare imunohistochimice, este completată printr-un studiu care realizează evaluarea profilului clinico-morfologic al unui lot semnificativ de pacienți (date nepublicate la momentul redactării tezei).

Contribuțiile personale în urma acestor cercetări au fost publicate în: *Rom J Morphol Embryol* (IF: 1,411).

Capitolul 7 translează cercetarea ştiinţifică către partea non-tumorală lezională tiroidiană, abordând patologia autoimună, care asociază variate entităţi lezionale, dar şi agregări cu alte tipuri de patologii autoimune care pot conduce către multiple disfuncţionalităţi şi o insuficienţă multiorganică. Astfel, plecând de la o prezentare teoretică a tiroiditelor autoimune (etiopatogeneză, mecanisme imunologice, caracteristici clinice şi ultrasonografice, aspecte citologie, histopatologice, profil imunohistochimic, diagnostic diferenţial), cercetarea ştiinţifică se adresează unei asocieri rare lezionale, repectiv tiroidita Hashimoto şi miastenia gravis, precum şi evaluării, într-un studiu experimental, a unui posibil factor etiopatogenic implicat în apariţia leziunilor autoimune, respectiv a seleniului – ca element esential pentru sănătate. Cercetările de morfologie planimetrică reprezintă un pionerat în abordarea morfometrică a patologiei experimentale tiroidiene.

Contribuțiile personale în urma acestor cercetări au fost publicate în: *Rom J Morphol Embryol* (IF: 0,912 şi 1,033), *Environ Eng Manag J* (IF: 1,096) şi *Exp Ther Med* (IF: 2.447).

Secţiunea III prezintă proiectele de viitor în plan profesional, academic şi de cercetare.

Direcția de cercetare prioritară va fi orientată către patologia tumorală tiroidiană, abordând carcinoamele slab diferentiate, anaplazice şi medulare tiroidiene, cu un prognostic rezervat, dar cu posibile şanse de identificare a unor noi markeri moleculari cu valoare prognostică în progresia tumorală. În paralel, voi intensifica interesul către patologia tumorală digestivă, urmărind studiul expresiei unor proteine asociate cu tumorigeneza, angiogeneza şi tranziţia epitelio-mezenchimală.

Secţiunea IV prezintă un număr de 800 referințe bibliografice aferente studiilor prezentate, multe din ele din ultimii cinci ani, demonstrând astfel actualitatea subiectelor abordate.

SECTION I.
OVERVIEW OF PERSONAL PROFESSIONAL, ACADEMIC, AND SCIENTIFIC ACHIEVEMENTS

I.1. INTRODUCTION

To have a teaching career in the field of medicine means to accept that, along this path, you can convey the responsibility, involvement, passion, compassion, perseverance, understanding, curiosity, and vision of those who develop around you. It means explicit training as well as the implicit transmission of the core values which ensure the continuity and evolution of medical education. Moreover, the teaching career in the medical field involves the integration of educational principles and tools in the broad and dynamic dimension of science and research. This requirement certifies the role of the educational system as a promoter of socio-economic and cultural development. Having a teaching career in medicine demands high levels of performance and professionalism for which personal motivation must include clear goals, strategy, and planning.

The habilitation thesis entitled **"The thyroid between malignancy and autoimmunity: the pathological way from diagnosis to research"** presents the clinical research I have conducted since the successful completion and defense of my PhD thesis "Cytological and Histological Correlations in Nodular Lesions of the Thyroid Gland", supervised by Professors Lorica Gavriliţă and Maria Sultana Mihailovici (Diploma E0002781, No. 1226/22.09.2006).

The focus of my doctoral thesis was thyroid nodules, the formation of which is the most common pathogenic process in thyroid pathology. The rationale behind the research was mainly epidemiological, as the historical region of Moldavia is well-known as an endemic region for goiter, with an increased frequency of thyroid nodules. Moreover, the Chernobyl disaster generated one more "black zone" in this pathology. In this context, the thesis was a survey of the thyroid nodular lesions occurring in the area, as well as of the techniques, concepts, and markers used to diagnose thyroid nodular pathology. It enrolled 3,380 patients diagnosed in the Pathology Department of "Sf. Spiridon" Clinical Emergency County Hospital, Iasi. The results revealed an upward trend in the incidence of thyroid nodular pathology in the area and demonstrated the value of fine needle aspiration biopsy as a standard screening method. The research also contributed to the refinement of pathological diagnosis in medullary thyroid carcinoma and thyroid metastases using immunohistochemical techniques.

Presently, I am an Associate Professor at the Pathology Discipline, Department of Morpho-Functional Sciences I, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi. Concurrently, I am a consultant pathologist at one of the largest hospital in the Moldavian region, "Sf. Spiridon" Clinical Emergency County Hospital Iasi, where I head the Pathology Department.

My professional, academic, and scientific pursuits are closely interconnected, and I strive to give each due attention.

I.2. PROFESSIONAL ACTIVITY

I graduated in 1993 from "Gr. T. Popa" University of Medicine and Pharmacy Iasi, Romania, Faculty of General Medicine (Diploma L1213/no.13/30.09.1993), and the following year I completed an internship in four medical specialties: Obstetrics-Gynecology at the "Cuza Vodă" Clinical Hospital, Pediatrics at "Sf. Maria" Clinical Hospital, Internal Medicine and Surgery at "Sf. Spiridon" Clinical Emergency County Hospital, Iasi (01.02.1994 - 31.01.1995).

After the 1994 Residency Competition, I opted for graduate training in Pathology at the "Sf. Spiridon" Clinical Emergency County Hospital, Iasi. In the four years of residency (31.01.1995-01.03.1999), I received a thorough education attending courses and seminars in Immunopathology, Human Genetics, Laboratory Medicine, Oncologic Cytology, and Pathology. During the residency I also participated in national conferences in Iasi, Târgu-Mureș, Cluj-Napoca, and Bucharest. I graduated as a specialist pathologist in October 1998 (Certificate A2371 No.1487/30.01.2001), and in 2003 I advanced to consultant pathologist (Certificate A22863 No. 12065/28.10.2003).

In 2000, I started working as a pathologist in the Pathology Department of "Sf. Spiridon" Clinical Emergency County Hospital Iasi, part-time at first, and in 2012 I was appointed Head of Service, a coordinating role I have fulfilled ever since.

Moreover, I have continuously enriched my professional expertise and personal abilities by participating in postgraduate training courses in relevant fields of interest in different cities of Romania (Iasi, Timișoara, Cluj-Napoca, Bucharest), but also in other countries (Poland, Portugal, Italy, United Kingdom, and Germany).

Appreciating the value of professional networking and representation, I have been an active member in important scientific societies: the Romanian Society of Normal and Pathological Morphology (Societatea Română de Morfologie Normală și Patologică), the Romanian Society of Clinical Cytology (Societatea Română de Citologie Clinică), the Romanian Society of Physicians and Naturalists (Societatea Română de Medici și Naturaliști), and the European Society of Pathology.

To grow, share, and apply my expertise in the field of pathology, I have participated in a range of national and international projects.

During 2010-2013, I was a member of the management team of a Project ANATOMOPAT "Pathology Laboratory – Professional and organizational training through the implementation of quality management" (POSDRU/81/3.2/S/58942). The project was addressed to 200 higher education and post-secondary education staff from pathology laboratories in Romania (consultant physicians, specialists, technicians), in order to implement state-of-the-art technologies and facilitate the accreditation of more such laboratories. The project provided a complex training program organized in 9 work packages (professional training courses, workshops), as well as internships in European laboratories and exchanges of experience through interregional cooperation, documentation, and implementation of quality management according to SR EN ISO 15189:007. On this occasion, I benefited from an internship in Pathology (New Technologies in Pathology Field) at the University of Turin, Molinette Hospital, Turin, Italy.

In 2018, I participated in the project "Alignment of Romanian medical practice with legal and qualitative requirements for respecting patient's rights and safety", and in the workshop "Compliance of medical practice with applicable legal requirements" organized by MedRight Experts.

I was the scientific consultant in the project IMAGO Mol "Institutional strengthening and increasing the visibility of the Innovative Regional Cluster of Molecular and Structural Imaging North-East, support framework for increasing the competitiveness of SMEs in the

field in Romania", implemented by "Grigore T. Popa" University of Medicine and Pharmacy Iasi, SMIS code 49820, Financing contract no. 1 /CLT800.014/14.05.2014. The objectives of the project were to increase the competitiveness of medical imaging service providers through intelligent specialization in the biomedical field, and to improve the diagnostic-therapeutic medical process by using an integrated medical data management system (USMED). The system was implemented by all the medical or related partners in the cluster.

The practical professional experience thus gained has helped me in my teaching career, giving the opportunity to contextualize theoretical knowledge when passing it on to future generations of doctors.

I.3. ACADEMIC ACTIVITY

I started my teaching career in 1999 as an Assistant Professor at the Pathology Discipline, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi. Initially, I developed my teaching skills by delivering practical lessons and seminars to 3rd year medical students and 2nd year nursing students, and by contributing to their examination process during exams sessions, when I assisted my coordinating professors. Gradually, my didactic involvement in delivering undergraduate curricula grew to include conducting practical work sessions, final revision sessions before the practical exam (full reviews of microscopic and macroscopic preparations), and coordinating scientific student papers, and events.

Over the years, the teaching approach improved continuously by the introduction of PowerPoint presentations, the selection of paraffin blocks or organs with eloquent pathologies to demonstrate the changes that underlie various diseases or live projection of microscopic images of the slides by using a microscope equipped with a video camera. Throughout my academic career, I have strived to make classes attractive to students by involving them in practical activities aimed to develop their capacity for observation, analysis, and expression.

In 2008, I was promoted to Assistant Professor/Lecturer and a decade later, in 2019, I became an Associate Professor at the Pathology Discipline, Department of Morpho-Functional Sciences I, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, where I am currently working. Once I became a lecturer, I started teaching specialized courses and conducting final examinations for 3rd year medical students studying in Romanian, 2nd year dentistry students studying in Romanian and in English, students enrolled in Nursing, Clinical Laboratory, Nutrition and Dietetics, Radiology and Medical Imaging study programs, as well as for residents in Pathology, Dermatovenerology, Oncology, and Forensic Medicine. To improve my own and the institution's capacity to deliver medical education in the English language, in 2010 I completed a specialized course organized by the Centre of Languages and Continuing Education at the "Alexandru Ioan Cuza" University of Iasi (Certificate of Attainment Council of Europe Level B2 No. 591/29.06.2010).

The teaching experience thus gained enabled me to design didactic materials and continuously update them: course/practical lessons materials (in Romanian and English), case presentations, and image galleries available on the E-learning platform of the University, aimed to facilitate the understanding of the theoretical notions communicated in lectured and seminars.

My academic collaborations with specialists in various medical fields have resulted in the authoring and co-authoring of several books, treatises, monographs, training materials, and guides (one book as first author and 14 as author chapters). The featured areas of interest

include endocrine, dermatological, hepatic, cervico-vaginal and breast pathology, as well as histochemistry and immunohistochemistry techniques applied in pathology.

During my academic activity I have coordinated or been lecturer at 14 postgraduate courses, and I have supervised over 27 bachelor's degree theses.

I am often invited to act as a member in different admission, competition, and examination committees regarding bachelor programs, the appointment of specialist/consultant ship, doctoral scientific reporting and presenting, employment and advancement of faculty members. In addition, I have been regularly involved in coordinating the teaching and scientific activities of residents and students, and I have supervised 19 original papers presented at student conferences, of which some received awards. Last but not least, as appointed tutor, I try to foster open communication with students, to be receptive to their needs and demands, and to give them all my support.

I.4. SCIENTIFIC ACTIVITY

The results of my scientific and research activity have been published in articles indexed by the Web of Science Core Collection and in other international databases. I have also disseminated the results at local, national, and international congresses, conferences, seminars, and workshops.

In my PhD thesis, I studied nodular lesions of the thyroid gland. The new methods of planimetric morphometry and cytochemistry, which were first implemented in our country, represented an effort to enable cytological diagnosis prior to surgery, a significant step in the case of follicular lesions suspected of being malignant. The results proved useful especially in clinically controversial cases, when an interventionist procedure comes with a higher risk, the patients being metabolically imbalanced, or the surgery presents the risk of postoperative complications.

The immunohistochemical techniques were used equally successfully in case of medullary carcinomas and secondary thyroid tumors. The diagnosis of a thyroid medullary carcinoma implied the use of a strategy characteristic to the tumoral type, and the initiation of screening aimed at spotting endocrinological pathologies and family aggregations. The secondary thyroid tumors turned out to be a diagnostic clue, especially in cases when thyroid impairment was the first manifestation of the malign tumoral process.

As previously mentioned, I published one book as principal author and I contributed to the publication of 14 book chapters.

My international visibility in medical journals can be summarized quantitatively as follows:

- Web of Science Clarivate Analytics H-index: 8;
- total number of citations without self-citations: 168;
- in extenso ISI papers: 41, of which 17 as lead author;
- in extenso IDB papers: 59.

I participated at national and international scientific congresses, conferences, courses, and symposia, of which: 32 were national events (e.g., National Symposium on Normal and Pathological Morphology and Annual Session on Pathology, Continuing Education Course "Tumor and Non-Tumor Endocrine Pathology", Regional Conference on Dermatology "Gheorghe Năstase", Congress of the Romanian Society of Endocrinology, National Conference on Clinical Cytology and Clinical Cytology Instruction Symposium, Francophone Symposium on Endocrinology, National Congress on Microscopic Morphology, National Conference of "Clinical Hospital CF Iasi","CONFER" Multidisciplinary Approach in the Management of Neuroendocrine Tumors", International Course on Digestive System

Pathology) and 4 were international (European Congress of Pathology 2010, 2013, 2014, and 2016).

During 2014-2016, I was a member of the research team implementing the internal grant "The effect of selenium supplementation on the antioxidant status, hormonal profile, autoimmune and thyroid ultrasound to euthyroid subjects with chronic autoimmune thyroiditis" awarded after a competition process at the "Grigore T. Popa" University of Medicine and Pharmacy Iasi (contract 29243/2013), coordinated by Professor Cristina Preda, PhD. The project aimed to investigate the potential protective role of selenium in thyroid autoimmunity (AIT) by administering inorganic Se (sodium selenite) to adult Wistar rats with iodine-induced AIT and tracking the effects on thyroid morphology and follicular cytology.

During 2016-2017, I coordinated the internal grant "Molecular stratification algorithm with prognostic value in papillary thyroid carcinoma" awarded by the "Grigore T. Popa" University of Medicine and Pharmacy Iasi (contract 31584/2015). The main scientific objective of the project was to build and implement an algorithm for papillary thyroid carcinoma (PTC) stratification based on a protein (molecular) profile which included "candidate" prognosis markers, thus validating substantially more sensitive differentiation criteria for the assessment of neoplastic evolution potential. The relevance of the goal set derived from the promising capacity of identifying, within PTC (defined through the standard histological criteria), subcategories (or subclasses) of PTC with different evolutions, founded on subtle differences in the carcinogenic pathogenic mechanism, which had a direct impact upon prognosis.

Since 2020, I have been a part of the national team involved in the Horizon 2020 project "REVERT – taRgeted thErapy for adVanced colorEctal canceR paTients" (848098 — REVERT — H2020-SC1-BHC-2018-2020/SC1-2019-Two-Stage-RTD) which will be implemented over a period of 4 years (2020-2024). In this project, I am the scientific coordinator representing "Sf. Spiridon" Clinical Emergency County Hospital. The REVERT project is managed by a consortium that involves many renowned European research institutes, as well as several certified biobanks from different countries (Italy, Spain, Germany, Romania, Luxembourg, Sweden). The REVERT project address the specific challenge of understanding at artificial intelligence system level the pathophysiology of metastatic colorectal carcinoma (mCRC) in patients responding well or poorly to therapies, in order to design optimal strategy for mCRC on a case-by-case basis, with therapeutic interventions modulated depending on patient's features. Accordingly, REVERT will build up an innovative artificial intelligence (AI)-based decision support system using the experience and the real-world data of several general hospitals operating in the EU healthcare system ultimately aimed at developing a new and improved model of combinatorial therapy that can identify in a personalized manner the most efficient and cost-effective therapeutic intervention for patients with unresectable mCRC.

I.5. FINAL REMARKS

In my view, a ***career plan*** is an essential prerequisite for coherent and robust personal and professional development. Exercising accountability for one's progression, monitoring and continuously improving the different aspects of one's actions generate an upward spiral of sustainable, harmonious evolution. These general considerations must be tailored to the specialty. For instance, Pathology is one of the most dynamic disciplines in present-day medicine, as the volume of generated information is growing exponentially, the connections with other disciplines are multiplying, and local perspectives are gaining global relevance. In this context, prompt and flexible adaptation is no longer an option, but a *sine-qua-non* condition for professional pathologists such as myself.

SECTION II.
THE THYROID BETWEEN MALIGNANCY AND AUTOIMMUNITY

CHAPTER 1.
STATE OF THE ART

Abnormalities of the thyroid gland are the most common lesions encountered in endocrine surgical practice. They include both non-neoplastic and neoplastic disorders. The main clinical and paraclinical manifestation in thyroid pathology is **thyroid nodule (TN),** defined as *"a circumscribed tumefaction relatively well delimited, with variable size, different morphology and echoic structure from by the adjacent thyroid tissue"* [Zbranca et al., 2008; Goel, 2015]. In the general population TNs are frequently identified, creating a permanent diagnostic dilemma. The prevalence of TN is between 5-50% depending on the investigated population and the identification method: 3.7-7% clinically, 42-67% on ultrasound and around 50% on autopsy [Nikiforov et al., 2017; Fridrihsone et al., 2018]. The incidence of TN is higher in females than in males (4/1 ratio) [Wilhelm, 2014; Lloyd et al., 2017]. The prevalence of TN increases linearly with age (in children not exposed to radiation it is around 1.5%, increasing about 0.1% per year), with radiation exposure, and iodine deficiency [Lloyd et al., 2017; Iglesias, 2017].

The clinical significance of TN founded on ultrasound in subjects who are suspected of thyroid disease is difficult to assess, however, a small number of nodules belonging to this category may be malignant [Ahn et al., 2018]. In fact, although only 5-15% of all clinically detectable nodules with cold scintigraphic pattern are malignant [Lim et al., 2016], and the prognosis of thyroid carcinomas (TC) is quite good (with a 5-year survival rate of 98.3%) [National Cancer Institute, 2021], the critical problem of TN consists precisely in differentiating their benign or malignant character, due to increasing the number of nodules identified by the detection systems [Hoang, Nguyen, 2017; Fridrihsone et al., 2018], and increase worldwide TC incidence (2.3% of all cancers, with an estimated new number of cases by 2021 of 44280) [National Cancer Institute, 2021]. In TN the purpose of investigations is to identify its nature, for the number of undiagnosed carcinomas and surgeries to be as small as possible, due to the postoperative complications, such as hypoparathyroidism, or vocal cord paralysis [Cannizzaro et al., 2017].

TC are the most common endocrine tumors and they account for 70% of all endocrine malignancies, approximately 1-5% of malignancies in women and 2% in men [Sherman et al., 2003; De Lellis, Williams, 2004; Kilfoy et al., 2009; Ferlay et al., 2010, Nikiforov et al., 2017; Miranda-Filho et al., 2021]. Differentiated thyroid carcinomas (DTC) represent more than 90% of TC, originating in the follicular cells. Two main entities are included in this tumoral category, according to the histopathological criteria: papillary thyroid carcinomas (PTC) and follicular thyroid carcinomas (FTC). PTCs are the most frequent DTCs, representing more than 70% of thyroid malignancies [De Lellis, Williams, 2004; Toniato et al., 2008; Sciuto et al., 2009; Jemal et al., 2009, Forman et al., 2014]. Medullary thyroid carcinoma (MTC) appears from parafollicular C cells, and accounts for less than 2-3% of all thyroid malignancies. Other malignant tumor identified in thyroid are Hurthle cell

carcinomas, poorly differentiated thyroid carcinoma (PDTC) or anaplastic thyroid carcinoma (ATC) with a very low incidence [Erhamamci et al., 2014; Romero-Rojas et al., 2015; Lloyd et al., 2017; Nikiforov et al., 2017].

Recent studies have confirmed an increase in the TC incidence worldwide, with clear reports in the oncological records of most European countries, the United States (which doubled or quadrupled in the last 3 decades) and Canada (four and five times as much), mainly affecting the young population [Kitahara et al., 2016; Bray et al., 2018; National Cancer Institute, 2021]. The incidence of TC has risen as diagnosis methods have become more reliable by the large implementation of high-resolution medical imaging or fine needle aspiration biopsy (FNAB), as well as by the refinement of histopathological and immunohistochemical methods [Chung et al., 2009; Pellegriti et al., 2013; Vaccarella et al, 2015].

The etiopathogenesis of TC is different depending on the tumor subtype.

PTC etiopathogeny incriminates environmental, hormonal and genetic factors [De Lellis, Williams, 2004; Nikiforov et al., 2017]. Environmental factors are related to radioactive iodine (RAI) and external radiation with a genotoxic effect or hormonal stimuli (thyroid stimulator hormone - TSH type) with nongenotoxic effect. The effect of the Chernobyl nuclear disaster (1986) on children is well-known, PTC incidence being 30 times as much in Belarus and 100 times as much in Gomel (which is closer to Chernobyl) [Williams et al., 2002; De Lellis, Williams, 2004; Takamura et al., 2016; Nikiforov et al., 2017; UNSCEAR, 2018]. For the PTC, the malignancy risk is 4 times higher for patients having a TC family history, with an ultra-high risk of association of PTC, breast cancer or paraganglioma [Kuratomi et al., 1994; Hemminki, Li, 2003]. In PTC, 4 categories of genetic mutations are described: rearrangements of the RET/PTC gene (80% of cases), TRK rearrangements (10% of cases), RAS gene mutations (10% of cases) and the BRAF gene mutations (more than 70% of cases, being directly reflected in the prognosis) [Nikiforov et al., 2017].

The continuous stimulation of the thyroid tissue through an increased secretion of TSH (as a result of the iodine deficiency) mainly generates FTC [Harach et al., 2002]. The evolution from DTC to the more aggressive PDTC and ATC is triggered by the existence of additional mutations, such as those of the p53 and the telomerase reverse transcriptase (TERT) genes [Liu et al., 2013; Tavares et al., 2016; Ulisse et al., 2021]. Same as for other types of solid cancers, the genetic instability is supposed to denote the driving force by which transformed thyreocytes accumulate additional gene mutations during disease progression [Ulisse et al., 2021]. In fact, a successive increase in chromosomal abnormalities was observed from well-differentiated PTC to PDTC and ATC, in terms of both number and frequency of detectable abnormalities [Wreesmann et al., 2002].

Genetic susceptibility is also demonstrated in medullary thyroid carcinomas (MTC), frequently associated with MEN2A or 2B syndromes [Nikiforov et al., 2017].

In Romania thyroid pathology is vast, the goiter zones reported in the North-Eastern and Central areas of the country being validated in time by extensive practical experience in endocrinology. An increase in the TC incidence by 10 times in the last decades has been documented, mostly on youth and PTC, possibly related to the Cernobyl nuclear disaster [Piciu et al., 2012; Cătană et al., 2012; Piciu et al., 2014; Globocan, 2020], or related to the iodine salt administrated as a food supplement through a national programme [Găleşanu et al., 1989; Mogoş et al., 1995; Ivan et al, 2002; Buzdugă et al., 2011]. Unfortunately, at national level, the research is focused mainly on epidemiologic and clinical elements. The study of immunohistochemical and molecular profile of the TC is insufficiently tackled, while report dissemination and visibility in mainstream publications is rather scarce [Nechifor Boilă et al., 2013; Nechifor Boilă et al., 2014; Nechifor Boilă et al., 2015; Nechifor Boilă et al., 2016].

The World Health Organization (WHO) published the WHO Classification of Tumors series to provide the international standards for diagnosis and cancer research, and updates the series on a regular basis, describing and illustrating the characteristics of each cancer type, including diagnostic criteria, pathological features, and associated molecular alterations. The latest endocrine tumor classification was published in the fourth edition of the WHO series in 2017 [Lloyd et al., 2017]. Compared with the third edition of the WHO classification published in 2004 [De Lellis, Williams, 2004], the most significant updates in the 2017 WHO classification of thyroid tumors involve: molecular and genetic characterization of follicular-derived thyroid tumors, a new classification for encapsulated well-differentiated follicular tumors, changing the international classification of diseases for oncology (ICD-O), identification of new variants of PTC, sub-classification of FTC into minimally invasive, encapsulated angioinvasive and widely invasive types, reclassification of Hürthle cell lesions and PDTC as distinct entities and changing the term carcinoma showing thymus-like differentiation to intrathyroid thymic carcinoma [Bai et al., 2020].

The new class of borderline tumors (hyalinizing trabecular tumor, noninvasive follicular thyroid neoplasm with papillary-like nuclear features - NIFTP, and tumors of uncertain malignant potential) is an important amendment in thyroid pathology practice [Carney et al., 2008; Nikiforov et al., 2016; Kakudo et al., 2018; Bai et al., 2020]. With this update, the profile of thyroid tumors could be structured into three risk groups depending on tumoral progression by the presence of recurrences or metastasis: negligible risk (<0.1%) in benign tumors, very low risk (<1%) in borderline tumors, and high risk in malignant tumors [Lloyd et al., 2017]. So, the new definition of follicular adenoma (FA) became *"benign, encapsulated, noninvasive neoplasm showing evidence of thyroid follicular cell differentiation, without nuclear features of PTC"* [Lloyd et al., 2017]. In this way, the noninvasive encapsulated follicular pattern tumors with PTC-like nuclear features are excluded from FA and are reclassified in NIFTP or well-differentiated tumor of uncertain malignant potential (WDT-UMP) [Lloyd et al., 2017].

In almost every edition of the WHO classification of thyroid tumors there were revisions on diagnostic criteria for PTC. In the last one, a borderline tumor entity was incorporated to reduce the diagnostic discordance, and a nuclear score guide for RAS type PTC was delivered. It was also aimed to reduce overdiagnosis and overtreatment of low-risk PTCs. The fourth edition supplementary modified the definition of PTC and stated that "*PTC is a malignant epithelial tumor showing evidence of follicular cell differentiation and a set of distinctive nuclear features. PTC is usually invasive. Papillae, invasion, or cytological features of PTC are required*." These changes were also intended to decrease overdiagnosis and overtreatment of low-risk PTCs, and the majority of encapsulated PTCs without clear-cut invasion were relegated to the borderline tumor category, including WDT-UMP and NIFTP [Nikiforov et al., 2017; Bai et al., 2020].

Fifteen histological variants are described for PTC, as follows: classical or conventional, follicular (FPTC), oncocytic, tall or columnar cells variants, with clear cells, diffuse sclerosing form, solid/trabecular, cribriform/morular, with prominent "hobnail" cells, with fasciitis-like stroma, papillary microcarcinoma (occult papillary carcinoma), encapsulated, with spindle cell and "Warthin-like" [De Lellis, Williams, 2004; Lloyd et al., 2011; Nikiforov et al., 2017]. Even if the majority of PTCs are well differentiated with a low rate of local invasion, recurrences, or metastases, there is a small group of tumors which show heterogenity with more aggressive variants, with distinct clinical, pathological, and molecular features. These pathological subtypes are considered in the latest American Thyroid Association (ATA) guidelines to be an intermediate risk of recurrence [Haugen et al., 2016]. Among the most aggressive variants of PTC are the diffuse sclerosing variant, tall and columnar cell variant, solid variant, and hobnail variant [Kilfoy et al., 2009; Sciuto et al.,

2009; Roman et al., 2013; Lloyd et al., 2017; Coca-Pelaz A, 2020]. These variants have been associated with higher rates of recurrence and metastasis, and in some cases absence of avidity to RAI therapy and may have lower survival rate [Lam et al., 2005]. Given the lack of knowledge of the natural history of these more aggressive variants, the treatment of these patients is often inadequate or suboptimal [Kim et al., 2013].

FTC is the second common malignancy with follicular cell differentiation, which "*lacks the diagnostic nuclear features of PTC"* [Nikiforov et al., 2017]. In the 2017 WHO classification, minimally invasive FTC is additionally classified as minimally invasive, widely invasive, and encapsulated angioinvasive. There is no amendment in the diagnostic criteria for widely invasive FTC, which extensively occupies the thyroid gland and extra-thyroidal soft tissue and often displays extensive vascular invasion [Nikiforov et al., 2017].

Clinical recommendations for accurate treatment in patients with DTC can differ according to the tumor-node-metastasis (TNM) staging system [Edge et al., 2010; Haugen et al., 2016]. Since the publication of the sixth edition of the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) TNM staging system in 2002, the TNM staging system for thyroid cancer has remained unchanged. In 2016, the AJCC/UICC published the eighth edition of the cancer staging system [Amin et al., 2017]. The most remarkable changes to the TNM staging system include the definition of T3 classification and the cutoff age in DTC. In the seventh edition of the TNM staging system (TNM7), the presence of both minimal and massive extrathyroidal extension (ETE) was an important factor in the definition of T3 tumors [Edge et al., 2010]. However, several studies showed similar clinical outcomes in patients with minimal ETE and those with no ETE [Ito et al., 2006]. Overstaging of DTC was a concern for patients with minimal ETE, including microscopic invasion when their primary tumor size was fewer than 4 cm. In the eighth edition of TNM staging system (TNM8), T3 was defined as a primary tumor bigger than 4 cm limited to the thyroid (T3a) or gross ETE invading strap muscles only (T3b) [Amin et al., 2017]. A significant number of patients with minimal ETE can thus be reclassified as T1 or T2 under TNM8 based on the primary tumor size. Moreover, the cutoff age for patients with DTC changed from 45 to 55 years in TNM8 [Amin et al., 2017]. There is increasing validation that a cutoff age of 45 years has led to overstaging in many patients with DTC, and using 55 years as a cutoff value in the TNM staging system may prevent overstaging in low-risk patients [Nixon et al., 2016]. This new ordering can also provide a more accurate assessment of disease mortality in high-risk patients. A significant number of patients between the ages of 45 and 55 years can be reclassified to stage I or II by applying TNM8.

Thyroid cancer is treated with surgery, followed by RAI ablation and suppression of thyrotropin (TSH) using levothyroxine (LT4). The sensitivity of tumors to RAI treatment varies, some tumors being less sensitive or even resistant to this therapy [Kim et al., 2013; Sethi et al., 2010]. The therapy is adapted according to: the TNM used in the international system of reporting thyroid carcinoma (UICC), the ATA (American Thyroid Association) or ETA (European Thyroid Association) recommendations. The assessment of PTC patients is based on a scoring system which leads to 3 risk categories: low, medium or high, with implicitly reserved prognosis. RAI is adaptable, selective use in certain classes is possible, the decision being up to the radiotherapist or oncologist [Sethi et al., 2010]. On the other hand, there are contradictory and incomplete data related to the benefit of ablational RAI in tumor recurrences related to the progress of patients to death [Kim et al., 2013]. The secondary effect of these radiations, namely the appearance of secondary tumors more frequently in young persons, is known, which turns RAI into a genuine oncological problem [Dean et al., 2000].

Numerous fields of research have established a strong association between chronic inflammation and increased predisposition to neoplastic transformation and cancer

development. It has been estimated that up to 20% of all tumors arise from conditions of persistent inflammation such as chronic infections or autoimmune diseases [Virchow et al., 1956; Balkwill et al., 2001; Franks et al., 2012]. A direct association between these two conditions has been established particularly in the gastrointestinal tract [Bozec et al., 2010]. The association of autoimmune thyroiditis (AIT) and TC was first documented in 1955 [Babli et al., 2018]. Over time the synchronicity of AIT and PTC has been well recognized in the literature. Some authors believe that coexistent thyroiditis is associated with lower tumor stage and better prognosis of thyroid cancer [Schäffer et al., 1998; Loh et al., 1999; Paparodis et al., 2014]. If this relationship is causative or just accidental remains a point of dispute, with three possible mechanisms: AIT is induced as a response to a pre-existing PTC, PTC is induced or facilitated by a pre-existing chronic inflammatory process, or common mechanisms are responsible for both diseases [Antonaci et al., 2009; Macejova et al., 2019].

Hashimoto's thyroiditis (HT) is the most common autoimmune inflammatory pathology of the thyroid and is the main cause of autoimmune hypothyroidism. It was first termed by Hakaru Hashimoto in 1912 as "lymphomatous struma" [Hashimoto, 1912]. It is characterized by an infiltrate of immune cells able to determine the destruction of the gland, its fibrous involution and consequent hypothyroidism [Lal, Clark, 2005]. Global incidence of HT is assessed between 0.3 and 1.5 cases out of 1000 individuals per year, commonly among members of the female gender (5/20,1) between 30 and 50 years of age [Davies et al., 2006, Muzza et al., 2010]. There are two different clinical variants: the diffuse form and the nodular form [Lal, Clark, 2005]. The main pathological feature is infiltration with lymphoid cells, structured in lymphoid follicles that often show prominent germinal centers, Huthle cell metaplasia, atrophy of thyreocytes, and interstitial fibrosis [Caturegli et al., 2013]. The frequency of AIT has been evaluated as 0.3–2% in children and 4–9.6% in adolescents and is rising [Zdraveska et al., 2012]. The childhood incidence of AIT peaks in early to mid-puberty, with a female majority of 2:1 [Zdraveska et al., 2012]. AIT is considered to be a premalignant lesion, with an increased prevalence in PTC mainly in the nodular variant with normoechogenic irregular background of the thyroid gland on US scans [Januś et al., 2018].

Due to the location in the immediate vicinity of the thyroid gland, other related parathyroid pathologies are identified simultaneously with thyroid glandular ablation. Similar to the other endocrine organs, abnormalities of the **parathyroid glands** include both hyperfunction and hypofunction [Phitayakorn, McHenry, 2006]. Tumors of the parathyroid glands, in contrast to thyroid tumors, usually come to attention because of excessive secretion of parathyroid hormone (PTH) rather than mass effects. Hyperparathyroidism is caused by elevated PTH and is classified into primary, secondary, and tertiary types. Primary hyperparathyroidism usually resulting from an adenoma or hyperplasia of parathyroid tissue [Beard et al., 1989]. Secondary hyperparathyroidism is caused by any condition that gives rise to chronic hypocalcemia, which, in turn, leads to compensatory overactivity of the parathyroid glands. Renal failure is by far the most common cause of secondary hyperparathyroidism, although several other diseases, including inadequate dietary intake of calcium, steatorrhea, and vitamin D deficiency, may also cause this disorder. Hypoparathyroidism is far less common than is hyperparathyroidism [Abate, Clarke, 2017]. Acquired hypoparathyroidism is almost always an inadvertent consequence of surgery; in addition, there are several genetic causes of hypoparathyroidism [Abate, Clarke, 2017].

This brief review of the main theoretical notions that define tumor and autoimmune thyroid pathology makes the bridge with the report of personal achievements. My research activity has focused primarily on thyroid pathology. This interest is justified by the continuity with the knowledge growth from the doctoral period and the expertise development in thyroid disease diagnosis, benefiting from the wide range of cases addressed to "St. Spiridon"

Clinical Emergency County Hospital Iasi, as a center of excellence in endocrinology for the North-Eastern region of Romania. Several papers published in the main stream, as well as some research grants, presented below, have resulted from studies focused on tumor and non-tumor thyroid pathology.

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- **Ciobanu Apostol D**, Giuşcă SE, Căruntu ID, Lozneanu L, Andriescu EC, Moscalu M. Relationships between clinicopathological prognostic factors in papillary thyroid microcarcinoma: a refined analysis based on 428 cases. *Int J Clin Exp Pathol* 2017; 10(8): 8944-8956.

Grants

- Molecular stratification algorithm with prognostic value in papillary thyroid carcinoma (internal research project 31584/2015, U.M.F. "Grigore T. Popa") - coordinator
- The effect of selenium supplementation on the antioxidant status, hormonal profile, autoimmune and thyroid ultrasound to euthyroid subjects with chronic autoimmune thyroiditis (internal research project 29243/2013, U.M.F. "Grigore T. Popa") – team member
- Cluster institutional strengthening and increasing the visibility of innovative regional and structural molecular imaging Northeast (IMAGO-MOL), frame-support for increasing capacity RDI members and competitiveness of SMEs in the area of Romania (POSCCE project, 1CLT/800.014) – team member

CHAPTER 2.
THE RELATIONSHIP BETWEEN THYROID CANCER AND NUCLEAR DISASTER OF CHERNOBYL

2.1. INTRODUCTION

With the mean incidence increasing by 6.2% per year, TC is the fifth most common malignancy diagnosed in women, and the most common cancer in women younger than 25 years [Carling, Udelsman, 2011]. The globally increasing incidence of TC has not been accompanied by an increasing mortality rate, which remarkably has in fact declined or remained stable [Miranda-Filho et al., 2021]. Data from the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program in the United States confirm that patients with PTC that behave aggressively (i.e., distant metastases at presentation) are rare and numerically constant [Colonna et al., 2015]. Additionally, the mortality of patients with metastatic PTC at the time of diagnosis during 1992–2017 was low (0.059 per 100,000 population per year) [Li et al., 2020]. The reason for this inconsistent outcome is mostly due to the growing number of small-PTC (≤1 cm) cases that are being diagnosed, potentially leading to overtreatment [Jegerlehner et al., 2017; Dal Maso et al., 2018].

For every type of cancer in every population worldwide, age is a key determinant of the risk of developing the disease [Armitage, Doll, 1954]. The age-standardized incidence rate (ASIR) is the summary rate that would have been observed, given the schedule of age-specific rates, in a population with the age composition of some reference population, called the standard population [Bray et al., 2014]. Calculation of the standardized rate is an example of direct standardization, whereby the observed age-specific rates apply (directly) to a theoretical standard population. ASIR in TC showed an upward trend worldwide [Deng et al., 2020]. The three highest incidence countries are China: 11,016 patients in 1990 and 41,511 in 2017, United States: 10,833 patients in 1990 and 25,896 in 2017 and India: 7369 patients in 1990 and 25,675 in 2017 [Deng et al., 2020]. An increased incidence was seen in 34% cases of high socio-demographic index areas [Deng et al., 2020], whilst the cases of relative mortality in TC are inversely proportional to the socio-demographic index [Goodarzi et al., 2019]. Increased incidence has also been reported in Europe with an overall 5-year age-standardized relative survival of 88% in women and 81% in men [Dal Maso et al., 2017].

The numbers of deaths per 100,000 population are influenced by the age distribution of the population. Two populations with the same age-specific mortality rates for a particular cause of death will have different overall death rates at different age distributions. Age-standardized death rates (ASDR) account for differences in the age distribution of the population by applying the observed age-specific mortality rates for each population to a standard population [Lopez et al, 2006]. ASDR in TC studied all over Europe showed that, in our country, results for ASDR were similar from 2000 (0.33 for men and 0.43 for women) to 2010 (0.3 for men and 0.43 for women), meaning that deaths caused by TC were stationary over time [La Vecchia et al., 2015].

In addition to inherited molecular changes for risk factors associated with increased incidence of TC, it also includes molecular changes caused by radiation [Ruggeri et al., 2008; Bazyka et al., 2018]. The worst nuclear disaster in history was the Chernobyl fallout in 1986,

with the radioactive cloud traveling 250,000 km^2 to North-Western Europe, leading to exposures of nearby populations mainly from beta and gamma radiation [UNSCEAR, 2018]. The radioactive materials were deposited mostly in Belarus, Russia, Ukraine, but also in many other European countries depending on wind speed and direction, and rainfall. In the initial days and weeks after the disaster the short half-life radionuclides were the most important contributors to the extrinsic and intrinsic effects of radiation [Iodine-131 (^{131}I) especially]. Later the significance of Cesium radio nuclides [^{134}Cs (Caesium 134), especially ^{137}Cs (Caesium 137)] started to grow, and it became the most important factor, especially over large distances [Open University, 2013; UNSCEAR, 2018]. Due to wind, precipitation patterns at that time and geometric characteristics, radioactive deposits vary from region to region [Begy et al., 2017]. Many of the children and adolescents received high thyroid doses, almost entirely because they drank fresh milk containing I-131 in the first few weeks following the disaster. The average absorbed thyroid doses of evacuated children and adolescents, and of non-evacuated children and adolescents (at the time of the disaster) in the so-called "contaminated areas" of the former USSR was about 900 mGy and 170 mGy, respectively [UNSCEAR, 2018]. Our country is located in the immediate vicinity of Chernobyl, more precisely to the Western part of Ukraine, and the North-Eastern part of Romania, where we conducted this study, is positioned about to 600 km from the epicenter of the disaster [Open University, 2013].

Aim

Considering that current information, after the Chernobyl fallout, showed that the incidence of TC is increased in those exposed to nuclear radiation [Bazyka et al., 2018], our aim was to analyze whether the incidence of TC in the North-Eastern region of Romania in the past 10 years was related to Chernobyl radiation exposure or not. Starting from our published results based on the 7th edition of AJCC reporting system, our study was developed in this Habilitation Thesis by the re-assessment of data in accordance with the changes in the 8^{th} edition AJCC reporting system, regarding the age cutoff, as prognostic factor. The main reason for this information is to complete the worldwide map with updated results.

2.2. MATERIALS AND METHODS

We performed a retrospective study that included patients diagnosed with TC over a period of 10 years (2009 – 2019) at "St. Spiridon" Clinical Emergency County Hospital, Iași. The research was approved by the Ethics Committee of the "St. Spiridon" Clinical Emergency County Hospital Iasi, pursuant to the ethical standards of Helsinki declaration regarding the patients' informed consent for the use of their medical information for scientific purpose.

Patients

Data were collected from information system (InfoWorld) using the key word TC that represented the admission diagnosis for patients. In settlement with WHO standards, pathologies were coded according to the International Classification of Diseases for Oncology (ICD-O-3) [Fritz A, 2013]. For our analyses, we used primary invasive thyroid cancer cases (ICD-O-3: C73) recorded between 2009 and 2019. After that, TC were grouped by: (i) histological subtype, as follows: papillary – ICD-O-3 codes M8050/0, M8050/2, M8050/3, M8050/6, M8260, M8341, M8342, M8344, M8350; non-papillary – ICD-O-3 codes M8020, M8021, M8030, M8031, M8032, M8033, M8041, M8290, M8330, M8331, M8332, M8335, M8345, M8346, M8347, M8510, M8511 and M8512; rare (<1%) (others/unknown); (ii) tumor stage (early, advanced) according to recent oncology guidelines, by using the TNM classification provided by the American Joint Committee on Cancer

(AJCC) Cancer Staging Manual [Compton et al., 2012; Haugen et al., 2016; Greene et al., 2017; Jegerlehner et al., 2017]. Firstly, the 7th edition of AJCC reporting system was used to make uniform reported data between our study group and literature, considering that the main references used this edition. Secondly, the 8th edition AJCC reporting system was taken into account, to translate the age cutoff at diagnosis from 45 years to 55 years. Inclusion criteria contained all malignant TC and the exclusion criteria were benign thyroid pathologies, or cases with partial data.

Age-adjusted incidence was obtained using public statistical data obtained from the Romanian National Institute of Statistics using interrogations on Tempo Online Platform [National Institute of Statistics, 2021].

Statistical analysis

The database with hospitalization dates and histopathological results was analyzed by using statistical methods. Rates were age-standardized to the Romanian standard population as published by the National Institute of Statistics. We figured annual thyroid cancer incidence rates stratified by gender, histological subtype and tumor stage. We fitted a linear regression model to estimate the annual mean absolute and relative changes in the standardized rates, with calendar year as predictor variable. A Pearson's chi-squared ($\chi 2$) test was performed to determine the differences in age stratification and histopathological factors between the groups. Continuous outcomes were analyzed using independent t-tests for groups of two and one-way analysis of variance among groups of three of more. $p < 0.05$ was considered significant. Statistical analyses were performed with Excel.

2.3. RESULTS

We included 1159 patients following key word: TC search (C73). Of them, 191 (16.48%) were men and 968 (83.52%) women.

Stratifications by counties highlight that a majority of 529 (45.64%) were from Iasi, where the Endocrinology academic center of the North-Eastern region in Romania is located, followed by neighboring counties. Age-adjusted incidence of TC displayed increased age-adjusted results for Iasi county (55.82/100,000 population), followed by Vaslui (44.26/100,000 population), Suceava (24.40/100,000 population), Neamţ (23.19/100,000 population), Botoşani (21.59/100,000 population), Bacău (17.26/100,000 population), Galaţi (7.20/100,000 population), and Vrancea (3.04/100,000 population). The first two counties have the most patients diagnosed with thyroid carcinoma per 100,000 inhabitants (Figure 2.1).

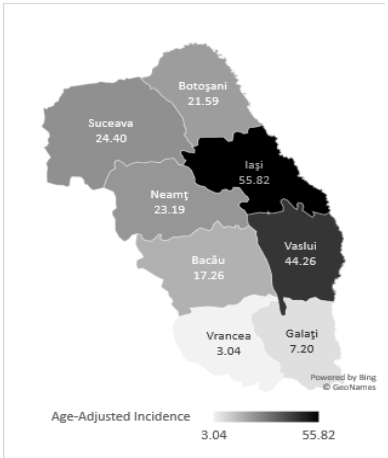


Figure 2.1. Age-adjusted thyroid carcinoma incidence in North-Eastern counties of Romania [National Institute of Statistics, 2021]

The histopathological results, available for 1068 patients, showed that PTC was most common in a total of 63.10% (classic variant of PTC and multiple endocrine neoplasia type 1), followed by follicular carcinoma (14.7%), follicular variant of PTC (10.67%), medullary carcinoma (6.74%) (sporadic medullary carcinoma and multiple endocrine neoplasia type 2), Hürthle cell carcinoma (2.43%), anaplastic thyroid carcinoma (1.02%), thyroid primary lymphoma (0.56%) and poorly differentiated thyroid carcinoma (PDTC) (0.28%). Considering the association of endocrine syndromes and TC, multiple endocrine neoplasia (MEN) 1 presented PTC in 0.18% and MEN 2 had medullary carcinoma in 0.28% (Table 2.1).

Table 2.1. Incidence of histopathological characteristics in 1068 patients

Histological Characteristics	**Number of Patients (%)**
Papillary thyroid carcinoma:	
Classical variant	672 (62.92)
Follicular variant	114 (10.67)
MEN 1 associated	* 2 (0.18)
Medullary thyroid carcinoma:	
Sporadic	69 (6.46)
MEN 2 associated	* 3 (0.28)
Follicular thyroid carcinoma:	
Classical variant	157 (14.7)
Hürthle cell thyroid carcinoma	26 (2.43)
Anaplastic thyroid carcinoma	11(1.02)
Poorly differentiated thyroid carcinoma	3 (0.28)
Primary thyroid lymphoma	6 (0.56)

* Considering that final genetic diagnosis is missing for MEN, those patients will be counted in papillary and medullary sections.

The histopathological exam revealed specific features for the PTC diagnosis. In classic/conventional PTC, the characteristic architecture was represented by branching papillae with central fibrovascular stalk, covered by cells with eosinophilic cytoplasm (Figures 2.2 – 2.4). The nuclei were larger than normal, often overlapping, looking "empty" ("Orphan Annie" nuclei), intranuclear inclusions and nuclear grooves. Cell polarity was completely lost. Squamous metaplasia was present 5% of the cases. The psammoma bodies (concentric, lamellar, calcified spheres composed in part of thyroglobulin) were identified in 50% of the cases (Figures 2.2 - 2.3). Some tumors also contained multinucleated giant cells. On the pushing edges of PTC in the adjacent thyroid tissue we found infiltrative foci (Figure 2.5).

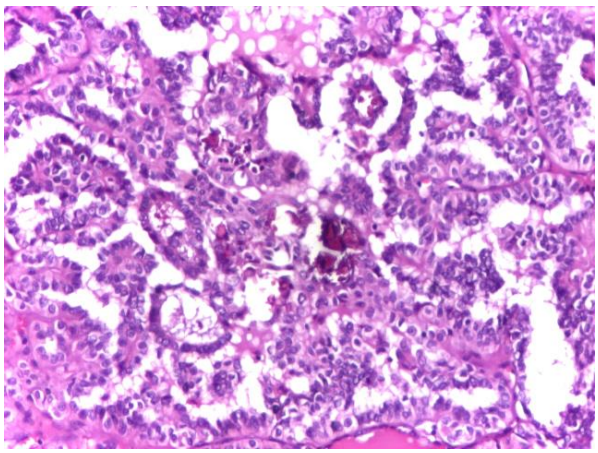


Figure 2.2. Branching papillae covered by cells with "Orphan Annie" nuclei, and psammoma body (HE, x 400)

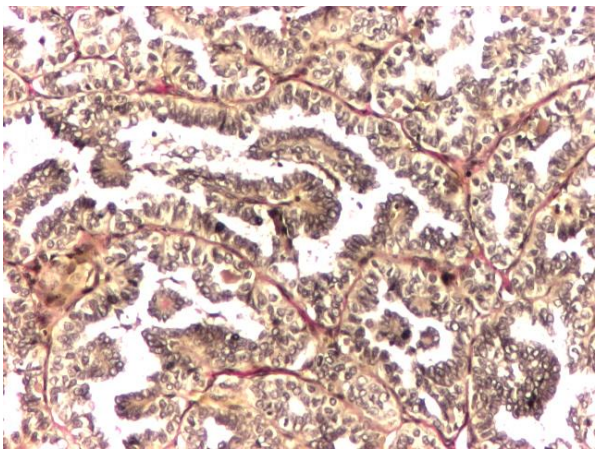


Figure 2.3. Branching papillae with a central fibrovascular stalk (VG, x 400)

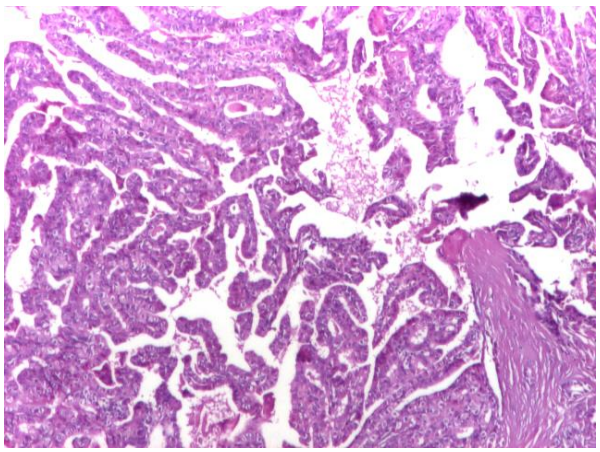


Figure 2.4. Tumoral cells with eosinophilic cytoplasm (HE, x400)

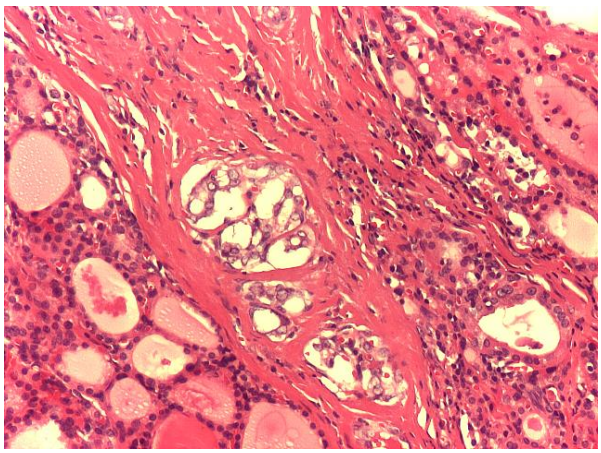


Figure 2.5. Infiltration of adjacent thyroid (HE, x100)

Highest annual incidence (120 cases) was found for PTC in 2009 – the first year of the assessed period; after that, incidence suffered some peaks over the years, as shown in Figure 2.6. The other histological subtypes were in plateau.

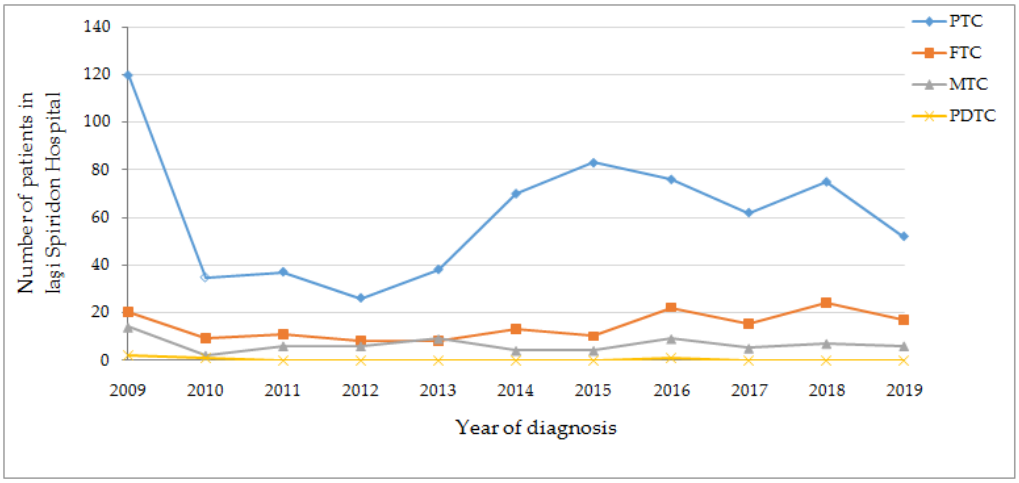


Figure 2.6. Thyroid carcinoma's histopathological picture of the North East region (2009–2019).
PTC—papillary thyroid carcinoma; FTC—follicular thyroid carcinoma; MTC—medullary thyroid carcinoma; PDTC—poorly differentiated thyroid carcinoma.

Table 2.2 provides a comparative picture of the main characteristics of the cases diagnosed each year. Our data showed that over the studied period, the mean age of the patients with TC was between 52 and 57.4 years (the 6th decade), with a stable male-to-female ratio over time (Table 2.2). No statistically significant results were found for age, gender and histological type of TC, between the subgroups of patients diagnosed in each year of the study (Table 2.2).

Considering that the peak of TC incidence was in 2009, mean age in that year was 51.7 for female and 53.9 for males. Those patients were about 28.7 years old for female and 30.9 years old for males at the time of the Chernobyl disaster. Consequently, we identified 48 cases in 2009 who were children and young adults at the time of the nuclear fallout (0–20 years old) (Table 2.2).

Table 2.2. Timeline characteristics of this study (age, gender, histopathology)

	2009	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	***Chi-square test***
Age at diagnosis												
Overall	52.0	52.1	55.5	53.7	54.6	53.3	55.9	55.8	57.0	55.1	57.4	1.000
Male	53.9	57.6	58.8	57.5	54.6	53.7	57.3	55.5	51.3	55.3	56.2	1.000
Female	51.7	50.9	54.6	52.6	54.7	53.2	55.5	55.9	57.9	55.0	57.6	1.000
p-value	0.911	0.590	0.773	0.717	0.909	0.956	0.949	0.886	0.462	0.939	0.812	
Gender												
Male	27	14	17	18	19	17	22	23	14	11	9	0.528
Female	177	64	68	62	74	89	89	102	89	94	60	0.998
Male-to-Female ratio	0.15	0.22	0.25	0.29	0.26	0.19	0.25	0.23	0.16	0.12	0.15	
Total	204	78	85	80	93	106	111	125	103	105	69	
0–20 years old in 1986 (26.91%)	48	16	23	18	16	33	29	39	27	39	24	
Pathological classification												
Papillary	120	35	37	26	38	70	83	76	62	75	52	0.259
Follicular	20	9	11	8	8	13	10	22	15	24	17	
Medullary	14	2	6	6	9	4	4	9	5	7	6	
PDTC*	2	1	0	0	0	0	0	1	0	0	0	

* PDTC - poorly differentiated thyroid carcinoma
**p-value <0.05 was considered to be statistically significant*

PTC has consistently remained at the forefront of the TC category, with a PTC/FTC ratio ranging from 3.05/1 (2019) to 8.3/1 (2015) and a percentage of differentiated TC (DTC) from 71.26% (2017) at 95.36% (2019) (Table 2.3).

Table 2.3. Incidence of PTC and PTC/FTC ratio between 2009–2019

Year of diagnosis	**PTC**	**FTC**	**PTC/FTC**	**PTC % DTC**
2009	**120**	20	6/1	85.71%
2010	35	9	3.8/1	79.54%
2011	37	11	3.36/1	77.08%
2012	26	8	3.25/1	76.47%
2013	38	8	4.75/1	82.60%
2014	70	13	5.38/1	84.37%
2015	83	10	**8.3/1**	89.24%
2016	76	22	3.45/1	77.55%
2017	62	15	4.13/1	71.26%
2018	75	**24**	3.12/1	75.75%
2019	52	17	3.05/1	**95.36%**

The percentage of TC identified in people aged 0 to 20 years at the time of the Chernobyl nuclear disaster in relation to the total number of diagnosed cases ranged from 17.2% (2013) to 37.14% (2018) (Table 2.4).

Table 2.4. Incidence of patient with TC with 0-20 years at time of Chernobyl disaster

Year of diagnosis	**Number TC**	**Number TC (0-20 year)**	**%**
2009	204	48	23.5%
2010	78	16	20.5%
2011	85	23	27.05%
2012	80	18	22.5%
2013	93	16	17.2%

Year of diagnosis	Number TC	Number TC (0-20 year)	%
2014	106	33	28.30%
2015	111	29	26.17%
2016	115	39	31.2%
2017	103	27	26.21%
2018	105	39	**37.14**%
2019	69	24	34.78%

Out of 971 patients (90.91%) with well differentiated thyroid carcinomas, 240 patients (24.71%) received radioactive iodine treatment.

TNM staging results showed 504 evaluated results, of which the most common was pT1 (34.92%), followed by pT3 (18.45%), pT2 (4.16%) and pT4 (0.39%). Lymph node involvement was found as N1a in 12.3% cases and N1b in 0.39% cases. Secondary lesions were encountered in 0.39% patients with valid histopathological results.

To investigate the influence of patients' age and genders on histological subtype of cancer, we divided all patients by age and gender groups. This classification displayed that per both genders, PTC was the most commonly encountered. The peak of TC was detected in the age group of 51–60 years old (296 patients): 40 males and 256 females. Papillary carcinoma was found in 75% for both genders (Table 2.5).

Table 2.5. Histopathological incidence by gender and age groups

	<10		**11–20**		**21–30**		**31–40**		**41–50**		**51–60**		**61–70**		**71–80**		**≥81**	
Both genders	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Papillary	0	0	6	85.7	16	72.7	71	75.5	135	75.4	222	75	162	71.4	51	67.1	11	84.6
Follicular	0	0	0	0	2	9.1	15	16	31	17.3	48	16.2	46	20.3	13	17.1	2	15.4
Medullary	0	0	1	14.3	4	18.2	8	8.5	12	6.7	22	7.4	16	7	9	11.8	0	0.0
Anaplastic	0	0	0	0	0	0	0	0	0	0	2	0.7	3	1.3	2	2.6	0	0.0
PDTC*	0	0	0	0	0	0	0	0	1	0.6	2	0.7	0	0	1	1.3	0	0.0
Total	0	0	7	100	22	100	94	100	179	100	296	100	227	100	76	100	13	100
Males																		
Papillary	0	0	0	0.0	3	60.0	6	85.7	18	78.3	27	67.5	25	67.6	11	50.0	0	0
Follicular	0	0	0	0.0	0	0.0	0	0.0	3	13.0	8	20.0	10	27.0	5	22.7	0	0
Medullary	0	0	1	100.0	2	40.0	1	14.3	2	8.7	3	7.5	2	5.4	5	22.7	0	0
Anaplastic	0	0	0	0.0	0	0.0	0	0.0	0	0.0	2	5.0	0	0.0	1	4.6	0	0
PDTC*	0	0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0
Total	0	0	1	100	5	100	7	100	23	100	40	100	37	100	22	100.0	0	0
Females																		
Papillary	0	0	6	100.0	13	76.5	65	74.7	117	75.0	195	76.2	137	72.1	40	74.1	11	84.6
Follicular	0	0	0	0.0	2	11.8	15	17.2	28	18.0	40	15.6	36	19.0	8	14.8	2	15.4
Medullary	0	0	0	0.0	2	11.8	7	8.1	10	6.4	19	7.4	14	7.4	4	7.4	0	0.0
Anaplastic	0	0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	3	1.6	1	1.9	0	0.0
PDTC*	0	0	0	0.0	0	0.0	0	0.0	1	0.6	2	0.8	0	0.0	1	1.9	0	0.0
Total	0	0	6	100.0	17	100.0	87	100.0	156	100.0	256	100.0	190	100.0	54	100.0	13	100.0

* PDTC - poorly differentiated thyroid carcinoma

**p-value <0.05 was considered to be statistically significant*

According to TNM staging in 7th AJCC edition, with a cutoff value of 45 years old, there were statistically significant results for PTC in groups under 45 and over 45 years old. There were no differences in the distributions of gender, lymph node involvement and metastasis (Table 2.6).

Table 2.6. Characteristics of thyroid cancer patients aged ≤ 45 and > 45 years as in TNM staging by 7th AJCC edition.

Clinicopathological characteristics	**<45**	**≥45**	**Total**	**χ2/F**	***Chi-square test***
Gender					
Male	47	144	191	0.318	p = 0.573
Female	220	748	968		
Histological Type					
Papillary	153	521	674	104.903	**p ≤ 0.001**
Follicular	27	130	157		
Medullary	20	52	72		
Anaplastic	0	7	7		
PDTC	0	4	4		
Others	92	255	347		
LN involvement					
Yes	18	44	62	1.434	p = 0.231
No	44	159	203		
Metastasis					
Yes	0	2	2	0.600	p = 0.439
No	1	3	4		

PDTC—poorly differentiated thyroid carcinoma, LN—lymph node
**p-value <0.05 was considered to be statistically significant*

On the other hand, according to TNM staging in 8th AJCC edition, with a cutoff value of 55 yo, there were significant statistical results for PTC and anaplastic thyroid carcinoma, and lymph nodes metastases, in groups under 55 and over 55 yo (Table 2.7).

Table 2.7. Characteristics of thyroid cancer patients aged ≤ 55 and > 55 years as in TNM staging by 8th AJCC edition.

Clinicopathological characteristics	**<55**	**≥55**	**Total**	***Chi-square test***
Gender				
Male	77	114	191	p = 0.191
Female	440	528	968	
Histological Type				
Papillary	301	373	674	**p = 0.019**
Follicular	54	93	157	
Medullary	31	41	72	
Anaplastic	0	7	7	
PDTC	1	3	4	
Others	169	178	347	
LN metastasis				
Yes	34	28	62	**p = 0.031**
No	80	123	203	
Metastasis				
Yes	0	2	2	p = 0.809
No	1	3	4	

**p-value <0.05 was considered to be statistically significant*

However, regarding the distribution of histological types, the extended age group classification table using 45 yo cutoff (7th AJCC edition) showed that statistically significant results were found for papillary subtype with lymph node invasion and distant metastasis (Table 2.8). Lymph node invasion was due to medullary carcinoma in nine patients, whilst rest of the cases suffered papillary lymph node metastasis.

Table 2.8. Characteristics of thyroid cancer patients by age groups related to the Chernobyl fallout.

Clinicopathological characteristics	**<21**	**21–45**	**46–59**	**≥60**	**Total**	**χ2/F**	***Chi-square test***
Gender							
Male	3	46	56	86	191	8015	p = 0.046
Female	9	227	380	352	968		
Histological Type							
Papillary	6	161	253	254	674	110,375	**p ≤ 0.001**
Follicular	0	30	58	69	157		
Medullary	1	20	23	28	72		
Anaplastic	0	0	1	6	7		
PDTC	0	0	2	2	4		
Others	5	89	133	119	346		
LN metastasis							
Yes	1	19	22	20	62	55.880	**p ≤ 0.001**
No	1	46	74	82	203		
Metastasis							
Yes	0	0	1	2	3	254.545	**p ≤ 0.001**
No	0	4	0	0	4		

PDTC—poorly differentiated thyroid carcinoma, LN – lymph node
**p-value <0.05 was considered to be statistically significant*

Regarding the histological distribution using 55 yo cutoff (8th AJCC edition), statistically significant results were identified in females ($p \leq 0.0399$) and PTC ($p \leq 0.001$) (Table 2.9).

Table 2.9. Characteristics of thyroid cancer patients by age groups related to the Chernobyl fallout.

Clinicopathological characteristics	**<21**	**21-55**	**56-70**	**>70**	**Total**	**Chi-square test**
Gender						
Male	3	79	79	30	191	**p ≤ 0.0399**
Female	9	467	401	91	968	
Histological Type						
Papillary	6	319	287	62	674	**p ≤ 0.001**
Follicular	0	70	72	15	157	
Medullary	1	33	29	9	72	
Anaplastic	0	0	5	2	7	
PDTC	0	1	2	1	4	
Others	5	177	132	33	347	
LN metastasis						
Yes	1	34	22	5	62	p = 0.223
No	1	86	100	16	203	
Metastasis						
Yes	0	0	2	0	2	p = 0.659
No	0	2	2	0	4	

**p-value <0.05 was considered to be statistically significant*

2.4. DISCUSSION

Our results are in agreement with previous studies that have shown close outcomes regarding histological findings [Dal Maso et al., 2017]. As in EUROCARE-5 [Dal Maso et al., 2017]. We identified that the most common TC is papillary carcinoma, followed by follicular type, medullary type and undifferentiated carcinomas. Similar results showed that 63.10% were papillary carcinomas in our study compared to a total of 71% in EUROCARE-5

[Dal Maso et al., 2017]; also, 14.7% were follicular carcinomas compared to a total of 15% in the European study, displaying almost identical result between these two types of carcinomas. Medullary carcinoma was reported in 6.74% of cases in our study, more cases compared to the total of 5% in the European study [Dal Maso et al., 2017]. Comparing with Eastern Europe's results, our numbers showed that papillary carcinoma incidence was similar in our region as in Estonia and Latvia, and follicular carcinoma incidence was similar in our region as in Bulgaria, Czech Rep., Latvia, Estonia and Slovakia [Dal Maso et al., 2017]. Medullary carcinoma incidence was similar to that of Estonia, Latvia and Poland [Dal Maso et al., 2017]. Anaplastic carcinoma was identified in 1.02% of those in our study, fewer cases than EUROCARE-5, which reported a total of 3%, and a similar result with the 2% encountered in Lithuania [Dal Maso et al., 2017]. We were not able to include more histological subtypes, such as: follicular variant of papillary carcinoma, Hürthle cell carcinomas, poorly differentiated carcinomas and MEN, because of the relatively small group of patients in this study compared to the large groups included in EUROCARE-5 [Dal Maso et al., 2017].

We also classified our cases by TNM, and the most common stage that we found was pT1 34.92%, fewer cases than those reported by Lim et al. (67.4%), those results being explained by the huge difference in patient numbers included in each study [Lim et al., 2017]. Comparative results, considering the patient number gap, were obtained for pT2 (4.16% in our study and 7.6% in the other study) and pT3 (18.45% in our study compared to 12%). We observed similar results to Lim et al. regarding the sequence of TNM percent: pT1 and then pT3 [Lim et al., 2017].

Gender incidence (83.52% cases in women and 16.48% cases in men) is also in accordance with the literature. These results are similar to those encountered by our colleagues from Cluj in Romania: 88.8% females and 11.2% males [Piciu et al., 2014]. Our female incidence is slightly higher than the worldwide incidence (77.1%) and male incidence is lower in our region than the worldwide incidence (29.9%) [Goodarzi et al., 2019].

Age-adjusted incidence shown that Iasi and Vaslui counties had the most patients per 100,000 inhabitants diagnosed with TC. In Iasi is located the only academic Endocrinology Clinic in the North-Eastern region of Romania, so most of the patients were addressed here, especially those from neighboring counties, such as Vaslui. Other close counties had a lower age-adjusted incidence because they might have referred to other Endocrinology centers (Cluj, Bucharest) due to more fluid infrastructure. Furthermore, for Botosani county, infrastructure is problematic; therefore, patients lack proper medical surveillance.

These results complete the work of our colleagues that highlights the same events reported between 1970 and 1985 before and after the Chernobyl fallout exposure during 1986–1992. Before the Chernobyl disaster they encountered a total of 94 thyroid carcinomas: 33.84% papillary, 34.78% follicular, 7.52% medullary and 12.22% anaplastic. After Chernobyl exposure (1986–1992) numbers changed, as they encountered 101 thyroid carcinomas: 47.47% papillary, 16.16% follicular, 4.04% medullary and 34.34% anaplastic. Major change consists in increased undifferentiated histology after the Chernobyl fallout, and over the years this histology was not connected to the radioactive disaster, the main reason being increased addressability after this nuclear disaster [Mogoş et al., 1995].

Another study conducted in our endocrinology department showed that between 2001 and 2004 125 patients were diagnosed with papillary carcinoma and after that, between 2005 and 2008, 276 patients were diagnosed with papillary carcinoma [Buzduga et al., 2011]. Unfortunately, we were confronted with a lack of information regarding thyroid carcinoma's incidence between the years 1993 and 2000.

Combined results of these three studies, conducted in the same Endocrinology Department, show that papillary carcinoma has considerably increased incidence over time, followed by follicular carcinoma in a smaller proportion, as we also seen in Miranda-Filho

reported data [Miranda-Filho et al., 2021]. Medullary and anaplastic carcinomas remained in plateau in our analysis (Figure 2.7).

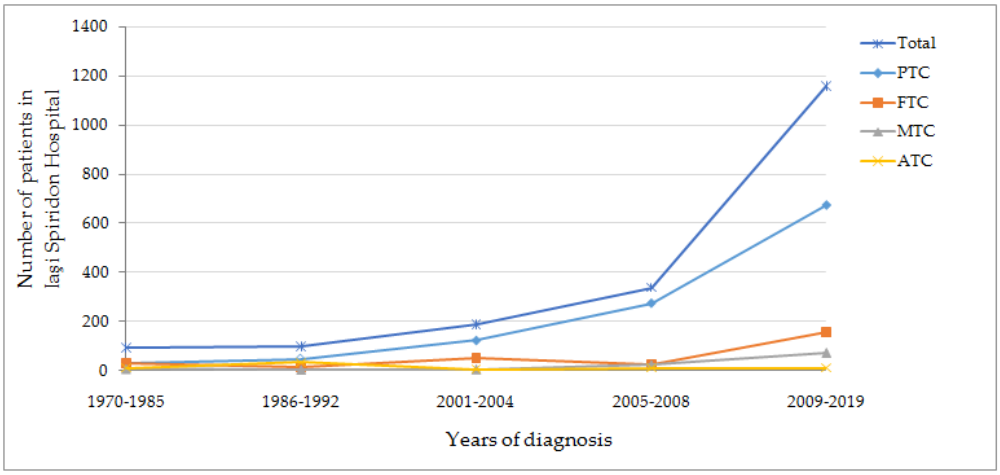


Figure 2.7. Thyroid carcinoma's evolutionary timeline before and after Cernobyl nuclear fallout exposure. Combined results of Mogos et al. (1970–1992), Buzduga et al. (2001–2008) and our results. The period of 1993–2000 is missing from this figure [Mogoş et al, 1995, Buzduga et al., 2011]

PTC—papillary thyroid carcinoma; FTC—Follicular thyroid carcinoma; MTC—medullary thyroid carcinoma; ATC—anaplastic thyroid carcinoma.

All Romanian studies of TC incidence were reviewed, and the results showed that the literature reports an increased incidence of 2–5 times depending on each author. Maximum incidence of radiogenic TC occurred within 5–10 years from the disaster, with a larger potential of 1–20 years, depending on the exposure rate. Our data complete these results showing a constant increase in thyroid carcinoma over 30 years after the Chernobyl fallout [Stanciu et al., 2015; Gábora et al, 2018]. Piciu et al. show a vast increase of thyroid carcinoma cases in Romania since 1970, with a peak of 511% between the years 2001 and 2010 in contrast to 1970–1980. Our highest incidence was in 2009, 23 years after this nuclear disaster [Piciu et al., 2014].

It should be noted that the number of TC cases in Moldavia area has increased exponentially, from 94 TC diagnosed before 1986 over 15 years (1970-1985), to 101 cases diagnosed in 7 years (1986-1992) and respectively 1068 patients in the 11-year study period (2009-2019) [Mogoş et al., 1995; Buzduga et al., 2011]. The upward trend of diagnosed TC cases overlaps with the general trend of the country, if we compare the number of diagnosed cases in the central area, where 99 cases were identified in the period 1990-1999 and 434 cases in the period 2000-2009 [Cătană et al., 2012]. The percent of PTC in the category of differentiated tumors is not as spectacular as that recorded in the central area of the country where the PTC/FTC ratio increased in the two time intervals investigated from 7.8/1 to 29.5/1 [Cătană et al., 2012]. The data identified in Romanian study are summarized in Table 2.10.

Age stratification highlights that 50.94% were between 40 and 59 year old followed by 32.41% of people 60–80 years old. The many cases in the first category were children and young adults (6–25 yo) at the time of the Chernobyl fallout. Mean age at diagnosis in 2009, the year with most frequent cases, was 50 years old, meaning that at the time of exposure they were 30 years old.

Our result also highlights that about 312 (26.91%) of total patients were between 0 and 20 years old at the time of the Chernobyl disaster. It should be noted that category (0-20 yo) represented constantly1:4 of the PTCs diagnosed in Moldavia area, with a peak of 37.14% and 34.78% identified in 2018 and 2019 (Table 2.4.). Radiation exposure in

childhood involves a great risk in developing thyroid carcinoma and studies have shown that children and young adults were most affected after this nuclear disaster [WHO, 2016].

Table 2.10. Characteristics of thyroid cancers in Romania by number, gender distribution and mean age

Study	Area	Time period	No TC	No PTC DTC	Women/Men ratio	Mean Age	Mean age woman	Mean age men
Mogoş et al., 1995	Moldavia	1970-1992	195	80 PTC	No	No	No	No
Szanto et al., 2009	Targu Mures	1984-2007	288	213 PTC	241/47 (5.12/1)	> 40	No	No
Buzduga et al., 2011	Moldavia	2001-2008	No	401 PTC	No	No	No	No
Cătană et al., 2012	Targu Mures	1990-2009	524	409 PTC	456/68 (6.7/1)	No	48.91	49.75
Piciu et al., 2013	Cluj	1970-2012	4779	4167 DTC	8/1	46.7	No	No
Stanciu et al., 2015	Sibiu	2011-2013	61	48 PTC	46/15 (3/1)	> 60	No	No
Teodoroiu et al., 2021	**Moldavia**	**2009-2019**	**1068**	**674 PTC**	**6.7/1**	**50**	**51.7**	**53.9**

The comparative study with the translated threshold age values from 45 to 55 yo brings new information in the distribution of TC. Increased incidence of thyroid carcinomas in the 40-59 age group supports the change in the threshold value for reporting carcinomas to 55 yo age (8^{th} AJCC edition) and reveals statistically significant values of PTC vs anaplastic carcinoma, and significant data for identification TC on female gender and lymph node metastases. According to 8th AJCC edition there is increasing evidence that a cutoff age of 45 years has led to overstaging in many patients with DTC, and using 55 years as a cutoff value in the TNM staging system may prevent overstaging only in low-risk patients [Greene et al., 2017]. A significant number of patients between the ages of 45 and 55 years can be reclassified to stage I or II by applying 8^{th} AJCC edition [Greene et al., 2017].

Patients exposed to fallout radioisotopes were monitored over time, and comparative studies between radiogenic TC and sporadic ones arise. Many studies came from Ukraine, the group coordinated by Bogdanova monitored patients that were under 4 years old at the time of the disaster, living in the exposed area, and found that TC were more aggressive than sporadic ones with a trabecular solid pattern. The comparison was made with patients born after the Chernobyl disaster. Fusion oncogene drivers may confer higher tumor aggressiveness than point mutation in young patients, and that may be the response to radiogenic aggressiveness [Bogdanova et al, 2021].

Further studies showed that radioactive iodine exposure in utero may affect children in a manner of dose dependent. Patients exposed in utero were monitored and some of them developed nodular goiter with significant results of nodule dimensions increased by over 1 cm. Some of them had TC, but these results were not statistically significant. These publications highlight that we should include as a risk factor I-131 in utero exposure for nodular goiter [Hatch et al, 2020].

Thirty years after Chernobyl, nearly 11,000 TC cases have been reported among those who were children or adolescents at the time of the disaster in Belarus, Ukraine and the most contaminated regions of Russia [WHO, 2016].

There has unquestionably been a substantial dose-dependent excess risk of Iodine-131 related radiogenic TC, many with a distinctive histopathology RET/PTC3. RET fusions are reported to be the most common observed alteration in children TC, and appeared to occur in

approximately 25–30% of sporadic pediatric PTC; these results further increase to nearly 45% in patients exposed to radiation [Paulson et al, 2019].

Results of TC incidence following exposure in utero have suggested that the risk is the same, or larger, than that following similar exposures in infancy. Investigation of clean-up workers has produced new data on TC risk in exposed adults, suggesting that the risk observed in exposed adults is not much lower than those observed in children [Hatch et al., 2017].

Also, a retrospective study was conducted in Romania that investigated the potential in utero risk factor for TC in the pediatric population. Children included in this study were born after 1986. The study found that TC had a peak at ages of 10–13 years old after the nuclear disaster [Ștefan et al., 2020].

Follow-up after Chernobyl exposure was done in the affected areas, and over a period of 23 years (1989–2012), age-standardized thyroid carcinoma incidence in females increased by 3.7 times in the high exposure region, and by 2.9 times in the low exposure region. Males also suffered an increase by 3.5 times in high areas, and 1.82 times in low areas. Substantial differences were observed in the age group 40–49 years old. These results are similar to our findings concluding that amongst patients who were children at the moment of the exposure a high dose radiation may trigger over time a TC [Bazyka et al., 2016].

In Romania, few studies covered the Chernobyl radiogenic TC subject, but an important follow-up was made in Targu Mures, an area that was affected by radiation. They found that between 1984 and 2007, in two patient groups, the pre-Chernobyl disaster group and the post-Chernobyl disaster group, PTC increased over time. After a period of 5–8 years from the radiogenic event, they marked first 10 children diagnosed with PTC, noting that before disaster there were no children diagnosed. The incidence of TC increased 3–5 times in 1993–1998 compared to data registered before exposure to ionizing radiation from Chernobyl. Our findings supplement these results showing that PTC is still increased over time, and most diagnosed patients were children and young adults at the time of the exposure [Szanto et al., 2009].

Caesium 137 was measured in our country by specialists using γ spectrometric measurements on soil samples collected from 153 locations. Results show that Caesium had an average of 8.3 ± 0.2 $kBqm^{-2}$, with higher values in the mountain areas (18.3 ± 0.6 $kBqm^{-2}$) compared to the hills and plains (2.6 ± 0.1 $kBqm^{-2}$). This conclusion covered only one region of Romania, and the North-Eastern region was not studied at the time [Begy et al., 2017].

Another result of Caesium 137 exposure over Europe's map show that Romania has an exposed background of 2–10 kBq m^{-2} and some peaks of 10–40 kBq m^{-2} in our region of interest: Center and North [Evangeliou et al., 2016; European Environment Agency, 2021].

A timeline study focused on the 2009–2019 period, 23-33 years after the Chernobyl disaster, comes in line with the half-life of the other radioisotopes such as Caesium-137 and Strontium-90, not specifically involved in TC, but may be involved in other carcinogenesis. This study is the beginning of our research in the field of synchronous and metachronous cancers linked to radiogenic events.

2.5. FINAL REMARKS

Our work brings details on patients' age and potential exposure to the Chernobyl fallout, filling many gaps in our country's cancer registry. The TC profile established in Moldavia area identifies PTC as the most common subtype of TC, present mostly on the female population, with an increase in age from 50 yo in 2009 to 57.4 yo in 2019, and a stable ratio over time among females and males. Application of the threshold 55 yo maintains the same statistical differences between histological subtypes, but also brings up the new relationship between age and lymph node involvement.

CHAPTER 3.
PAPILLARY THYROID MICROCARCINOMA – A PECULIAR ENTITY WITHIN THE PAPILLARY THYROID CARCINOMA

3.1. INTRODUCTION

The 2004 WHO classification of thyroid tumors defines papillary thyroid microcarcinoma (PTMC) as a tumor with a diameter of or less than 1 centimeter [Hedinger et al., 1989; LiVolsi et al., 2004; Rosai et al., 2017]. The introduction of the term of PTMC came to replace and make uniform a variety of labels and definitions mostly descriptive which confused and rendered the diagnosis more difficult, such as small papillary carcinoma with a diameter of less than 1.5 cm, occult papillary carcinoma and incidentaloma. The latter designated the incidentally discovered tumors in total thyroidectomies for other thyroid pathology [Gemsenjager et al., 1999] or in the event of an autopsy [Sampson et al., 1970]. PTMC accounts for up to 30% of the total cases diagnosed as papillary thyroid carcinoma (PTC) [Giordano et al., 2010; Liu et al., 2014].

To endorse non-aggressive surgical approach and save patients' psychological suffering of cancer diagnosis, a new term for indolent PTMC – namely noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) was introduced [Hung et al., 2018]. However, the term NIFTP in histological diagnosis is strictly inflexible [Amendoeira et al., 2018]. For example, the tumor has to present encapsulation, purely follicular architecture, presence of nuclear features of PTC, and absence of capsular and vascular invasions [Hung et al., 2018; Amendoeira et al., 2018].

Several studies published in the last years underline the characteristics of PTMC and its relationship with PTC. Within this context, two reports that offer a detailed image of these two entities in South Korea are relevant. The analysis of the incidence rate of TC in South Korea between 1999–2008 shows that the proportion of PTC increased continuously, from 87.4% in 1999 to 97.4% in 2008, whereas the average tumor diameter was decreasing, from 18 mm in 1999 to 8 mm in 2008 [Park et al., 2016]. In parallel, the assessment of clinicopathological features and prognostic changes in TC, during a period of 40 years, found that the proportion of PTMC increased from 6.1% in 1962 to 9% in 1990, 54% in 2005 and 43.1% in 2009, respectively [Cho et al., 2013].

It is worth to mention that the prevalence of incidental PTMC in both malignant and benign thyroid diseases is reported to be between 7.1%–16.3% [Vasileiadis et al., 2013; Slijepcevic et al., 2015]. It may be incidentally found in up to 22% of cases operated for exclusively benign diseases of the thyroid and also in 0.5 to 5.2% in autopsy studies of patients with non-thyroidal diseases [Carlini et al., 2006; Ito et al., 2007]. Consequently, the true prevalence of incidental PTMC is not really known, as the literature up to date gives variable data related to geographic region, base disease (benign or malignant) and type of study (on live patient or postmortem diagnosis). The continuously increasing prevalence of PTMC in living people, despite an unchanged mortality rate, appears to be related to the development of diagnosis methods and screening programs for thyroid diseases [Chung et al., 2009].

The TNM staging system has undergone alterations, i.e. if thyroid tumors of less than 2 cm were initially classified as T1 stage, as of 2006 the T1a category was added, and it is the

counterpart of PTMC [LiVolsi et al., 2004; Compton et al., 2006]. The lymph nodes in the head and neck area were divided into seven levels according to the standard classification proposed by the American Head and Neck Society (AHNS) and the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) [Haugen et al., 2016; Greene at el., 2017]. The central lymph node compartment, corresponding to level VI, is the most frequent location for lymph node metastasis in PTC patients [Liu et al., 2014].

Most of PTMC have a favorable evolution [LiVolsi et al., 2004; Rosai et al., 2017; Ito et al., 2018], rarely reporting metastasis, and less than 1% mortality rate [Chow et al., 2003; Hay et al., 2008; Noguchi et al., 2008; Roti et al., 2008]. Despite the indolent behavior generally encountered in PTMC, a small number of cases have an aggressive clinical trajectory, similar to PTC [Pelizzo et al., 2004; Noguchi et al., 2008; Lee et al., 2014]. These cases are characterized by secondary involvement of lymph nodes [Chow et al., 2003; Besic et al., 2008; Mercante et al, 2009; Lombardi et al., 2010], a high rate of recurrence (up to 20%), distant metastasis and mortality [Chow et al., 2003; Ito et al., 2004; Pelizzo et al., 2004; Noguchi S et al., 2008; Kim et al., 2014].

The management of PTMC varies from monitoring without surgical therapy to total thyroidectomy, with or without radioactive iodine treatment. Given the morbidity associated to thyroidectomy that cannot be decreased under 1-3%, even in specialized centers [Leboulleux et al., 2016], extensive surgical treatment for all cases of diagnosed PTMC is not justified; nowadays, even if still under debate and only used for selected cases, active surveillance gains an important place in the management of PTMC [Lim et al., 2009; Leboulleux et al., 2016; Vaccarella et al., 2016; Ito et al., 2018].

The guidelines of the American Thyroid Association (ATA) advise a risk-directed approach in the management of TC lobectomy, or an active surveillance protocol based on repetitive imaging studies and thyroglobulin measurements, is now suggested for PTMC without known preoperative risk factors [Haugen et al., 2016]. The routine dissection of central lymph nodes is yet another controversial theme, especially as the long-term lack of benefit has been demonstrated, and this has been supported by possible post-surgery complications such as transitory hypocalcaemia [Ito et al., 2004; Ito et al., 2014; Leboulleux et al., 2016].

Contrary to the clear definition of the prognostic factors for PTC [LiVolsi et al., 2004] which includes age (with a threshold at 55 years old (yo) according to the last UICC TNM classification [Brierley et al., 2017], tumor size, capsule invasion, histologic variants (diffuse sclerosing, tall or columnar cell, micropapillary/hobnail) and differentiation degree, local or distant metastasis, and surgical resection of the lesion, the prognostic factors for PTMC are still contentious. The PTMC characteristics which indicate a high risk of metastasis are little known [Bradley et al., 2017], some studies considering that metastasis of central lymph nodes is directly involved in tumor relapse, other data sustaining the prognostic role of histological variants with aggressive behavior (oncocytic, sclerosing or tall cell) or of associated lesional background [Roti et al., 2008; Bircan et al., 2014].

Aim

Within this context, we analyzed the clinicopathological profile of two consecutive groups of PTMC, aiming to identify the cases with increased oncological risk.

Our analysis focused on the possible relationships between a set of clinicopathological characteristics and four parameters expressing tumor extension and aggressiveness (namely lympho-vascular invasion, thyroid capsule invasion, extrathyroidal extension and lymph node metastasis). Thus, supplementary data may be considered as supporting the refinement and accuracy of PTMC prognostic factors, by a broader perspective on tumor behavior.

3.2. MATERIAL AND METHODS

The retrospective study comprised two consecutive group of PTMC investigated between 2010-2016 and 2016-2018, respectively, at the "Sf. Spiridon" Clinical Emergency County Hospital, Iasi. The study was approved by the Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy and "Sf. Spiridon" Clinical Emergency County Hospital Iasi, based on the patients' informed consent on the usage of their biologic material leftover after diagnostic testing, in accordance with the ethical standards of Helsinki declaration.

Patients

The first group (G1) comprised 428 patients diagnosed with PTMC during 2010-2016 (168 patients with thyroidectomy and cervical lymph node dissection and 260 cases PTMC incidentally found after surgery for non-tumor thyroid pathology). The second group (G2) included 612 patients operated for benign thyroid diseases in the course of 2016-2018, in which 144 cases of PTMC were identified.

Medical files and histopathological reports were retrospectively reviewed in order to document the main clinicopathological characteristics in each patient. No familial cases were registered. Our database included information regarding gender, age (< 55 and respectively ≥ 55 yo), tumor size, histological variant (papillary, follicular, oncocytic), location (subcapsular, intraparenchymal), unilateral or bilateral involvement, number of foci (two or more foci for multifocality, taking into account the diameter of largest foci for tumor size), lympho-vascular invasion, thyroid capsule invasion (defined as microscopic presence of tumor cells into the thyroid capsule), extrathyroidal extension (defined as microscopic presence of tumor cells into perithyroidal soft tissues: adipose tissue, skeletal muscle, sizable vessels and nerves), and lymph node metastasis (including the number of positive lymph nodes). On the histological evaluation we also noted the associated thyroid pathology.

In G1 group (428 patients), the total thyroidectomy was performed due to bilateral nodular benign thyroid lesions with cervical compression signs. The collected data revealed 3 recurrences and 7 deaths, not related to thyroid malignancy. Central compartment lymphadenectomies were performed in 168 patients, and lymph node metastases were identified in 23 cases. The three patients who presented recurrence in the lymph node compartments (corresponding to level VI and VII) were female, aged over 55 yo, with the following characteristics of the primary tumor: tumor size of 5, 7 and 9 mm, subcapsular location, conventional PTMC (2 cases) and oncocytic PTMC (1 case), lympho-vascular invasion and extrathyroidal extension; all have been treated by thyroidectomy without cervical lymph node dissection.

In G2 group (612 patients), the total thyroidectomy was performed in 125 cases (86.8%), because of either the surgical indication or the expressed preference of the patient (in cases where only lobectomy was indicated). Thyroid lobectomy was performed in 14 cases (9.7%) diagnosed with a single nodule with benign clinical, ultrasound and FNAB features (fine-needle aspiration biopsy). A later completion thyroidectomy was done in 5 cases (3.4%), due to unfavorable pathological report (extrathyroidal invasion, vascular invasion, lymphatic emboli, and perineural invasion). Central compartment lymphadenectomies were also performed in these cases, concomitant with the completion of a thyroidectomy. In other 5 cases, lymphadenectomy was performed, following an interval of 11 to 23 months after thyroidectomy, for lymphatic recurrence (3 cases in the central compartment and 2 cases in the both central and homolateral compartments). Overall, a total of 8 cases (5.6%) were pathologically proven to have lymph node metastases. No distant metastases were found in G2 group of patients.

Immunohistochemical exam

Immunohistochemical techniques were performed to confirm the diagnosis of PTMC, using a panel of antibodies: anti-CK19 (Cytokeratin 19), anti-HBME1 (Hector Battifora and Mesothelioma 1), anti-galectin 3 and anti-CD56 (CD56-cluster of differentiation 56) antibodies. Table 3.1 summarizes the main information on these immunohistochemical markers.

For each case, a paraffin-embedded tissue fragment was chosen. The 3-μm thick sections obtained from the blocks were placed on silanized slides, dewaxed in xylene, rehydrated in consecutive descending concentrations of ethanol (100%, 90%, 80%, and 70%), and rinsed in distilled water. For antigen retrieval, we used Heat Induced Epitope Retrieval (HIER) technique: slides were placed in citrate buffer pH 6 and heated in a water bath, at 98^0C, for 30 minutes. The slides were immersed in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase, and incubated with the primary antibodies for anti-Cytokeratin 19 (CK19) (clone A53-B/A2.26, Thermo Scientific, 1:100 dilution), anti-galectin 3 (clone 9C4, Cell Marque, 1:50 dilution), anti-HBME1 Mesotelial Cell (clone HBME1, Dako, 1:100 dilution), and anti-CD56 (123C3, Dako, 1:75) overnight, at 4^0C.

The immunoreaction was amplified with the suitable secondary and tertiary antibodies of the UltraVision Quanto Detection System HRP DAB (Thermo Scientific, USA) and developed with 3.3'-diamino-benzidine (DAB) tetrahydrochloride chromogen (Thermo Scientific, USA). The counterstaining of the sections was done with Lillie's modified Hematoxylin. Positive and negative controls have been simultaneously run in order to verify the accuracy of the technique.

The evaluation of antibodies was based on the specific membrane or cytoplasmic label for each antibody. The typical model for the positive diagnosis of PTMC was CK19, galectin 3 and HBME1 positive and CD56 negative (with positive internal control thyroid tissue).

Table 3.1. The antibodies used for immunohistochemical PTMC confirmation

Antibody	Manufacturer	Clone	Antigen retrieval	Class	Dilution	Cellular localization
Anti-Cytokeratin 19 (CK19)	Thermo Scientific, USA	A53-B/A2.26	Citrate, pH 6	Mouse monoclonal	1:100	Cytoplasm
Anti-galectin 3	Cell Marque, USA	9C4	Citrate, pH 6	Mouse monoclonal	1:50	Cytoplasm
Anti-HBME1	Dako, Denmark	HBME1	Citrate, pH 8	Mouse monoclonal	1:100	Membrane
Anti-CD56	Dako, Denmark	123C3	Citrate, pH 9	Mouse monoclonal	1:75	Membrane

Statistical analysis

Data were analyzed using the SPSS V.22-SPSS Inc. (IBM Corporation, Chicago, IL, USA). The results of the univariate analysis were reported as mean ± standard deviation (SD) for continuous variables. Total count and percent were reported for categorical variables. Chi-square test (Maximum-Likelihood, Yates, Mantel-Haenszel) was performed for categorical variables and Kruskal-Wallis test for continuous variables. Correlations between predictor and outcome variables were determined using univariate analysis (Spearman Rank test, Gamma) and multiple logistic regression. The significance level (*p*-value) was considered to be 0.05 (5%); a confidence interval of 95% shows that the decision is correct.

3.3. RESULTS

3.3.1. Clinicopathological profile and prognostic factors in PTMC - G1 group (2010-2016)

General clinicopathological characteristics

Among the 428 patients in the study, 364 (85.04%) were female and 64 (14.96%) male, with a mean age of 54.64 ± 11.12 years. 245 patients (57.25%) were over 55 yo at the time of the diagnosis, whereas 183 were under 55 yo (42.75%). The mean diameter of the tumor was 3.76 mm ± 2.50 mm with a median value of 3 mm and a range between 0.1 and 10 mm.

The histopathological exam identified three histologic variants of PTMC, namely conventional (155 cases - 36.22%) (Figures 3.1 - 3.2), follicular (244 cases - 57%) (Figure 3.3), and oncocytic (29 cases - 6.78%) (Figure 3.4).

Other histological variants were excluded, because specific features that could define such variants appeared in less than 10% of tumor areas (meaning tall cells in 8 cases, clear cells in 4 cases).

Accompanying thyroid pathology included nodular goiter - 225 cases (52.57%), colloid goiter - 111 cases (25.93%), Hashimoto thyroiditis - 68 cases (15.89%), Basedow disease - 17 cases (3.97%), and thyroid adenoma - 7 cases (1.63%).

Most of the tumors had intraparenchymal location (257 cases - 60.04%) as opposed to subcapsular (171 cases - 39.96%).

Multifocality was found in 130 cases (30.37%) of PTMC, almost half of them (64 cases - 49.23%) with bilateral involvement of the thyroid. The number of foci varied between 2 and 5, and were distributed as follows: 85 patients (65.39%) had 2 tumor foci, 34 cases (26.15%) - 3 foci, 9 (6.92%) - 4 foci and 2 patients (1.54%) - 5 foci.

Histopathological exam also showed lympho-vascular invasion in 18 cases (4.20%), perineural invasion in 7 cases (1.63%), thyroid capsule invasion in 93 cases (21.73%), and extrathyroidal extension in 74 cases (17.29%) (Figures 3.5 – 3.7).

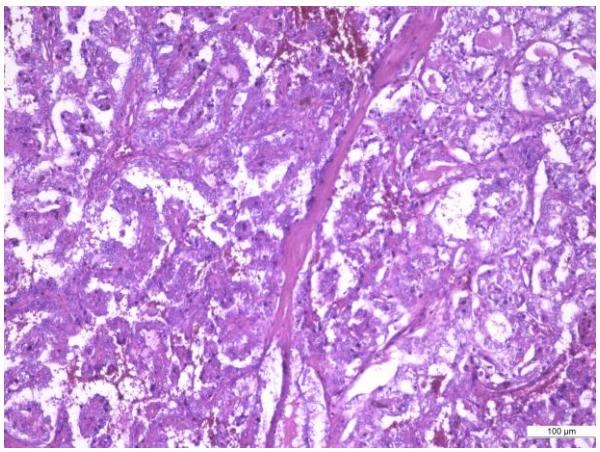


Figure 3.1. Conventional, papillary variant of PTMC, with "Orphan Annie" nuclei (HE, x 40)

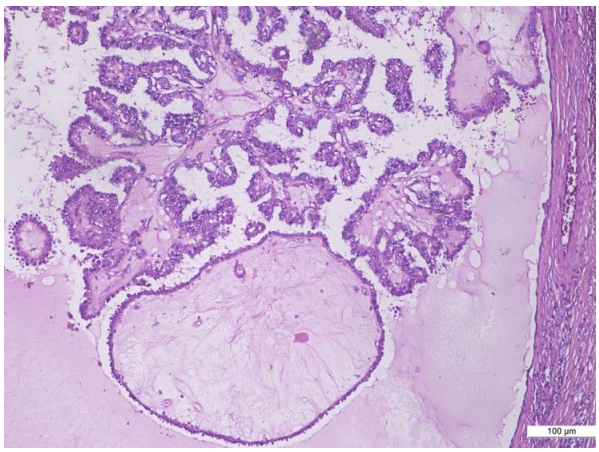


Figure 3.2. Conventional, papillary variant of PTMC, with edematous stromal cores (HE, x 100)

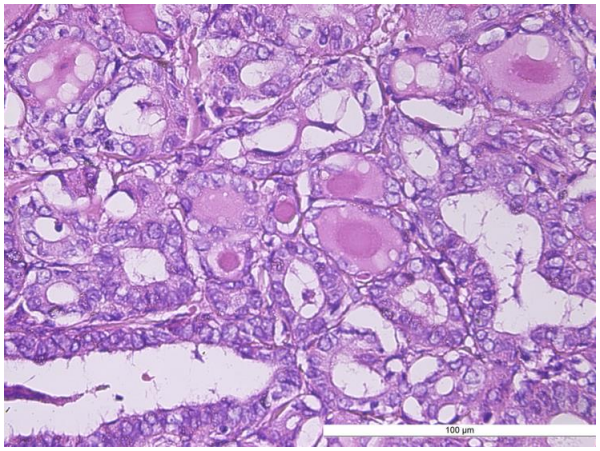


Figure 3.3. Follicular variant of PTMC, nuclei with ground glass appearance, grooves and irregular contour (HE, x 400)

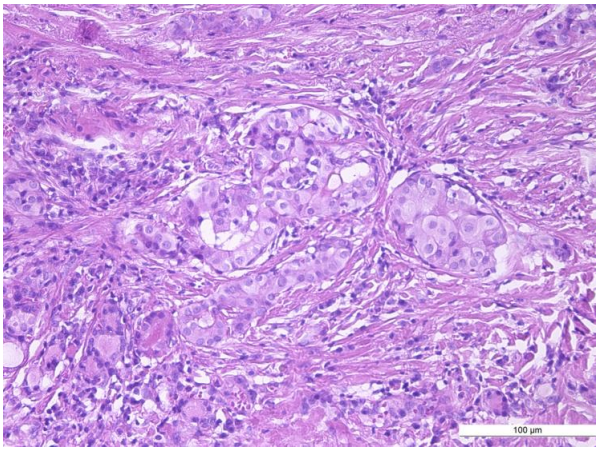


Figure 3.4. Oncocytic variant of PTMC - oxyphilic cells with abundant eosinophilic granular cytoplasm and typical papillary carcinoma nuclei (HE, x 40)

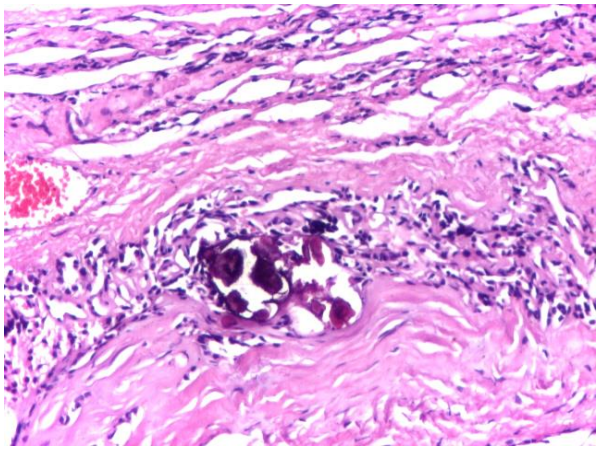


Figure 3.5. Capsular invasion associated with psamoma bodies (HE, x 200)

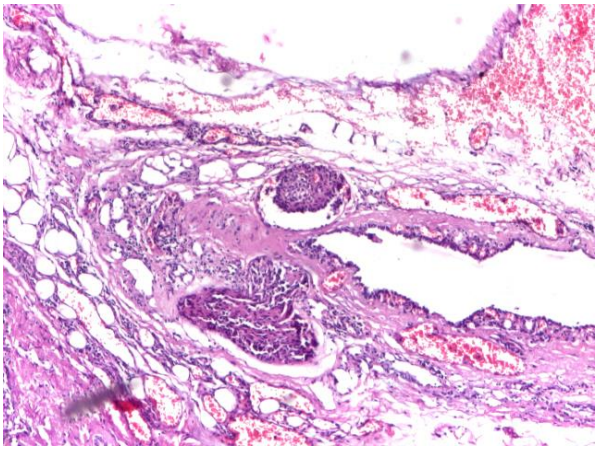


Figure 3.6. Lympho-vascular invasion (HE, x 40)

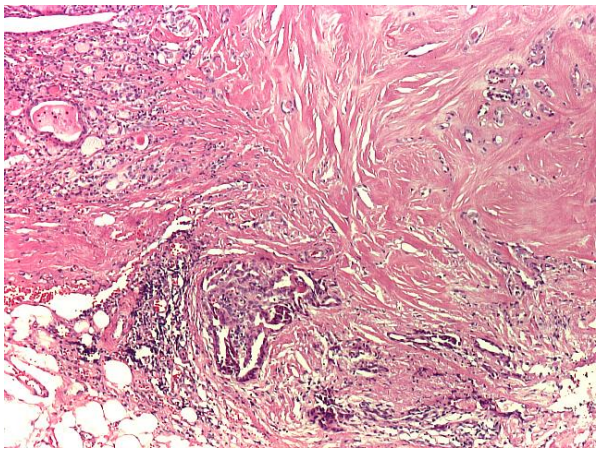


Figure 3.7. Extrathyroid extension (HE, x 40)

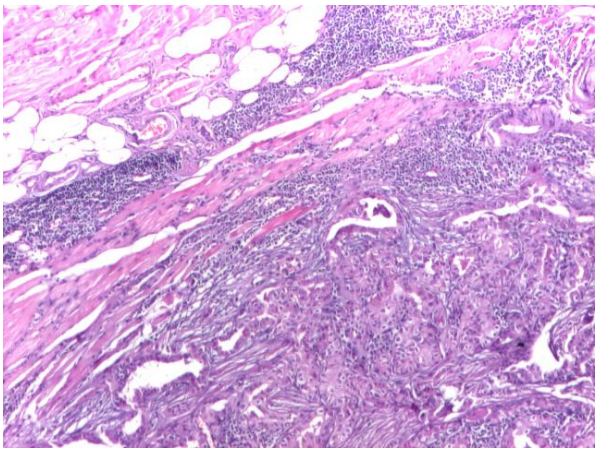


Figure 3.8. Lymph node metastases (HE, x 40)

PTMC confirmed by immunohistochemical reactions showed an intense positive staining on cytoplasm (CK19 and galectin 3) or membrane (HBME1) and a negative membrane staining for CD56 (with positive control in non-tumor thyroid tissue) (Figures 3.9 – 3.12).

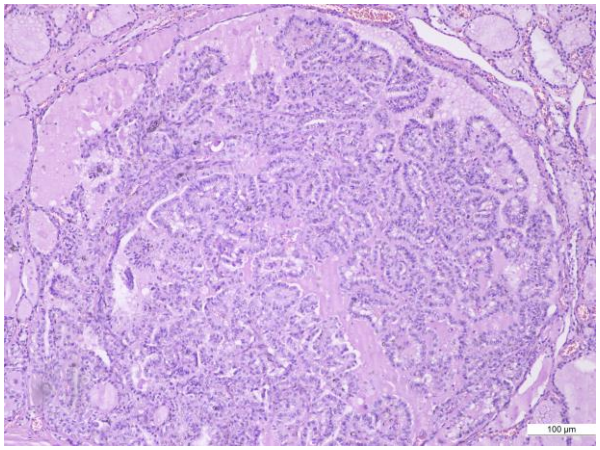


Figure 3.9. Conventional PTMC with papillae covered by cells with eosinophilic cytoplasm (HE, x 100)

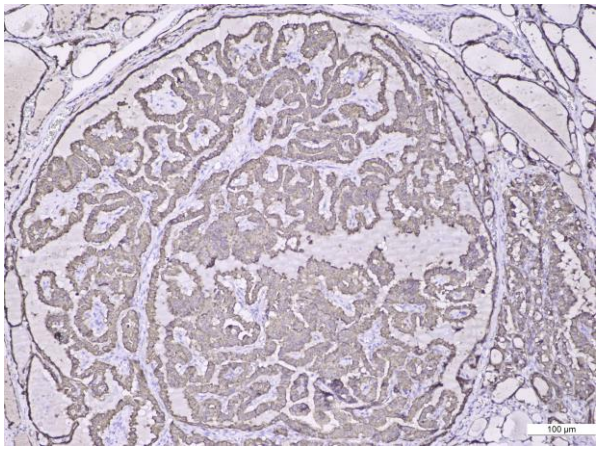


Figure 3.10. Strong cytoplasmic expression in PTMC (IHC, anti-CK19, x 100)

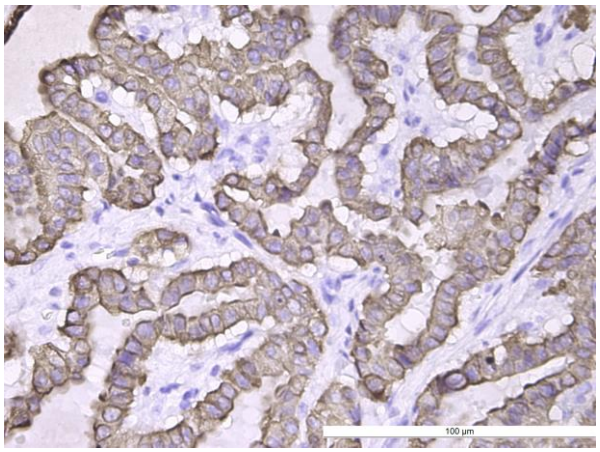


Figure 3.11. Moderate cytoplasmic expression for galectin 3 in PTMC (IHC staining anti-galectin3, x 400)

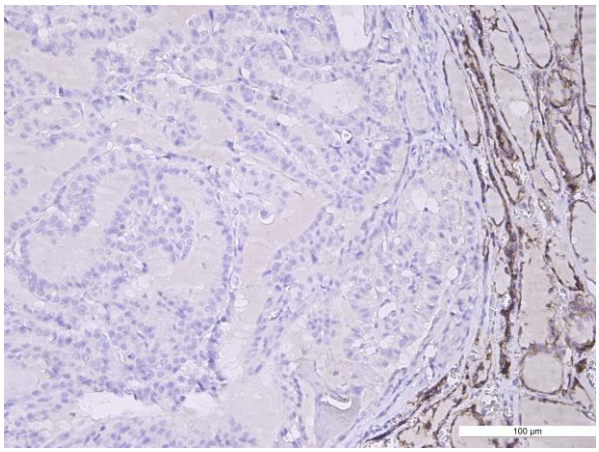


Figure 3.12. Negative expression for CD56 in PTMC, with positive control in non-tumoral thyroid tissue (IHC staining anti-CD56, x 200)

Lymph node dissection performed in 168 patients showed lymph node metastases in 23 cases - 13.69% (18 cases N1a and 5 cases N1b) (Figure 3.8). The number of positive lymph nodes varied between one and ten, as follows: 14 cases with one node, 2 cases with three nodes, 2 cases with four nodes, whereas the remaining 5 cases presented two, six, seven, eight, and ten nodes, respectively. For these 23 cases the location of the primary tumor was variable: multifocal and bilateral (10 cases), or limited to a thyroid lobe in upper third (5 cases), middle third (3 cases) and lower third (5 cases).

Relationships between clinicopathological prognostic factors – univariate analysis

The statistical analysis addressed the possible relationship between several clinicopathological characteristics and four parameters nominated to indicate tumor extension and aggressiveness, namely: lympho-vascular invasion, thyroid capsule invasion, extrathyroidal extension and lymph node metastasis.

Age

Our results indicate no statistically significant association between the patients' age (related to the threshold of 55 yo) and lympho-vascular invasion, thyroid capsule invasion, extrathyroidal extension or lymph node metastasis (including the number of the positive lymph nodes).

Tumor size

The tumor size was significantly larger in the patients with lympho-vascular invasion (p=0.009) (Table 3.1), thyroid capsule invasion (p<<0.001) (Table 3.2), extrathyroidal extension (p<<0.001) (Table 3.3), presence of lymph node metastasis (p=0.00006) and number of metastatic lymph nodes (p=0.020) (Table 3.4).

Location particularities

The subcapsular location was significantly associated with lympho-vascular invasion (p=0.0019, OR=5.64) (Table 3.1), thyroid capsule invasion (p<<0.001, OR=12.3) (Table 3.2), extrathyroidal extension (p<<0.001, OR=9.38) (Table 3.3) and lymph node metastasis (p=0.0065, OR=3.53) (Table 3.4).

Histologic type

Statistical analysis performed for each histological variant in comparison with the others showed a significant association between the conventional type and presence of lympho-vascular invasion (p=0.0027, OR=4.91) (Table 3.1). All three histological subtypes significantly associated with thyroid capsule invasion and extrathyroidal extension, the highest risk being registered for the oncocytic variant (p=0.0079, OR=2.77, and p=0.0023, OR=3.26, respectively) (Table 3.3). Concurrently, the sub-unitary values of OR indicated that the follicular variant displayed the lowest risk for lympho-vascular (OR=0.2) (Table 3.1) and thyroid capsule invasion (OR=0.46) (Table 3.2), as well as extrathyroid extension (OR=0.34) (Table 3.3). We demonstrated a significant association (p<<0.001, OR=4.39) with lymph node metastasis only for oncocytic variant (Table 3.4).

Focality and bilaterality

Multifocality and bilaterality were significantly associated with thyroid capsule invasion (p=0.0014, OR=2.18, and p=0.0032, OR=3.17, respectively) (Table 3.2) and extrathyroidal extension (p=0.0018, OR=2.28, and p=0.012, OR=2.80, respectively) (Table 3.3). In parallel, we noticed that thyroid capsule invasion and extrathyroidal extension are directly influenced by the number of tumor foci. Thus, for the capsule invasion, the presence of 3 foci causes an OR of 2.58, which grows exponentially for a number of more than 4 foci to 8.28 (Table 3.2). Similarly, for extrathyroidal extension the presence of 3 foci causes an OR of 2.69, which grows significantly for a number of more than 4 foci to 7.74 (Table 3.3). We found no significant association between multifocality (regardless of the number of foci) and lympho-vascular invasion or lymph node metastasis; similarly for bilaterality. However, statistical analysis showed a significant correlation between unifocality and the number of metastatic lymph nodes (p=0.0264).

Associated endocrine pathological background

By considering all accompanying thyroid lesions, colloid goiter and Hashimoto thyroiditis were significantly associated with extrathyroidal extension (p=0.0393, and p=0.0384, respectively) (Table 2.3).

The association between the clinicopathological characteristics and the four parameters expressing tumor extension and aggressiveness are summarized in Tables 3.1-3.4.

Table 3.1. Clinicopathological characteristics of G1 patients according to lympho-vascular invasion

Clinicopathological characteristics	**Lympho-vascular invasion**		**Univariate analysis**	**OR (95% CI)**
	absent (n=410)	present (n=18)		
Age at diagnosis	54.7±11.03	53.6±13.41	p=0.845	
<55 years old	174 (95.1%)	9 (4.9%)	p=0.695	1.36 (0.48-3.81)
≥55 years old	236 (96.3%)	9 (3.7%)		
Tumor size (mm)	3.68±2.46	5.85±2.60	**p=0.009***	

Clinicopathological characteristics	Lympho-vascular invasion		Univariate analysis	OR (95% CI)
	absent (n=410)	present (n=18)		
Location				
subcapsular	157 (91.8%)	14 (8.19%)	**p=0.0019***	5.64 (1.7-20.68)
intraparenchymal	253 (98.4%)	4 (1.6%)		
Histopathologic type				
conventional	142 (91.6%)	13 (8.4%)	**p=0.0027***	4.91 (1.59-16.12)
follicular	240 (98.4%)	4 (1.6%)	**p=0.00506***	0.20 (0.06-0.67)
oncocytic	28 (96.5%)	1 (3.5%)	p=0.7881	0.80 (0.12-6.94)
Focality of the tumor				
unifocal	286 (96%)	12 (4%)	p=0.782	0.87 (0.29-2.66)
multifocal	124 (95.4%)	6 (4.6%)		
Multifocality – number of tumor foci			p=0.652	
2	82 (96.5%)	3 (3.5%)	p=0.913	0.87 (0.19-3.42)
3	31 (91.2%)	3 (8.8%)	p=0.4008	2.31 (0.49-9.48)
≥4	11 (100%)	0 (0%)	p=0.996	2.17 (0.39-12.64)
Unilateral or bilateral involvement				
unilateral	64 (96%)	2 (3%)	p=0.647	2.13 (0.32-17.49)
bilateral	60 (93.7%)	4 (6.3%)		
Coexisting thyroid pathology				
thyroid adenoma	7 (100%)	0 (0%)	p=0.7711	3.36 (0.14-23.57)
Graves' disease	17 (100%)	0 (0%)	p=0.7706	1.35 (0.06-8.25)
colloid goiter	104 (93.7%)	7 (6.3%)	p=0.2006	1.86 (0.66-4.97)
nodular goiter	217 (96.4%)	8 (3.6%)	p=0.4810	0.71 (0.26-1.87)
Hashimoto's thyroiditis	65 (95.6%)	3 (4.4%)	p=0.9265	1.06 (0.23-3.50)

*OR: odd ratio; CI: confidence interval; *p-value <0.05 was considered to be statistically significant.*

Table 3.2. Clinicopathological characteristics of G1 patients according to thyroid capsule invasion

Clinicopathological characteristics	Thyroid capsule invasion		Univariate analysis	OR (95% CI)
	absent (n=335)	present (n=93)		
Age at diagnosis	54.6±11.13	54.8±11.17	p=0.559	
<55 years old	147 (80.3%)	36 (19.7%)	p=0.372	0.81 (0.49-1.33)
≥55 years old	188 (76.7%)	57 (23.3%)		
Tumor size (mm)	3.16±2.23	5.96±2.17	**p<<0.001***	
Location				
subcapsular	94 (55%)	77 (45%)	**p<<0.001***	12.34 (6.62-23.27)
intraparenchymal	241 (93.8%)	16 (6.2%)		
Histopathologic type				
conventional	113 (72.9%)	42 (27.1%)	**p=0.0427***	1.62 (1.09-2.65)
follicular	205 (84%)	39 (16%)	**p=0.00091***	0.46 (0.28-0.75)
oncocytic	17 (58.6%)	12 (41.4%)	**p=0.0079***	2.77 (1.19-6.42)
Focality of the tumor				
unifocal	246 (82.6%)	52 (17.4%)	**p=0.00149***	2.18 (1.32-3.60)
multifocal	89 (68.5%)	41 (31.5%)		
Multifocality-number of tumor foci				
2	63 (74.1%)	22 (25.9%)	P=0.0827	1.65 (0.90-3.03))
3	22 (64.7%)	12 (35.3%)	**p=0.0125***	2.58 (1.12-5.88)
≥4	4 (36.4%)	7 (63.6%)	**p=0.00058***	8.28 (2.08-15.13)
Unilateral or bilateral				

Clinicopathological characteristics	Thyroid capsule invasion		Univariate analysis	OR (95% CI)
	absent (n=335)	present (n=93)		
involvement				
unilateral	53 (80.3%)	13 (19.7%)	**p=0.00328***	3.17 (1.36-7.49)
bilateral	36 (56.3%)	28 (43.7%)		
Coexisting thyroid pathology				
thyroid adenoma	6 (85.7%)	1 (14.3%)	p=0.984	0.59 (0.02-4.10)
Graves' disease	16 (94.1%)	1 (5.9%)	p=0.1879	0.21 (0.01-1.23)
colloid goiter	84 (75.7%)	27 (24.3%)	p=0.4415	1.22 (0.72-2.02)
nodular goiter	180 (80%)	45 (20%)	p=0.3617	0.80 (0.50-1.28)
Hashimoto's thyroiditis	49 (72.1%)	19 (27.9%)	p=1.1761	1.40 (0.81-2.68)

*OR: odd ratio; CI: confidence interval; *p-value <0.05 was considered to be statistically significant; for P<0.00000001 we have noted P<<0.001.*

Table 3.3. Clinicopathological characteristics of G1 patients according to extrathyroidal extension

Clinicopathological characteristics	Extrathyroidal extension		Univariate analysis	OR (95% CI)
	absent (n=354)	present (n=74)		
Age at diagnosis	54.7±11.12	54.3±11.2	p=0.994	
<55 years old	151 (82.5%)	32 (17.5%)	p=0.925	1.02 (0.60-1.75)
≥55 years old	203 (82.9%)	42 (17.1%)		
Tumor size (mm)	3.34±2.33	5.85±2.26	**p<<0.001***	
Location				
subcapsular	111 (64.9%)	60 (35.1%)	**p<<0.001***	9.38 (4.85-18.41)
intraparenchymal	243 (94.5%)	14 (5.5%)		
Histopathologic type				
conventional	118 (76.1%)	37 (23.9%)	**p=0.00673***	2.00 (1.17-3.42)
follicular	218 (89.3%)	26 (10.7%)	**p=0.00002***	0.34 (0.19-0.59)
oncocytic	18 (62.1%)	11 (37.9%)	**p=0.0023***	3.26 (1.36-7.7)
Focality of the tumor				
unifocal (foci)	258 (86.6%)	40 (13.4%)	**p=0.00184***	2.28 (1.32-3.94)
multifocal (foci)	96 (73.9%)	34 (26.1%)		
Multifocality-number of tumor foci				
2	67 (78.8%)	18 (21.2%)	p=0.7896	1.73 (0.89-3.35)
3	24 (70.6%)	10 (29.4%)	**p=0.0136***	2.69 (1.11-6.44)
≥4	5 (45.5%	6 (54.5%)	**p=0.00017***	7.74 (1.97-13.99
Unilateral or bilateral involvement				
unilateral	55 (83.3%)	11 (16.7%)	**p=0.0127***	2.80 (1.15-6.96)
bilateral	41 (64.1%)	23 (35.9%)		
Coexisting thyroid pathology				
thyroid adenoma	6 (85.7%)	1 (14.3%)	p=0.8323	0.79 (0.03-5.48)
Graves' disease	17 (100%)	0 (0%)	p=0.3045	0.27 (0.01-1.53)
colloid goiter	86 (77.5%)	25 (22.5%)	**p=0.0393***	6.62 (1.13-14.42)
nodular goiter	192 (85.3%)	33 (14.7%)	p=0.1312	0.67 (0.40-1.12)
Hashimoto's thyroiditis	53 (77.9%)	15 (22.1%)	**p=0.0384***	6.41 (1.04-14.01)

*OR: odd ratio; CI: confidence interval; *p-value <0.05 was considered to be statistically significant; for P<0.00000001 we have noted P<<0.001.*

Table 3.4. Clinicopathological characteristics G1 patients according to lymph node metastasis

Clinicopathological characteristics	Lymph node metastasis		Univariate analysis	OR (95%CI)
	absent (n=145)	present (n=23)		
Age at diagnosis	53.1±11.97	52.6±11.93	p=0.915	
<55 years old	67 (83.7%)	13 (16.3%)	p=0.357	1.51 (0.58-4.01)
≥55 years old	78 (88.6%)	10 (11.4%)		
Tumor size (mm)	3.51±2.39	5.85±2.41	**p=0.00006***	
Location				
subcapsular	57 (78.1%)	16 (21.9%)	**p=0.0065***	3.53 (1.26-10.17)
intraparenchymal	88 (92.6%)	7 (7.4%)		
Histopathologic type				
Conventional	56 (82.4%)	12 (17.6)%	p=0.219	1.73 (0.66-4.57)
Follicular	82 (91.1%)	8 (8.9%)	p=0.052	0.41 (0.15-1.11)
Oncocytic	7 (70%)	3 (30%)	**p<<0.001***	4.39 (1.73-7.11)
Focality of the tumor				
unifocal (foci)	99 (88.4%)	13 (11.6%)	p=0.274	1.66 (0.62-4.41)
multifocal (foci)	46 (82.1%)	10 (17.9%)		
Multifocality-number of tumor foci				
2	25 (83.3%)	5 (16.7%)	p=0.601	1.33 (0.39-4.31)
3	13 (76.5%)	4 (23.5%)	p=0.214	2.14 (0.52-8.11)
≥4	8 (88.9%)	1 (11.1%)	p=0.789	0.8 (0.12-5.31)
Unilateral or bilateral involvement				
Unilateral	21 (84%)	4 (16%)	p=0.980	1.26 (0.26-6.27)
Bilateral	25 (80.7%)	6 (19.3%)		
Coexisting thyroid pathology				
thyroid adenoma	2 (100%)	0 (0%)	p=0.9017	3.11 (0.27-35.68)
Graves' disease	6 (75%)	2 (25%)	p=0.6696	2.20 (0.41-11.66)
colloid goiter	29 (82.9%)	6 (17.1%)	p=0.6954	1.41 (0.51-3.89)
nodular goiter	70 (89.7%)	8 (10.3%)	p=0.3268	0.57 (0.22-1.43)
Hashimoto's thyroiditis	38 (84.4%)	7 (15.6%)	p=0.8634	1.23 (0.47-3.22)

*OR: odd ratio; CI: confidence interval; *p-value <0.05 was considered to be statistically significant; for P<0.00000001 we have noted P<<0.001.*

Relationships between clinicopathological prognostic factors – multivariate analysis

The multivariate analysis was based on the prognostic clinicopathological factors which showed a significant association within the univariate analysis.

For lympho-vascular invasion, only tumor size (p=0.016, OR=1.28) and subcapsular location (p=0.041, OR=3.44) had predictive value.

For thyroid capsule invasion, 3 out of 6 factors presented predictive value, namely: tumor size (p=0.015, OR=1.28), subcapsular location (p=0.036, OR=3.54), and multifocality (p=0.024, OR=2.63).

For extrathyroidal extension, the negative predictive factors included: tumor size (p=0.018, OR=1.28), subcapsular location (p=0.037, OR=3.20), oncocytic histological variant (p=0.041, OR=1.95), and multifocality (p=0.021, OR=1.97). The presence of follicular variant (p=0.037, OR=0.49) significantly decreased the risk for extrathyroidal extension.

For lymph node metastasis, out of the four factors which showed significant associations in the univariate analysis only one maintained a predictive potential. We found that tumor size (p=0.003, OR=1.37) is a negative prognostic factor, which increases the risk of metastasis.

3.3.2. Clinicopathological profile and prognostic factors in PTMC – G2 group (2016-2018)

General clinicopathological characteristics

Among the 144 patients diagnosed with PTMC, 117 (81.2%) were female and 27 (18.8%) were male. With an overall mean age of 54.77 +/- 11.9 years, 79 patients (54.9%) were older than 55 years (mean age 63.65 years) and 65 (45.1%) were less than 55 years old, ranging from 25 to 54 years with a mean age of 43.67 years.

The overall mean diameter was 3.04 ± 2.2 mm and 109 patients (75.7%) had small tumors (less than 5 mm in their largest diameter). Fifty-one patients (35.4%) were diagnosed with multifocal tumors.

Regarding the pathological type, 110 tumors (76.4%) were the follicular variant, 19 (13.2%) were conventional, papillary and 15 patients (10.4%) were diagnosed with other variants of PTMC, as follows: 10 cases of tall cell variant (6.94%), 3 cases of hobnail PTMC (2.08%), and 2 cases of columnar PTMC (1.38%).

The underlying diseases for which the patients were referred to surgical treatment were: multinodular goiter in 106 (73.6%) cases, adenoma in 36 (25%) cases, 25 cases of Hashimoto thyroiditis (17.4%), Basedow disease and other types of hyperthyroidism in 7 cases (4.9%) and primary hyperparathyroidism (HPT) in 11 cases (7.6%), with a small percent presenting a combination thereof.

When assessing the histopathologic factors of aggressiveness, the final reports showed that 14.6% of PTMC had extracapsular invasion (21 cases), 5.6% (8 cases) presented perineural invasion and only 1 case (0.7%) had vascular invasion. Lymphatic emboli were found in 13 patients (9%) and lymph nodes metastasis in 8 patients (5.6%).

The main characteristics of the patients with PTMC in G2 group are presented by comparison to G1 group in Table 3.5.

Table 3.5. Clinical and pathological characteristics of groups (G1 and G2 patients)

Clinicopathological characteristics		**G1** **n, %**	**G2** **n, %**
Age	< 55 years	183 (42.75%)	65 (45.1%)
	≥ 55 years	245 (57.25%)	79 (54.9%)
Gender	Male	64 (14.96%)	27 (18.8%)
	Female	364 (85.04%)	117 (81.2%)
Mean diameter		3.76 mm ± 2.50	3.04 ± 2.2 mm
Diameter	Small (< 5 mm)		109 (75.7%)
	Large (≥ 5mm)		35 (24.3%)
Multifocality	No	298 (69.62%)	93 (64.6%)
	Yes	130 (30.37%)	51 (35.4%)
Variants	Follicular	244 (57%)	110 (76.4%)
	Conventional	155 (36.22%)	19 (13.2%0
	Oncoytic	29 (6.78%)	0
	Tall cell/hobnail/columnar	0	15 (10.4%)
Local invasion	Extracapsular	74 (17.29%)	21 (14.6%)
	Vascular	0	1 (0.7%)
	Perineural	7 (1.63%)	8 (5.6%)
	Lymphatic emboli	18 (4.20%)	13 (9.0%)
Positive lymph nodes	Yes	23 (13.69%)	8 (5.6%)
Base disease (including multiple lesions on the same patient)	Multinodular/colloid goiter	336 (78.50%)	106 (73.6%)
	Hashimoto thyroiditis	68 (15.89%)	25 (17.4%)
	Adenoma	7 (1.63%)	36 (25.0%0
	Basedow disease/hyperthyroidism	17 (3.97%)	7 (4.9%)
	Concomitant primary hyperparathyroidism	0	11 (7.6%)

n – number of cases, % - percentage

The mortality rate after surgical treatment was zero; 1 permanent unilateral paresis (0.69%) and four (2.7%) transitory paresis of the recurrent laryngeal nerve were recorded. One (0.69%) case of permanent hypocalcemia and 6 (4.1%) cases of transitory hypocalcemia were noted. Considering the ATA risk stratification, most of the patients (112 cases, 77.7%) were included in low risk category, whereas 32 patients (22.2%) had intermediate risk and no patient met the criteria for high risk category.

Relationships between clinicopathological prognostic factors – univariate and multivariate analysis

The statistical analysis addressed the possible correlations between the positive lymph nodes as the dependent variable and other histopathological markers of aggressiveness.

No relation with the patients' age and gender was found, but the presence of metastatic lymph nodes was positively correlated with the extracapsular invasion (moderate to strong correlation, $r = 0.587$, $p < 0.001$), lymphatic emboli (moderate to strong correlation, $r = 0.558$, $p < 0.001$) and perineural invasion (strong correlation, $r = 0.603$, $p < 0.001$). Moreover, extracapsular invasion was positively correlated with the presence of lymphatic emboli (moderate correlation, $r = 0.488$, $p < 0.001$), perineural invasion (moderate correlation, $r = 0.501$, $p < 0.001$), and vascular invasion (weak correlation, $r = 0.2$, $p = 0.01$). It was also positively correlated (moderate correlation, $r = 0.45$, $p < 0.001$) with the tumors larger than 5 mm and with the total diameter when the tumors were multicentric (weak correlation, $r = 0.33$, $p < 0.001$). The presence of lymphatic emboli was positively correlated also with large tumors (moderate correlation, $r = 0.453$, $p < 0.001$) and with total diameter in case of multicentric tumors ($r = 0.406$, $p < 0.001$) and negatively with small tumors (moderate correlation, $r = -0.443$, $p < 0.001$) (Table 3.6).

Table 3.6. Assessment of correlations between different markers of tumoral aggressiveness in G2 group

Correlations		Positive lymph nodes	Extracapsular invasion	Lymphatic emboli	Perineural invasion	Vascular invasion	Age	Male	Female
Positive lymph nodes	Pearson Correl	1.00	**0.587****	**0.558****	**0.603****	-0.020	-0.023	-0.039	0.039
	Sig. (2-tailed)		0.000	0.000	0.000	0.809	0.781	0.644	0.644
Extracapsular invasion	Pearson Correl	**0.587****	1	**0.488****	**0.501****	**0.202***	0.061	0.104	-0.104
	Sig. (2-tailed)	0.000		0.000	0.000	0.015	0.467	0.215	0.215
Lymphatic emboli	Pearson Correl	**0.558****	**0.488****	1	**0.347****	**0.265****	-0.023	0.035	-0.035
	Sig. (2-tailed)	0.000	0.000		0.000	0.001	0.789	0.678	0.678
Perineural invasion	Pearson Correl	**0.603****	**0.501****	**0.347****	1	-0.020	0.000	0.039	-0.039
	Sig. (2-tailed)	0.000	0.000	0.000		0.809	0.996	0.644	0.644
Vascular invasion	Pearson Correl	-0.020	**0.202***	**0.265****	-0.020	1	-0.041	0.174*	-0.174*
	Sig. (2-tailed)	0.809	0.015	0.001	0.809		0.628	0.037	0.037
Age	Pearson Correl	-0.023	0.061	-0.023	0.000	-0.041	1	0.110	-0.110
	Sig. (2-tailed)	0.781	0.467	0.789	0.996	0.628		0.191	0.191
Male	Pearson Correl.	-0.039	0.104	0.035	0.039	**0.174***	0.110	1	**0.590****
	Sig. (2-tailed)	0.644	0.215	0.678	0.644	0.037	0.191		0.000
Female	Pearson Correl.	0.039	-0.104	-0.035	-0.039	**-0.174***	-0.110	**0.590****	1
	Sig. (2-tailed)	0.644	0.215	0.678	0.644	0.037	0.191	0.000	

**. Correlation is significant at the 0.01 level (2-tailed) *. Correlation is significant at the 0.05 level (2-tailed)

Positive lymph nodes correlated positively with large tumors (weak to moderate correlation, r=0.365, $p < 0.01$), but not with the type of disease for which the patient was initially operated (Table 3.7). No correlations were found between the histological variant and any of the markers of aggressiveness (extracapsular invasion, vascular invasion, lymphatic emboli or neural invasion). Also, the presence of lymph nodes metastasis was not correlated with the histological variant of PTMC (seven cases of follicular and one tall cell variant).

Table 3.7. Assessment of positive lymph nodes in relation with general histopathological data in G2 group

Correlations		Positive lymph Nodes	Hashimoto	Adenoma	Multinodular Goiter	Basedow's Disease	Primary HTP	Small	Large
Positive lymph nodes	Pearson Correl	1.00	0.049	0.070	0.076	-0.055	-0.070	**-0.357****	**0.365****
	Sig. (2-tailed)		0.560	0.404	0.363	0.514	0.406	0/000	0.000
Hashimoto	Pearson Correl	0.049	1	-0.138	**-0.682****	-0.104	0.075	-0.039	0.047
	Sig. (2-tailed)	0.560		0.100	0.000	0.217	0.370	0.639	0.573
Adenoma	Pearson Correl	0.070	-0.138	1	-0.055	-0.131	0.075	-0.084	0.094
	Sig. (2-tailed)	0.404	0.100		0.516	0.119	0.369	0.316	0.260
Multinodular goiter	Pearson Correl	0.076	**-0.682****	-0.055	1	**-0.378****	-0.006	-0.009	-0.001
	Sig. (2-tailed)	0.363	0.000	0.516		0.000	0.945	0.918	0.990
Basedow disease	Pearson Correl	-0.055	-0.104	-0.131	**-0.378****	1	0.057	-0.022	0.026
	Sig. (2-tailed)	0.514	0.217	0.119	0.000		0.501	0.789	0.753
Primary hyperparathyroidism	Pearson Correl	-0.070	0.075	0.075	-0.006	0.057	1	0.102	-0.098
	Sig. (2-tailed)	0.406	0.370	0.369	0.945	0.501		0.224	0.241
Small	Pearson Correl	**-0.357****	-0.039	-0.084	-0.009	-0.022	0.102	1	**-0.981****
	Sig. (2-tailed)	0.000	0.639	0.316	0.918	0.789	0.224		0.000
Large	Pearson Correl	**0.365****	0.047	0.094	-0.001	0.026	-0.098	**-0.981****	1
	Sig. (2-tailed)	0.000	0.573	0.260	0.990	0.753	0.241	0,000	

**. Correlation is significant at the 0.01 level (2-tailed) *. Correlation is significant at the 0.05 level (2-tailed)

Logistic regression analysis (Table 3.8) identified the most influential variables associated with positive lymph nodes. On multivariate analysis, large tumors (Odds Ratio/OR 28.25; $p < 0.05$), perineural invasion (OR 73.88; $p < 0.05$) and the presence of lymphatic emboli (OR 55.28; $p < 0.05$) were independent predictors of lymph node metastasis located in the central compartment.

Table 3.8. Multivariate analysis – risk factors for positive lymph nodes in central compartment in G2 group

Parameters	Odds Ratio	95% Confidence Interval		p
		Lower	Upper	
Large tumors (≥5mm)	28,25	3,33	239,52	0,000
Lymphatic emboli	55,28	9,39	325,18	0,000
Extracapsular invasion	0,09	0,05	0,16	0,000
Perineural invasion	73,88	11,82	461,77	0,000
Vascular invasion	0,99	0,97	1,00	0,9

3.4. DISCUSSION

The dual behavior of PTMC, predominantly favorable and rather rarely aggressive [Pisanu et al., 2015], is currently a stirring topic in thyroid pathology, especially because PTMC incidence has been rising after its definition as a distinct diagnostic entity-undoubtedly due to the imaging techniques progress [Gao et al., 2016]. The major issue consists in the identification of those clinicomorphological characteristics which can distinguish between the two behavioral types. This focus is absolutely necessary since the percentage of cases with a central lymph node metastasis is also rising, varying from under 10% [Bircan et al., 2014] up to 61% [Lim et al., 2009; Kim et al., 2012; Zhou et al., 2012; Zhao et al., 2013; Liu et al., 2014; Lee et al., 2014]. A recent point of view contends that PTMC is essentially an early stage in the development of PTC [Noguchi et al., 2008; Park et al., 2010; Kim et al., 2014] and not a different entity. Consequently, a paradigm shift in the PTMC treatment is recorded in relation to risk stratification [Pisanu et al., 2015]. A certain trend recommends an initial rather aggressive treatment similar to PTC [Pisanu et al., 2015; Gao et al., 2016] motivated by the high percentage of central lymph node metastasis revealed through prophylactic central lymph node dissection [Zhou et al., 2012]. Nonetheless, such a treatment must be regarded as a bold decision, since criteria to anticipate further evolution are not really available, and the prognostic value of this therapy failed to be demonstrated even by randomized clinical trials [Noguchi et al., 2008; Kim et al., 2015].

The most studied potential prognostic factors are age, gender, finding modality (incidental versus non-incidental), tumor size, histological type, extension, multifocality, lymph node or distant metastasis upon diagnosis, type of surgical treatment, and ablative radioiodine therapy [Roti et al., 2008; Mantinan et al., 2012; Usluogullari et al., 2015]. Unfortunately, the identification of those prognostic factors meant to ensure the stratification of PTMC in two large classes, with high or low risk of recurrence, is far from being completed. The results until now are unconvincing.

Within this context, we define four parameters associated with tumor aggressiveness, namely: lympho-vascular invasion, thyroid capsule invasion, extrathyroidal extension, and lymph node involvement.

In the whole study group, lympho-vascular invasion was rather rare (G1 - 4.20%, G2 - 0.7%), and thyroid capsule invasion was identified in approximately one fifth or less of our cases (G1 - 21.73%, G2 - 14.6%). To the best of our knowledge, these two parameters are less studied as prognostic factors in PTMC in comparison with PTC.

The extrathyroidal extension, present in our groups in less than one fifth of our cases (G1 - 17.29%, G2 - 14.6%), is reported in several studies in a percentage which varies, largely, between 2 and 52% of PTMC [Chow et al., 2003; Pelizzo et al., 2004; Park et al., 2010; Krämer et al., 2010; Liu et al., 2014; Pisanu et al., 2015]. Nevertheless, its role of prognostic factor is a contradictory issue, with evidences in support [Vergez et al., 2010; Varshney et al., 2014; Pisanu et al., 2015; Bradley et al., 2017] or against it [Chow et al.,

2003; Pelizzo et al., 2004; Schönberger et al., 2007; Zhao et al., 2013].

The lymph node involvement was recorded for 13.69% of G1 and 5.6% G2 cases, lower than the percentages reported in the literature (i.e. 24.3-64%) [Wang et al., 2015]. Only in 5 of the 23 cases in G1 and 2 of 8 cases in G2 with lymph node metastasis, the primary tumor was located in the upper third of the thyroid lobe, so that the association between the lateral neck lymph node involvement and this location [Kwak et al., 2009; Zhang et al., 2012] cannot be confirmed. Some proofs argue that lymph node metastasis represents a negative prognostic factor as independent predictor of disease recurrence [Chow et al., 2003; Hay et al., 2008; Pisanu et al., 2009; Kim et al., 2014] or as risk factor for distant metastasis rarely present at the diagnosis time [Roti et al., 2008]. However, there are differences regarding the location of the involved lymph nodes; the prognostic value for metastasis in lateral neck lymph nodes is already ascertained [Kim et al., 2015], whereas for central lymph nodes is still under discussion [Giordano et al., 2010; Liu et al., 2014].

Concomitantly, we analyzed the prognostic value of the clinicopathological characteristics, as follows: age, tumor size, tumor location, histological type, focality and bilaterality, and associated thyroid pathology.

Age

The prognostic value of age has been intensively studied using a cutoff of 45 yo [Roti et al., 2008], with contradictory results [Kim et al., 2012; Zhang et al., 2012; Zhao et al., 2013; Kim et al., 2013]. For most studies, age is not a prognostic factor for lymph node metastasis, disease recurrence or survival [Chow et al., 2003; Liu et al., 2014; Kim et al., 2015]. On the other hand, age over 45 yo is more frequently correlated with the thyroid capsule invasion [Usluogullari et al., 2015], central lymph node metastasis [Cho et al., 2012; Iti et al., 2014; Kim et al., 2015; Guo et al., 2015] and nodal recurrence [Pisanu et al., 2015]. These controversial results allow for a major change in the 8th Edition of the TNM Classification of Malignant Tumours, applicable from January 2017, where the age for a poor prognosis in well-differentiated thyroid carcinoma has changed from 45 to 55 years of age [Brierley et al., 2017]. The implementation of the new cutoff of 55 yo in the AJCC/UICC staging system aims to prevent the low-risk category of patients from overstaging and consequently from overtreatment [Brierley et al., 2017].

Almost half of our patients were less than 55 yo (G1 - 42.75%, G2 - 45.1%), the raised age cutoff considered by the 8th edition of AJCC/TNM staging [Amin et al., 2017]. Even if it was to consider the previous cutoff age used until 2016 (45 years), more than a quarter of our patients (G1 - 17.3, G2 - 25.9%) would have been under that threshold, considered a risk factor for the disease progression [Mercante et al., 2019]. Unfortunately, our results cannot confirm a relationship between this age cutoff and the four considered parameters of aggressiveness. Therefore, we consider that the prognostic value of the age is still debatable.

Tumor size

Tumor size may have potential as independent predictive factor, although there are also contrary opinions [Bircan et al., 2014]. The cutoff is very variable [Chow et al., 2003; Lim et al., 2009; Lee et al., 2011; Kim et al., 2012; Zhou et al., 2012; Zhang et al., 2012; Kim et al., 2013; Usluogullari et al., 2015], most of the studies using a threshold of 5 mm [Bircan et al., 2014; Kim et al., 2015; Bradley et al., 2017]. Our studies demonstrated the association between tumor size larger than 5 mm and the aggressiveness parameters. The role of independent predictive factor was confirmed by multivariate analysis. These results agree with reported data, which certify that the tumor size is associated with multifocality [Wang et al., 2015], bilateral location [Zhou et al., 2012; Usluogullari et al., 2015], lympho-vascular invasion and extrathyroidal extension [Chow et al., 2003; Pelizzo et al., 2004; Park et al., 2010; Krämer et al., 2010; Usluogullari et al., 2015; Wang et al., 2015], lymph node

metastasis [Pisanu et al., 2015; Kim et al., 2015; Usluogullari et al., 2015; Wang et al., 2015; Guo et al., 2015; Kaliszewski et al., 2019], disease persistence [Gao et al., 2016] and distant metastasis during diagnosis [Roti et al., 2008].

Tumor location

To the best of our knowledge, there is little information on the prognostic value of the tumoral, subcapsular or intraparenchymal situs investigated in G1 study [Niemeier et al., 2012]. We formulated the hypothesis according to which the location of the tumor microfocus may influence the subsequent evolution in a negative way. The data obtained showed, beyond any doubt, that subcapsular PTMC has a high potential of lympho-vascular and thyroid capsule invasion, extrathyroidal extension and lymph node metastasis. The multivariate analysis emphasized the role as independent predictive factor for the subcapsular location in relation to the first three parameters of aggressiveness considered, but not for lymph node metastasis. We consider this a valuable result since PTMC can be identified by high-resolution medical imaging, and the location (subcapsular or intraparenchymal) may guide the therapeutic conduct towards either conservative or aggressive approaches.

Our results were in agreement with the study published in 2020 by Tallini et al., who demonstrate on multivariate analysis a significant difference between patients with large size PTMC, more than 5 mm and 0 mm distance of the edge of the tumor from the thyroid capsule, that showed the most aggressive features, and the patients with size under 5 mm and distance of the edge of the tumor from the thyroid surface more than 0 mm (small nonsubcapsular PTMC) the most indolent ones [Tallini et al., 2020]. In Tallini study group these aggressive tumors were characterized by tall cell histotype ($p<0.0001$), BRAF V600E mutation ($p<0.0001$), tumor fibrosis, aggressive growth with invasive features, vascular invasion, lymph node metastases ($p = 0.003$), and intermediate ATA risk [Tallini et al., 2020]. The tumors were associated more commonly with invasion of extrathyroidal tissue ($p < 0.0001$), high-grade features ($p = 0.029$), mitoses ($p = 0.049$), and intra- and peritumoral lymphoid cell infiltration ($p = 0.003$ and $p < 0.0001$, respectively). Microscopically, the tumors presented distinctive features, such as a larger number of pseudoinclusions ($p = 0.003$), nuclear grooves ($p = 0.004$), and nuclear membrane irregularities ($p = 0.0001$); and a greater proportion of tumor cells with eosinophilic cytoplasm ($p = 0.012$), tall cell features ($p = 0.0001$), and papillary or with solid/trabecular pattern of growth ($p = 0.0003$ and $p = 0.039$, respectively) [Tallini et al., 2020].

Histologic type

Relatively few studies are focused on the prognostic value of the histological configuration of PTMC. According to literature, the conventional, papillary variant represents 65-99% of the total cases, the follicular variant being reported in 0.3-31% of the cases, sclerosing variant in 5-11.7% of cases, whereas oncocytic and tall cell variants in 0.8% [Pelizzo et al., 2004; Roti et al., 2008]. The follicular variant is associated with distant metastasis at diagnosis [Roti et al., 2008], and the oncocytic or tall cells variants are considered more aggressive [Pelizzo et al., 2004]. The structure of the G1 group was largely different from already reported percentages. Thus, in G1 group we recorded higher percentages for the follicular (57%) and for the oncocytic variant (6.78%) than those reported (31% and 0.8%, respectively), while for the conventional variant, the percentage we noted, namely 36.22%, was below the 65% found in the literature [Pelizzo et al., 2004; Roti et al., 2008]. In G2 group also follicular variant of PTMC was the most identified category (76.4%), followed by conventional (13.2%) and other rare histological types of PTMC, tall, hobnail or columnar one (10.4%). These histologic findings could express regional features. At national level, the research on thyroid pathology is focused mainly on epidemiologic and clinical elements. An increase in the TC incidence by 10 times in the last decades (mostly on young population) and a high frequency for follicular variant of PTC have been documented,

possibly related to the Chernobyl nuclear accident [Piciu et al., 2012; Piciu et al., 2014], or to the iodine salt administrated as a food supplement [Ivan et al., 2002]. These features should be confirmed by extensive histological studies, as Romanian data about the histological variants of PTC and PTMC are currently very limited [Nechifor-Boila et al., 2013; Nechifor-Boila et al., 2015]. Although we noticed discrepancies in the frequency of histological variants, in G1 group the obtained results partially overlap the state of the art. Thus, the oncocytic variant, considered to be the most aggressive [Pelizzo et al., 2004], had the highest risk of associating with thyroid capsule invasion, extrathyroidal extension and lymph node metastasis. We also noted a significant correlation between the conventional variant and lympho-vascular invasion. Furthermore, the follicular variant had the lowest risk of lympho-vascular, thyroid capsule invasion, and extrathyroidal extension. While very few studies target the potentially prognostic value of the histologic type, we consider the multivariate analysis result to be important, and accordingly the oncocytic variant is an independent predictive factor for extrathyroidal extension.

Focality and bilaterality

The hypothesis of multifocal PTC arising from independent clonal origins of distinct tumor foci [Shattuck et al., 2005; Bansal et al., 2013] is not confirmed for PTMC. Several articles target the analysis of multifocality as a prognostic factor in PTMC, with contradictory results. Multifocality could be an independent predictor or risk factor for lymph node metastasis (central or not) [Shattuck et al., 2005; Lee et al., 2008; Roti et al., 2008; Mercante et al., 2009; Kim et al., 2012; Zhou et al., 2012; Zhao et al.,2013; Pellegriti et al., 2013; Kim et al., 2013; Liu et al., 2014; Guo et al., 2015; Gao et al., 2016; Gao et al., 2019; Kaliszewski et al., 2019], irrespective of age [Zhao et al., 2013], and relapse [Chow et al., 2003; Hay et al., 2008; Besic et al., 2008; Mercante et al., 2009; Mantinan et al., 2012; Zhao et al. 2013; Cho et al., 2015]. Likewise, multifocality is associated with extrathyroidal extension [Kim et al., 2014], thyroid capsule invasion and a significant risk of a contralateral tumor [Lim et al., 2009; Liu et al., 2014]. In contrast, complementary data show that there is no correlation between multifocality and prognosis, assessed through lymph node metastasis [Moon et al., 2011] and recurrence rate [Cappelli et al., 2007]. Our G1 study confirms the association of multifocality and bilaterality with thyroid capsule invasion and extrathyroidal extension. According to the multivariate analysis, multifocality is an independent predictive factor. For example, from 3 foci to over 4, the OR value increases exponentially, and this observation could represent a major decisional event in the assessment of prognosis. On the other hand, our study corroborates the report series [Cappelli et al., 2007; Guo et al., 2015; Moon et al., 2011; Kaliszewski et al., 2019] which dispute the role of multifocality and bilaterality as prognostic factors for lymph node metastasis. Similarly, in the data presented by ATA Guidelines, the risk of structural disease recurrence can vary from 1%–2% in unifocal PTMC, to 4%–6% in multifocal PTMC [Haugen et al., 2016]. These results may be explained either through the timing of PTMC diagnosis (made in more or less incipient stages), or through the behavioral differences of this tumor (indolent versus aggressive type).

Associated thyroid pathology

The variability of the reported data concerning the incidence of PTMC operated for benign diseases is large. A recent study on large cohort of 1793 patients discovered a 4.62% incidence of PTMC [Maturo et al., 2017]. As the percent of incidental PTMC in our cohort was higher (G1 - 60.74%, G2 - 23.52%), our results agree with two other studies that reported that PTMC in histological specimens after thyroid surgery for benign diseases was found in 22% respectively 27.4% of cases [Carlini et al., 2005; Lee et al., 2014].

Endocrine pathology in the thyroidal tissue observed concurrently with PTC is more frequently studied than in PTMC. Some data indicates a positive correlation of Hashimoto disease with disease free survival and overall survival [Lee et al., 2013]. The potential

prognostic role of Hashimoto thyroiditis [Bircan et al., 2014], Graves' disease [Phitayakorn et al., 2008], nodular goiter, and adenoma for PTC is controversial. In the case of PTMC, only Hashimoto thyroiditis is constantly identified [Phitayakorn et al., 2008; Vlassopoulou et al., 2016], although the relation with the central lymph node metastasis [Bircan et al., 2014; Guo et al., 2015] or the persistence of disease [Pisanu et al., 2015] is not confirmed.

Our study groups were characterized by a great variety of associated lesions. There was no correlation between thyroid pathology associated with PTMC and the considered parameters of aggressiveness, except for colloid goiter and Hashimoto thyroiditis versus extrathyroidal extension in G1 group, and none in G2 group. It is difficult to explain why these associated endocrine diseases in G1 group are correlated only with extrathyroidal extension and not with lympho-vascular invasion, thyroid capsule invasion or lymph node metastasis. Possibly this result reflects the peculiarity of the analyzed group, which had extrathyroidal extension and high frequency of colloid goiter (25 out of 111 cases, 22.5%) and Hashimoto thyroiditis (15 out of 86 cases, 22.1%). Consequently, we cannot recommend the associated thyroid pathology as a prognostic factor for PTMC. The future challenge is to demonstrate whether the pre-existent pathological background leads to the development of PTMC or not.

Lymph node involvement

Positive lymph nodes in the central compartment were found in 4.2% cases - G1 and 5.6% cases - G2, which is far lower than in other studies in which central compartment lymphadenectomy was routinely performed (in the absence of macroscopic lymph nodes), showing the high frequency of subclinical central lymph node metastasis in PTMC [Kim et al. 2012; Frangos et al., 2015].

Although the necessity of prophylactic central compartment lymphadenectomy is still not widely accepted for PTC (not only PTMC) [An et al., 2019], a recent meta-analysis shows that, in addition to thyroidectomy, it reduces the risk of local recurrence without increasing the incidence of laryngeal nerve injury (temporary or permanent) and the incidence of permanent hypocalcemia [Dobrinja et al., 2017]. Moreover, Xue et al. revealed that the sensitivity of ultrasound ranged from 22.6 to 55% in predicting central lymph node metastases, which means that almost half of the patients with metastases were incorrectly diagnosed [Xue et al., 2018]. Thus, in some centers, prophylactic central lymph node dissection is widely performed in PTMC to allow more accurate TNM staging and thus to decide the post-operative management [Yuan et al., 2017; Gao et al., 2019]. The incidence of lymph node metastases was highly reported in imagistic detection, and prophylactic lymph node dissection was supposed to lower the incidence of residual lymph node metastasis and to improve the overall prognosis [Lin et al., 2016]. As male gender and ultrasonographic tumor diameter were independent risk factors of lymph node metastases, surgeons would be considering these risk factors when they decide to extend surgical dissections [Lang et al., 2013; Lin et al., 2016].

The 2009 ATA risk stratification system classified all patients with thyroid differentiated carcinomas and loco-regional lymph node metastases in intermediate risk category, in which the risk of structural disease recurrence can vary from 4% in patients with fewer than five metastatic lymph nodes, 5% if all involved lymph nodes are under 0.2 cm, 19% if more than five lymph nodes are involved, 21% if more than 10 lymph nodes are involved, 22% if macroscopic lymph node metastases are clinically evident (clinical N1 disease), to 27%– 32% if any metastatic lymph node is more than 3 cm [Haugen et al., 2016].

Our surgical center performs elective central compartment neck dissection, based on imaging information and intraoperative aspects, and it was performed in 39.25% in G1 group and 6.9 % in G2 group. The obtained data are discordant and revealed positive lymph nodes in 13.69% (G1) and 80% (G2) of them. Consequently, the data obtained in the multivariate

analysis are not superimposable between the two groups. Although in the first G1 PTMC group for lymph node metastasis, out of the four factors which showed significant associations in the univariate analysis, only one maintained a predictive potential. We found that only a tumor in size larger than 5 mm diameter (p=0.003, OR=1.37) is a negative prognostic factor, which increases the risk of lymph node metastasis. In the G2 PTMC group, on multivariate analysis lymphatic emboli ($p < 0.05$, OR=55.28), perineural invasion ($p < 0.05$, OR=73.88) and large diameter of the tumors over 5 mm ($p < 0.05$, OR=28.25) are increasing the risk of lymph nodes metastases. The obtained data overlap with those in the field literature about these factors that correlate with each other and give, along with the positive lymph nodes, an increased risk of local recurrence for PTMC [Lee et al., 2008; Mercante et al., 2009; Kim et al., 2012].

3.5. FINAL REMARKS

PTMC affects a significant percentage of the patients considered to have benign thyroid diseases. The present study confirmed that the tumor size is a negative prognostic factor for lympho-vascular invasion, thyroid capsule invasion, extrathyroidal extension and lymph node metastasis. We also demonstrated the strong relationship between the subcapsular location, lympho-vascular and thyroid capsule invasion, and extrathyroidal extension. The multifocality was correlated only with capsule invasion and extrathyroidal extension. Regarding the histological variants, the only validated correlation was between the oncocytic variant and extrathyroidal extension. We cannot sustain the pre-existent pathological background associated thyroid pathology as a prognostic factor for PTMC.

Our work contributes to the validation of PTMC prognostic factors, useful in stratification of PTMC in high or low risk classes, and is able to explain the behavioral differences in tumor development.

CHAPTER 4.
PAPILLARY THYROID CARCINOMA – STRATIFICATION AND PROGNOSTIC FACTORS

4.1. INTRODUCTION

The differences between the PTC biological behaviors, with result in the low versus high metastasis potential of some cases, are grounded through sequences of the carcinogenic mechanism, namely: alterations of the cellular cycle and increase of proliferation, changes in the adhesive morphological status of cells, and influence of the tumoral microenvironment.

One challenging research topic in understanding the PTC aggressive behavior addresses molecular markers that include molecules involved in the cellular cycle regulation (*i.e.*, cyclin D1, p27, p53), or cell growth (Her2/neu), in cell adhesion (E-cadherin, β-catenin, claudin-1, tubulin), or in tumor microenvironment changes (fibronectin, periostin) [Dean et al., 2000; De Lellis et al., 2004; Sethi et al., 2010; Nikiforov et al., 2011; Xing et al., 2013; Radu et al., 2015; Radu et al., 2016]. Concurrently, the histological variants of PTC [Lloyd et al., 2017] sustain the stratification of PTC cases in high-risk or low-risk categories, starting from the time of diagnosis [Sherman et al., 2003; Kakudo et al., 2004; LiVolsi et al., 2011; Lloyd et al., 2011; Kakudo et al., 2014]. This ongoing process is critical, because the validation of new prognostic factors opens the perspective of PTC stratification, based on differences of aggressiveness at molecular level.

Proto-oncogene HER – 2/neu (C-erbB2), also known as CD340, is located on the 17q chromosome and it codifies the transmembrane tyrosine kinase receptor for the epidermal growth factor (EGF) [Yarden et al., 2001]. Its amplification or overexpression plays an important role in tumoral progression and aggressiveness, through direct effects on the cell cycle, angiogenesis, cellular motility and apoptosis [Yarden et al., 2001]. HER-2/neu is considered a new prognostic factor in numerous types of cancer. It is involved in tumor biology, with a key role in the uncontrolled cell growth. HER-2/neu overexpression, firstly demonstrated in breast and ovary cancer [Yokota et al., 1986; Berchuck et al., 1990; Iqbal et al., 2014] and later confirmed in gastric, colon, lung and bladder cancer [Heinmöller et al., 2003; Stephens et al., 2004; Coogan et al., 2004; Reichelt et al., 2007; Cervera et al., 2011; Ieni et al., 2013; Iqbal et al., 2014; Ieni et al., 2014; Ieniet al., 2015] is associated with poorly differentiated phenotype, high metastasis capacity and poor overall survival [Iqbal et al., 2014]. Moreover, HER-2/neu becomes a therapeutic target in the breast and gastric cancer [Iqbal et al., 2014; May et al., 2014; Bilici et al., 2014].

HER-2/neu overexpression in PTC has been identified in the aggressive forms with an increased potential of metastasis, allowing the supplementary Herceptin therapy for these patients, similar to breast cancer [Kremser et al., 2003; Iqbal et al., 2014]. However, data on HER-2/neu involvement in PTC progression are controversial. HER-2/neu overexpression is reported in a wide range of values, varying from 0% to 79% [Lemoine et al., 1990; Haugen et al., 1992; Kremser et al., 2003; Mondi et al., 2003; Balta et al., 2012; Sugishita et al., 2013; Mdah et al., 2014]. These discrepancies are due to the large differences in the assessment algorithm [Lemoine et al., 1990; Haugen et al., 1992; Sugg et al., 1998; Utrilla et al., 1999; Kremser et al., 2003; Mondi et al., 2003; Elliott et al., 2008; Qin et al., 2012; Wu et al., 2013;

Sugishita et al., 2013; Mdah et al., 2014; Ruggeri et al., 2016]. Consequently, there is no consensus on the prognostic and therapeutic value of HER-2/neu [Mdah et al., 2014; Ruggeri et al., 2016; Siraj et al., 2017].

E-cadherin, a calcium-dependent transmembrane cell adhesion molecule, is essential for the normal function of epithelial cells [Pećina-Slaus et al., 2003]. In the adherens junctions, the E-cadherin intracytoplasmic domain links to β-catenin that in turn connects to α-catenin jointed to the actinic cytoskeleton [Ozawa et al., 1989; McCrea et al., 1991]. Besides the role in the intercellular stability [Lilien et al., 2005], β-catenin acts as a signaling factor in the canonical Wnt pathway [Brembeck et al., 2006]. The decrease of E-cadherin expression is responsible for the loss of cell adhesion, tumor growth and proliferation, leading to metastasis [Bracke et al., 1996; Kefeli et al., 2005]. The diminution of E-cadherin has been observed in several malignancies, in association with tumoral advanced stages and disease progression [Guilford et al., 1999; Berx et al., 2001; Sobrinho-Simões et al., 2002; Hirohashi et al., 2003; Rocha et al., 2003; Ceyran et al., 2015; Liu et al., 2015].

In benign thyroid lesions, a high expression of E-cadherin in thyroid cells is reported [Liu et al., 2015]. E-cadherin expression in TC is generally reduced, with the following particularities: it is still present in DTC or in the minimally invasive TC, and completely absent in the undifferentiated ones. Several studies sustain that loss of E-cadherin expression is a decisive step in dedifferentiation, progression, and metastatic spread of TC, in relationship with a poor prognostic [Böhm et al., 2000; Garcia-Rostan et al., 2001; Rocha et al., 2001; Rocha et al., 2003; Lantsov et al., 2005; Ralhan et al., 2010; Liu et al., 2015; Ceyran et al., 2015; Lam et al., 2017; Caruntu et al., 2018]. On the other hand, the involvement of β-catenin in carcinogenesis is also documented in different types of tumors [Kawasaki et al., 2003; Khramtsov et al., 2010; Sinnberg et al., 2011; Tao et al., 2014; Shang et al., 2017], but few reports focus on its expression in TC, in relationship with a poor prognostic [Böhm et al., 2000; Garcia-Rostan et al., 2001; Rocha et al., 2001; Lantsov et al., 2005; Ralhan et al., 2010; Lam et al., 2017; Caruntu et al., 2018].

EpCAM (MOC-31), a transmembrane glycoprotein with a molecular weight of 40 kDa, is different from the four classical families of cell-adhesion molecules represented by cadherins, integrins, selectins, and members of the immunoglobulin superfamily. Its structure contains 314 amino acids, organized into an extracellular domain (EpEx-MOC-31) with 242 amino acids, a transmembrane domain with 23 amino acids and a short intracellular domain (EpICD) with 26 amino acids [Munz et al., 2009; Dai et al., 2017]. The extracellular domain consists of an epidermal growth factor (EGF)-like component, a thyroglobulin-like component [Baeuerle et al., 2007], which intervenes in the mechanism of cathepsin inhibition produced by tumor cells, with effects in the metastasis process [Nomura et al., 2005], and a cysteine-free component [Armstrong et al., 2003]. EpCAM is displayed at the adherens junctions' level on the basolateral membrane of the normal epithelial cells, including the thyroid tissue.

Changes in EpCAM expression occurs in a wide variety of cancers, as an early indicator for carcinogenesis [Balzar et al., 1999; Went et al., 2006]. EpCAM has the ability to annul the E-cadherin mediated cell-adhesion once interrupted the connection between the α-catenin and F-actin [Winter et al., 2003; Schmelzer et al., 2008]. Impaired intercellular adhesion is an important parameter in determining the prognostic impact in tumor pathology. However, EpCAM is poorly studied in TC, limited data sustaining the correlation between its expressions, with the decreasing of the overall survival [Ralhan et al., 2010]. EpCAM is also proposed as a potentially prognostic factor and therapeutic target with applicability for the clinical management of aggressive TC [Ralhan et al., 2010; Kunavisarut et al., 2012; Fong et al., 2014; Okada et al., 2014].

Microtubules are cytoskeletal proteins, made of two categories of α and β tubulin heterodimers, with multiple isotypes and with a varied, specific composition compared to the type of tissue and the intracellular functions. Microtubules contribute to maintaining the cellular form and also to the intracellular transport and chromosomal segregation during mitosis, with the consecutive formation of the mitotic spindle [Katsetos et al., 1993; Orr et al., 2003]. The class III of β-tubulin (TUBB3) is usually identified in cells of neuronal origin, where it contributes to the formation of dynamic microtubules which are essential in neurite growth and maintenance [Katsetos et al., 1993; Katsetos et al., 2003; Lebok et al., 2016]. TUBB3 is also expressed in other normal tissues – namely the testicle, small intestine and placenta [Leandro‑García et al., 2010].

Multiple studies proved the implication of TUBB in carcinogenesis. Higher levels of TUBB3 are reported in brain, lung, colorectal, ovarian, prostate and laryngeal carcinomas [Katsetos et al., 1993; Ferrandina et al., 2006; Koh et al., 2010; Leandro‑García et al., 2010; Hetland et al., 2011; Chen et al., 2012; Zhang et al., 2012; Zheng et al., 2012; Tsourlakis et al., 2014]. The analysis of TUBB3 expression in relation to therapy showed differences in response and resistance to treatment. TUBB3 overexpression is associated to a poor prognostic and, frequently, to chemoresistance to the neo-adjuvant therapy with taxane-class of neo-adjuvant chemotherapy with role in microtubules stabilization [Magnani et al, 2006], used in lung, uterus, ovary, colon or breast cancer [Ferlini et al., 2007; Tommasi et al., 2007; Seve et al., 2010; Mariani et al., 2012; Roque et al., 2013]. Strictly referring to the thyroid tumor pathology, literature review indicates few papers that analyze TUBB3 in anaplastic cell thyroid carcinoma and the treatment with anti-microtubule agents that inhibit mitosis by disrupting microtubules [Ain et al., 2004; Gómez Sáeza et al., 2015], and only two studies (published as abstracts) that focuses on TUBB3 expression in PTC [Colato et al., 2011; Colato et al., 2012].

As a component of the cellular matrix, periostin (PN) has been recently included in the list of putative prognostic markers. PN is a cellular adhesion molecule, initially identified within the osteoblastic cellular line in mice [Takeshita et al., 1993] and named according its identification in periosteum and periodontal ligamentum [Horiuchi et al., 1999]. In humans, PN is encoded by a gene located on chromosome 13(13q13.3) [Litvin et al., 2004]. Structurally, it is formed by one N-terminal constant domain, one cysteine-rich domain (EMILIN-like), four fasciclin-repetitive-Fas domains, and one C-terminal hydrophilic domain exhibiting a variable structure according to the isoform [Takeshita et al., 1993; Horiuchi et al., 1999; Litvin et al., 2004]. PN is secreted by fibroblasts [Norris et al., 2008; Hamilton et al.; 2008; Ruan et al., 2009] and belongs to fasciclin-I family of proteins, functioning in cell – cell and cell – extracellular matrix (ECM) interactions. It is located in fetal and normal adult organs, such as embryonic periosteum, placenta, heart valves, thyroid, adrenal glands, lung, stomach, colon, testicle, prostate, vagina, ovary, breast, and periodontal ligamentum [Gillan et al., 2002; Tai et al., 2005; Kudo et al., 2007; Nuzzo et al., 2014].

PN epithelial and stromal overexpression in tumor pathology has been studied according to tumor growth, angiogenesis, invasiveness, and metastasis [Kudo et al., 2007; Zhu et al., 2010; Morra et al., 2011; Nuzzo et al., 2014; Ratajczak-Wielgomas et al., 2015]. The published data are relatively limited but nevertheless they are supporting PN involvement in tumor progression in different locations, such oral [Siriwardena et al., 2006], head and neck [Chang et al., 2005; Kudo et al., 2006], breast [Shao et al., 2004; Puglisi et al., 2008; Zhang et al., 2010; Contie et al., 2011; Xu et al., 2012; Nuzzo et al., 2016], ovary [Gillan et al., 2002; Zhu et al., 2010; Zhu et al., 2011; Choi et al., 2011; Karlan et al., 2014], prostate [Tsunoda et al., 2009; Tischler et al., 2010; Sun et al., 2011; Nuzzo et al., 2012], kidney [Castronovo et al., 2006; Dahinden et al., 2010; Morra et al., 2011], pancreas [Baril et

al., 2007; Ben et al., 2011], stomach [Li et al., 2007; Kikuchi et al., 2014; Lv et al., 2014], colon [Bao, et al., 2004; Xiao et al., 2015], liver [Riener et al., 2010; Morra et al., 2011; Jang et al., 2016], lung [Sasaki et al., 2001; Soltermann et al., 2008; Morra et al., 2012; Hong et al., 2013], pleura [Schramm et al., 2010], brain [Sasaki et al., 2002; Tian et al., 2014; Zhou et al., 2015; Mikheev et al., 2015], and its association with aggressive phenotypes and poor prognosis [Kudo et al., 2007; Ruan ett al., 2009; Morra et al., 2011; Ratajczak-Wielgomas et al., 2015].

Aim

Based on this data, the aim of our study was to analize the expression pattern of several "candidate" biomarkers (HER-2/neu, E-cadherin, *β*-catenin, EpCAM, TUBB3 and periostin) and their relationship with the classical clinicopathological factors, in different histological subtypes of PTC.

4.2. MATERIALS AND METHODS

The retrospective study included selected cases of sporadic PTC, diagnosed at the "Sf. Spiridon" Clinical Emergency County Hospital, Iaşi, Romania, between 2006 and 2016. The research has been approved by the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, pursuant to the ethical standards of Helsinki declaration regarding the patients' informed consent for the use of their medical information for scientific purpose.

Patients

All patients underwent total thyroidectomy, and in some cases regional lymphadenectomy was also performed. The patient's clinicopathological characteristics (gender, age, tumor size, and lymph node metastases) were obtained from the medical files.

All cases were reviewed by three independent pathologists in order to establish the histological variant, tumor stages according to the 2017 WHO Classification, and TNM and AJCC criteria [Greene et al., 2002; De Lellis et al., 2004; Brierley et al., 2017; Lloyd et al., 2017; Amin et al., 2017], and to reassess the following characteristics: multifocality, lympho-vascular invasion, extrathyroidal extension (presence of tumor cells into perithyroidal soft tissues, beyond the thyroid capsule). All the cases were classified into low-risk and high-risk group. The low-risk group comprised the cases diagnosed as conventional, follicular, oncocytic, macrofollicular, and clear cell variants, characterized by indolent behavior and favorable prognosis [Sherman et al., 2003; Kakudo et al., 2004; LiVolsi et al., 2011; Lloyd et al., 2011; Kakudo et al., 2014], whereas the high-risk group consisted of the cases diagnosed as columnar and tall cell, follicular angioinvasive, cribriform-morular, hobnail, diffuse sclerosing, and solid subtype, conventional with dedifferentiation to squamous cell carcinoma and oncocytic with undifferentiated solid areas variants, known as aggressive subtypes in clinical course and poorer prognosis [Kakudo et al., 2004; LiVolsi et al., 2011; Kakudo et al., 2014].

The patients identified during this period constituted the basis for four groups in correspondence to the investigated molecular marker, PTC histological variant and aggressiveness, as follows: **group 1** with 120 patients (73 in low-risk group and 47 in high-risk group) – analyzed for Her2neu (C-erbB2) expression; **group 2** with 70 patients (45 in low-risk group and 25 in high-risk group) – analyzed for for E-cadherin, β-catenin and MOC31 expression; **group 3** with 70 patients (23 in low-risk group and 47 in high-risk group) – analyzed for β-tubulin 3 expresion; **group 4** with 50 patients (various histological subtypes) – analyzed for periostin expression.

Immunohistochemical exam

For each case, a representative paraffin-embedded tissue fragment was chosen. The 3-μm thick sections obtained from the blocks were placed on silanized slides, dewaxed in xylene, rehydrated in consecutive descending concentrations of ethanol (100%, 90%, 80%, and 70%), and rinsed in distillated water. For antigen retrieval, we used Heat Induced Epitope Retrieval (HIER) technique: slides were placed in citrate buffer pH 6 and heated in a water bath, at 98^0C, for 30 minutes. The slides were immersed in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase, and incubated with the primary antibodies for HER-2/neu (C-erbB2) (clone SP3, Thermo Scientific, 1:100 dilution), E-cadherin (clone EP700Y, Thermo Scientific, 1:100 dilution), *β*-catenin (clone *β*-catenin-1, Agilent–Dako, 1:300 dilution), EpCAM (MOC-31, DAKO, 1:200), β/beta-tubulin 3 (2G10, Thermo Scientific, 1:200) and periostin (F-10, Biotechnology Inc., Santa Cruz, 1:100) overnight, at 4^0C.

The immunoreaction was amplified with the suitable secondary and tertiary antibodies of the UltraVision Quanto Detection System HRP DAB (Thermo Scientific, USA) and developed with 3.3'-diamino-benzidine (DAB) tetrahydrochloride chromogen (Thermo Scientific, USA). The counterstaining of the sections was done with Lillie's modified Hematoxylin. Positive and negative controls have been simultaneously run in order to verify the accuracy of the technique.

Table 4.1 summarizes the main information on the immunohistochemical markers used.

Table 4.1. The antibodies used for immunohistochemical tehniques

Antibody	Manufacturer	Clone	Antigen retrieval	Class	Dilution
HER-2/neu (C-erbB2)	Thermo Scientific, USA	SP3	Citrate, pH 6	rabbit monoclonal	1:100
E-cadherin	Thermo Scientific, USA	EP700Y	Citrate, pH 6	rabbit monoclonal	1:100
β-catenin	Dako, Denmark	*β*-catenin-1	Citrate, pH 6	mouse monoclonal	1:300
EpCAM (MOC-31)	Dako, Denmark	MOC-31	Citrate, pH 6	mouse monoclonal	1:200
β/beta-tubulin 3	Thermo Scientific, USA	2G10	Citrate, pH 6	rabbit policlonal	1:200
Periostin	Biotechnology Inc Santa Cruz, USA	F-10	Citrate, pH 6	policlonal mouse	1:100

Semi-quantitative assessment

The semi-quantitative assessment was done by using adapted scores based on the literature reports that took into account the staining intensity (I) and the percentage of positive cells (P) of membranar or cytoplasmic immunostainng. The total score resulted by summing P with I, with different reporting intervals for each antibody. Specifically, for Her-2/neu was evaluated not only membranous, but also cytoplasmic immunopositivity [Kremser et al., 2003; Wu et al., 2013], E-cadherin was assessed on membranous expression, β-catenin and MOC-31 expressions were quantified both at membranous and cytoplasmic level [Rocha et al., 2001; Wiseman S et al., 2006], for TUBB3 we evaluated cytoplasmic expression [Powell et al., 2014], and PN expression has been separately assessed in tumor epithelial cells and in intratumoral stroma on cytoplasmic level [Choi et al., 2011; Jia et al., 2016; Sung et al., 2016].

The corresponding non-tumoral thyroid tissue within each PTC specimen has been constantly evaluated. This step allowed us to establish the basal level of thyroid tissue immunoreaction.

The threshold values for each marker were summarized in Table 4.2.

Table 4.2. Semiquantitative score used for PTC evaluation

Antibody	Tissue	Labelling	Positive cells	Staining intensity	Final score (P + I)	Interpretation
Her-e/neu (C-erbB2)	tumoral cells	membranous cytoplasmic	0 < 10%	1: weak	0: negative	0-8: negative
			1: 10%-25%	2: moderate	1-4: weakly positive	9-12: positive
			2: 25%-50%	3: strong	5-8: strongly positive	
			3:50-75%			
			4 > 75%			
E-cadherin	tumoral cells	membranous	0 < 5%	1: weak	1–4: low	1–4: low
			1: 6–25%	2: moderate	6–12: high	6–12: high
			2: 26–50%	3: strong		
			3: 51–77%			
			4 > 75%			
β-catenin	tumoral cells	membranous cytoplasmic	0 < 10%		1–3: low	1–3: low
			1: 10–30%		4–7: high	4–7: high
			2: 31–50%,			
			3: 51–70%,			
			4 > 70%.			
EpCAM (MOC-31)	tumoral cells	membranous cytoplasmic	0 < 10%	1: weak	1–4: low	1–4: low
			1: 10–30%	2: moderate	5–7: high	5–7: high
			2: 31–50%	3: strong		
			3: 51–70%			
			4 > 70%			
β/beta-tubulin 3	tumoral cells	cytoplasmic	1 ≤50%	1: weak	3: low	3: low
			2 > 50%	2: moderate	4: moderate	4: moderate
				3: strong	5: high	5: high
PN	tumoral cells	cytoplasmic	0 < 10%	1: weak	0 – 3: low	0 – 3: low
			1: 10–30%	2: moderate	4 – 6: high	4 – 6: high
			2: 31–60%	3: strong		
			3 > 60^			
	tumoral stroma	connective tissue	0 < 5%		0: negative	0: negative
			1 > 5%		1: positive	1: positive

Statistical analyses

Statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) *v.* 19 program (SPSS Inc., IBM Corporation, Chicago, IL, USA) and the χ^2 (chi-square) test (Maximum-Likelihood, Yates, Mantel–Haenszel). Statistical significance was considered for $p<0.05$.

4.3. RESULTS

4.3.1. HER-2/neu assessment in PTC

Clinicopathological features

The main clinicomorphological parameters that characterized G1 group are summarized in Table 4.3.

Table 4.3. The clinicomorphological parameters in G1 group

Variables	Number #	Percent %
Gender		
Female	93	77.5%
Male	27	22.5%
Age at time of diagnosis		
Mean age	53.11±14.87	
Mean tumoral diameter	27.06±20.34 mm	
Histological variants		
Low risk group	**73**	**60.83%**
Conventional	16	13.33%
Follicular	8	6.66%
Oncocytic	25	20.83%
PTMC	21	17.5%
Clear cell	3	2.5%
High risk group	**47**	**39.17%**
Columnar cell	3	2.5%
Tall Cell	15	12.5%
Cribriform	6	5%
Hobnail	1	0.83%
Diffuse sclerosing	5	4.16%
Solid	6	5%
Follicular angioinvasive	7	5.83%
Conventional with dedifferentiation to squamous cell carcinoma	3	2.5%
Oncocytic with undifferentiated solid areas	1	0.83%
Extrathyroid extension		
Yes	82	68.33%
No	38	31.67%
Focality		
Unifocal	70	58.34%
Multifocal	50	41.66%
Lymph-vascular invasion		
Yes	62	51.66%
No	58	48.34%
Lymph node metastases (68 lymphadenectomies)		
Yes	32	47.05%
N1a	21	30.88%
N1b	11	16.17%
No	36	52.95%
T stages		
T1	22	18.33%
T2	8	6.66%
T3	82	68.33%
T4	8	6.66%

HER-2/neu expression

HER-2/neu expression assessed with score values between 1 and 4, considered negative, was heterogeneous, weak, with cytoplasmic location and granular pattern, visible in the apical and lateral domains of the tumor follicular cells (Figure 4.1). In cases scored between 5 and 8, also considered negative, HER-2/neu expression was also heterogeneous, but moderate as staining intensity, with granular and diffuse cytoplasmic and discontinuous

membranous patterns (Figures 4.2 – 4.4). Cases scored between 9 and 12, considered positive, showed strong immunostaining of tumor cells, with both cytoplasmic and continuous, complete membranous distribution (Figures 4.5 – 4.6).

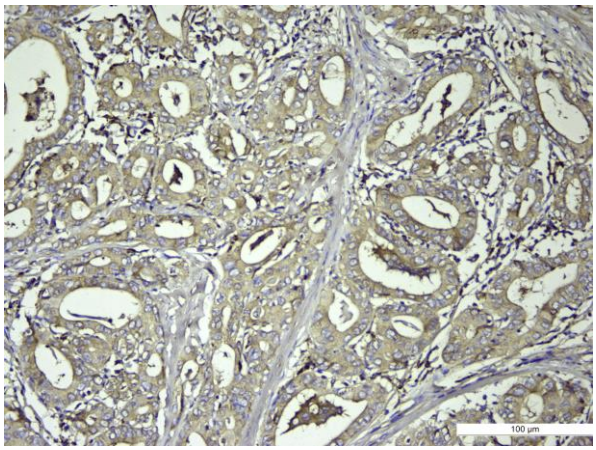


Figure 4.1. PTC, follicular variant: weak cytoplasmic granular pattern of HER-2/neu (IHC, anti-Her2, x 200)

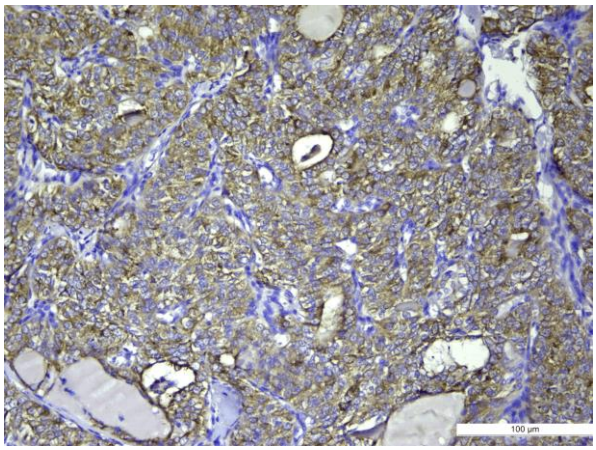


Figure 4.2. PTC, follicular variant: moderate staining with predominant cytoplasmic pattern and focal membranous immunoreactivity (IHC, anti-Her2, x 200)

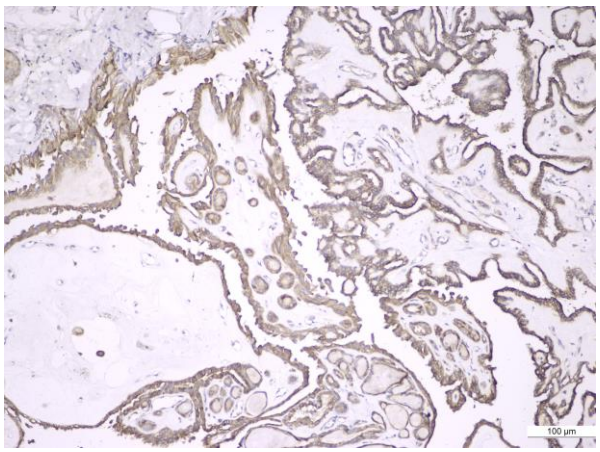


Fig.4.3. PTC, conventional variant: moderate HER-2/neu staining with predominant cytoplasmic and focal membranous immunoreactivity (IHC, anti-Her2, x 100)

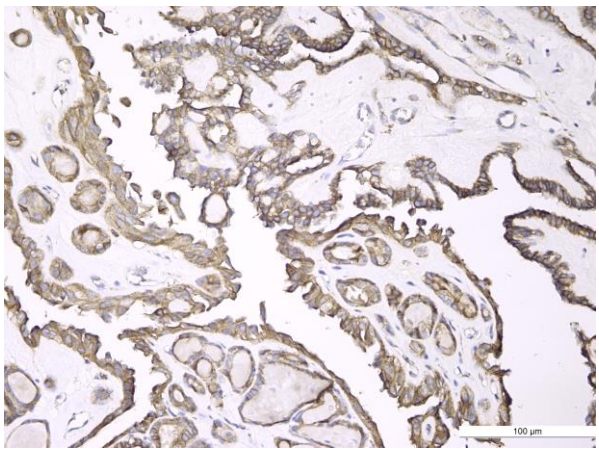


Fig. 4.4. PTC, conventional variant: moderate HER-2/neu staining with predominant cytoplasmic and focal membranous immunoreactivity (IHC, anti-Her2, x 200)

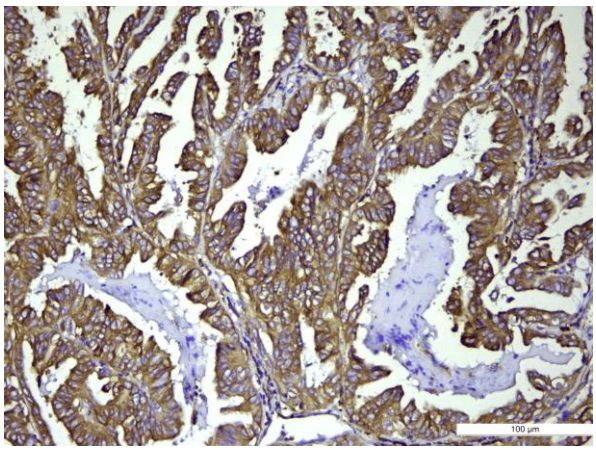


Figure 4.5. PTC, follicular variant: **s**trong HER-2/neu membranous and cytoplasmic pattern (IHC, anti-Her2, x 200)

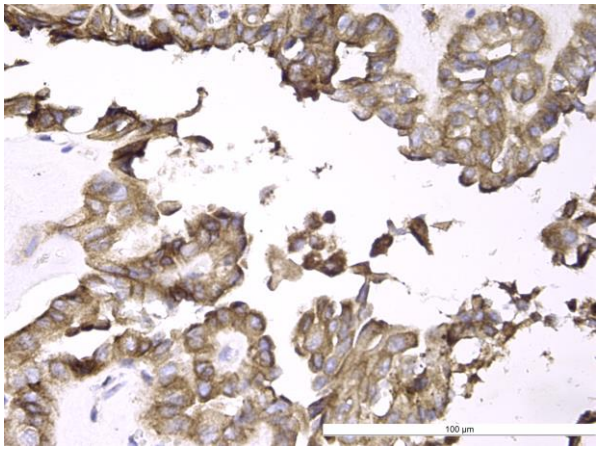


Fig. 4.6. PTC, conventional variant: strong HER-2/neu expression: cytoplasmic and membranous pattern (IHC, anti-Her2, x 400)

HER-2/neu positivity was noticed in 25 (20.8%) cases, from which 20 cases were classified as subtypes with low-risk and five with high-risk (Table 4.3). HER-2/neu was negative in 95 (79.2%) cases, distributed as follows: 53 cases as low-, and 42 cases, respectively, as high-risk subtypes (Table 4.4).

Table 4.4. HER-2/neu expression in G1 group with different subtypes of PTC

Histological subtypes	**HER-2/neu expression**	
	Positive (n=25)	***Negative (n=95)***
Low risk group	*20*	*53*
conventional (*n*=15)	5	11
follicular (*n*=8)	3	5
oncocytic (*n*=25)	6	19
papillary microcarcinoma (*n*=21)	6	15
clear cell (*n*=3)	0	3
High risk group	*5*	*42*
tall cell (*n*=15)	1	14
columnar cell (*n*=3)	0	3
cribriform morular (*n*=6)	0	6
diffuse sclerosing (*n*=5)	0	5
solid (*n*=6)	1	5
PTC with prominent hobnail features (*n*=1)	0	1
angioinvasive follicular (*n*=7)	1	6
conventional with dedifferentiation to squamous cell carcinoma (*n*=3)	2	1
oncocytic with undifferentiated solid areas (*n*=1)	0	1

4.3.2. Cell adhesion molecules in PTC

Clinicopathological features

The main clinicomorphological parameters that characterized G2 group are summarized in Table 4.5.

Table 4.5. The clinicomorphological parameters in G2 group

Variables	**Number #**	**Percent %**
Gender		
Female	55	78.57%
Male	15	21.42%
Age at time of diagnosis		
Mean age	49 yo	
< 55 yo	42	60%
> 55 yo	28	40%
Mean tumoral diameter	33.2 mm	
Histological variants		
Low risk group	**45**	**64.28%**
Conventional	16	22.85%
Follicular	9	12.85%
Macro-follicular	6	8.57%
Clear Cell	4	5.71%
Oncocytic	10	14.28%

Variables	Number #	Percent %
High risk group	**25**	**35.71%**
Tall Cell	8	11.42%
Cribriform	5	7.14%
Hobnail	1	1.42%
Diffuse sclerosing	3	4.28%
Solid	5	7.14%
Follicular angioinvasive	1	1.42%
Conventional with dedifferentiation to squamous cell carcinoma	1	1.42%
Oncocytic with undifferentiated solid areas	1	1.42%
Extrathyroidal extension		
Yes	49	70%
No	21	30%
Focality		
Unifocal	45	64.28%
Multifocal	25	35.72%
Lymph-vascular invasion		
Yes	46	65.71%
No	24	35.29%
Perineural invason		
Yes	12	17.14%
No	58	82.86%
Lymph node metastases (41 lymphadenectomies)		
Yes	22	53.65%
N1a	15	36.58%
N1b	7	17.07%
No		
T stages		
T1	18	25.71%
T2	27	38.57%
T3	25	35.71%
Relaps		
Yes	9	13%
No	61	87%

The association between the histological variants of PTC, stratified into low-risk and high-risk groups, and pT stages-revealed a relative equal distribution of the cases in low-risk group in all pT cathegory, whereas in high-risk group cases were diagnosed mainly in pT3 stage (Table 4.6).

Table 4.6. PTC histological variants and staging in G2 group

Histological variants – # (%)	T1 #	T2 #	T3 #
Low-risk group			
Conventional – 16 (22.85%)	6	4	6
Clear cell – 4 (5.71%)	0	3	1
Macrofollicular – 6 (8.57%)	4	2	0
Follicular – 9 (12.85%)	2	6	1
Oncocytic – 10 (14.28%)	3	4	3
High-risk group			
Conventional with squamous differentiation – 1 (1.42%)	0	0	1
Tall cell – 8 (11.42%)	2	5	1
Solid – 5 (7.14%)	0	0	5

Histological variants – # (%)	T1 #	T2 #	T3 #
Cribriform morular – 5 (7.14%)	1	0	4
Diffuse sclerosing – 3 (4.28%)	0	1	2
Follicular angioinvasive – 1 (1.42%)	0	1	0
Hobnail – 1 (1.42%)	0	0	1
Oncocytic with low-differentiated solid areas – 1 (1.42%)	0	1	0

E-cadherin, β-catenin and MOC31 expression

The intensity of E-cadherin membranous immune-expression was predominantly moderate or low in PTC, compared to the adjacent benign or normal thyroid tissue, strongly positive. Moderate membranous expression was observed not only in conventional and follicular subtypes, but also in some cases with a more aggressive course, like tall cell, cribriform and diffuse sclerosing variants. Low E-cadherin expression was noticed in PTC cases with high tumor extent (pT3) and lymph node metastases. In these cases, E-cadherin expression was heterogeneous, with 20–60% positive cells present in tumor areas.

Figures 4.7 - 4.9 illustrate different immunoexpression patterns of E-cadherin.

β-catenin immunostaining pattern in normal thyroid tissue or in the adjacent Hashimoto thyroiditis and nodular goiter was strong membranous, either circumferential in the cuboidal and columnar cells, either basal in the hypofunctional areas.

In PTC, *β*-catenin had a discontinuous membranous expression with the persistence of lateral membrane staining and the absence of immunostaining in apical and basal pole of the tumor cells. We also noted that in areas where the membranous staining was lacking, *β*-catenin was expressed in the cytoplasm of the tumor cells with moderate or even high intensity.

Some cases of conventional PTC preserved a strong, diffuse membranous staining, whereas in the follicular, tall cell and solid variants a large heterogenity was observed.

The nuclear staining of *β*-catenin was identified in PTC cribriform-morular variant in approximately 40% of the tumor cells together with moderate cytoplasmic expression and loss of the membranous one in more than 80% of the cells.

Figures 4.10 - 4.12 illustrate different immunoexpression patterns of *β*-catenin.

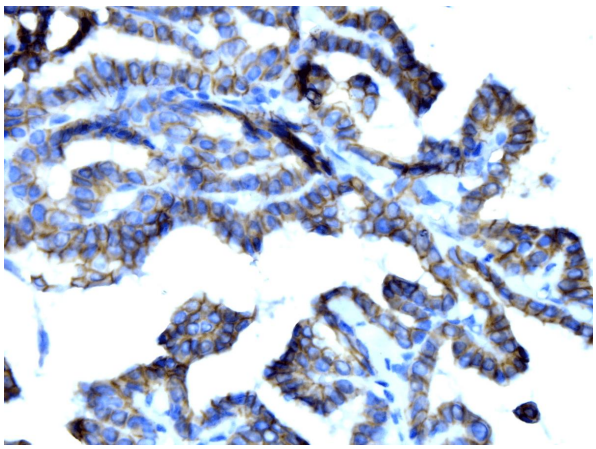


Figure 4.7. PTC, conventional variant: membranous E-cadherin expression with strong intensity (IHC, anti-E-cadherin, x 400)

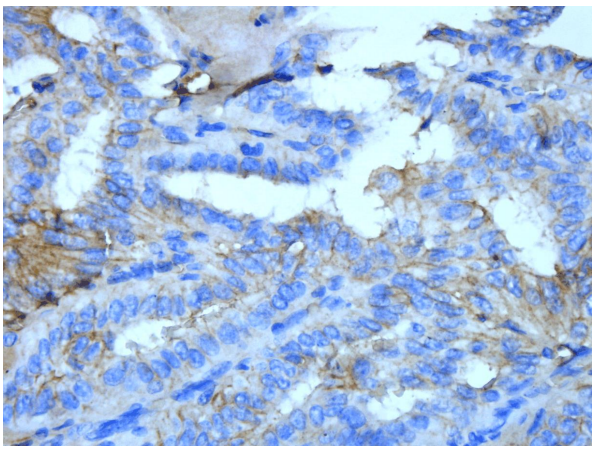


Figure 4.8. PTC, tall cell variant: low membranous E-cadherin expression with heterogeneous pattern (IHC, anti-E-cadherin, x 400)

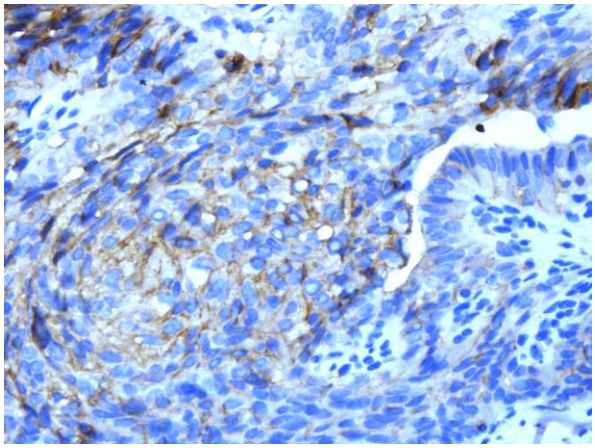


Figure 4.9. PTC, cribriform-morular variant: membranous E-cadherin expression with low intensity and heterogeneous pattern (IHC, anti-E-cadherin, x 400)

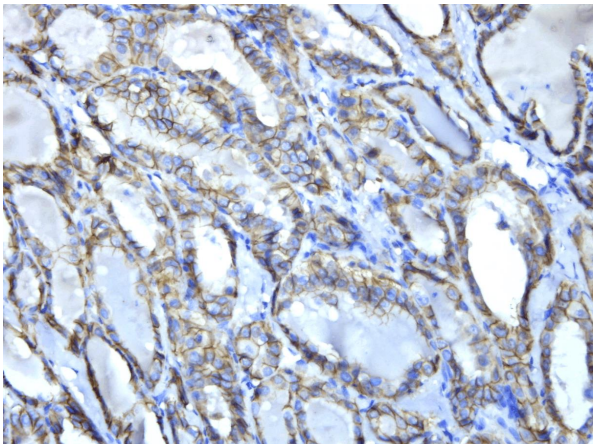


Figure 4.10. PTC, follicular variant: membranous β-catenin expression with moderate intensity and homogenous pattern (IHC, anti-β-catenin, x 100)

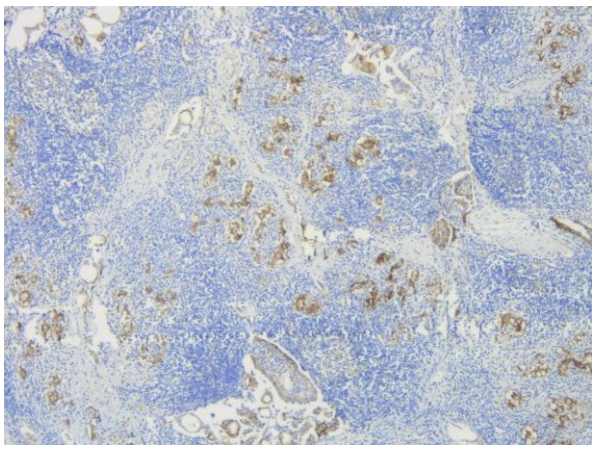


Figure 4.11. PTC, diffuse sclerosing variant: cytoplasmic β-catenin expression with high intensity (IHC, anti-β-catenin, x 50)

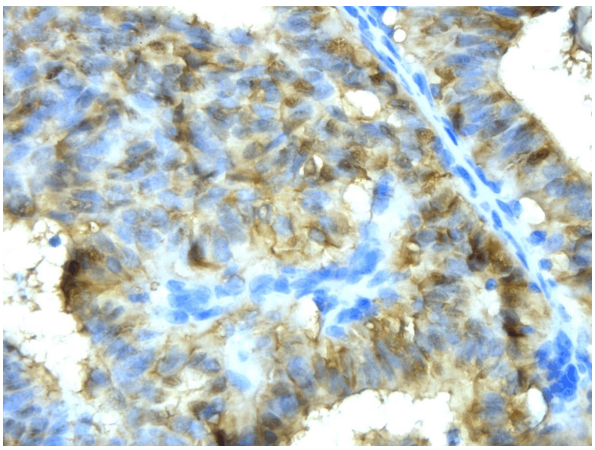


Figure 4.12. PTC, cribriform-morular variant: cytoplasmic β-catenin expression with moderate intensity, associated with isolate nuclear β-catenin immunostaining (IHC, anti-β-catenin, x 400)

In 65 of the 70 (93%) PTC cases, the MOC-31 expression was circumferentially membranous and cytoplasmic, with a fine granular aspect (Figures 4.13 – 4.17), whereas five (7%) PTC cases presented only circumferential membranous expression (Figure 4.18).

In the normal thyroid tissue and benign lesions adjacent to the tumor proliferation, MOC-31 showed strong, basolateral membranous immunoreaction, with negative cytoplasm.

The semi-quantitative assessment of MOC-31 in G2 group allowed the classification of cases as 47 (67%) cases with high score and 23 (33%) cases with low score.

All 47 cases presenting a MOC-31 high score showed both circumferential membranous and cytoplasmic expression in 30% to 100% of tumor cells, with moderate to strong intensity. 36 of 47 (77%) cases were comprised in the low-risk group (conventional, clear cells, follicular, macrofollicular, and oncocytic variants, including all PTMC), whereas 11 (23%) cases were comprised in the high-risk group (tall cell, cribriform morular, diffuse sclerosing, hobnail, follicular angioinvasive variants).

18 of 23 (78%) cases with MOC-31 low score were characterized by membranous circumferential and cytoplasmic expression, in 20–80% of tumor cells, with weak intensity of staining. Six (33.33%) cases were diagnosed as low-risk histological variants (clear cells, conventional, and follicular subtypes), the other 12 (66.66%) cases as high-risk histological subtypes (conventional with squamous differentiation, tall cell, solid and oncocytic with poorly differentiated solid areas subtypes). On the other hand, five of 23 (22%) cases with low score showed only membranous MOC-31 positivity in 9–40% of tumor cells, predominant with weak intensity. Three of these five cases belong to low-risk group (conventional, clear cell and follicular variants), whereas two cases – to high-risk group (solid and diffuse sclerosing subtypes). Considering both membranous and cytoplasmic expression with low score, nine cases have presented histological pattern classified as low-risk group, the remaining of 14 belonging to high-risk group.

Thus, the general image offered by this double framing (by score and risk category) revealed an association between low score and low risk in 80% of all cases, low score and high risk in 56% of the cases, high score and low risk in 36% of the cases and high score and high risk in 44% of the cases.

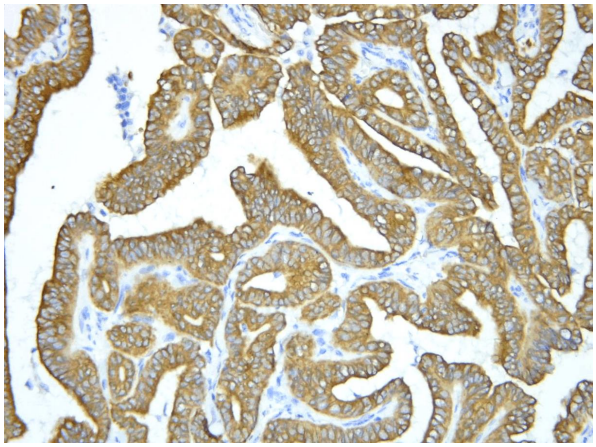


Figure 4.13. PTC, conventional variant: membranous and cytoplasmic MOC-31 expression (IHC, anti-MOC-31, x 200)

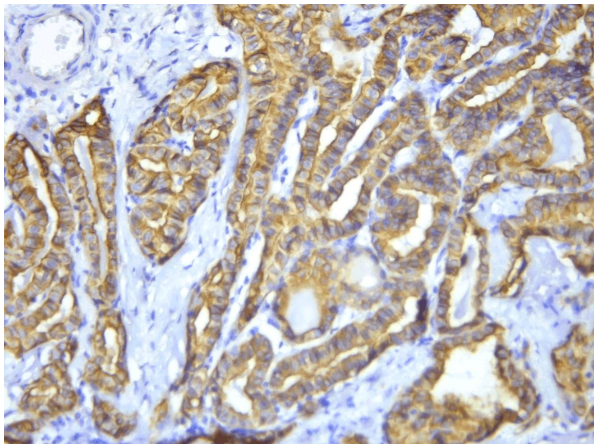


Figure 4.14. PTC, follicular variant: membranous and cytoplasmic MOC-31 expression (IHC, anti-MOC-31, x 200)

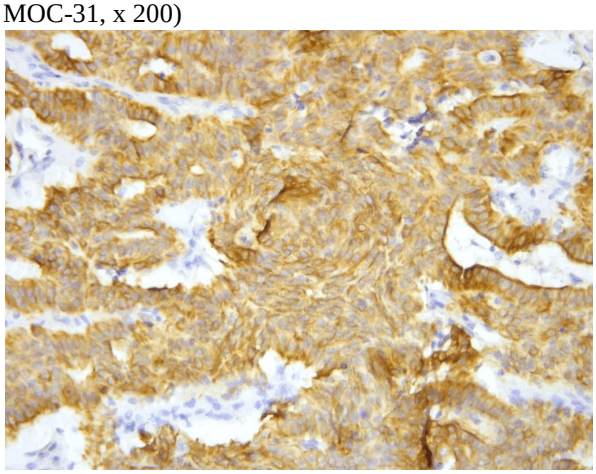


Figure 4.15. PTC, cribriform-morular variant: membranous and cytoplasmic MOC-31 expression (IHC, anti-MOC-31, x 200)

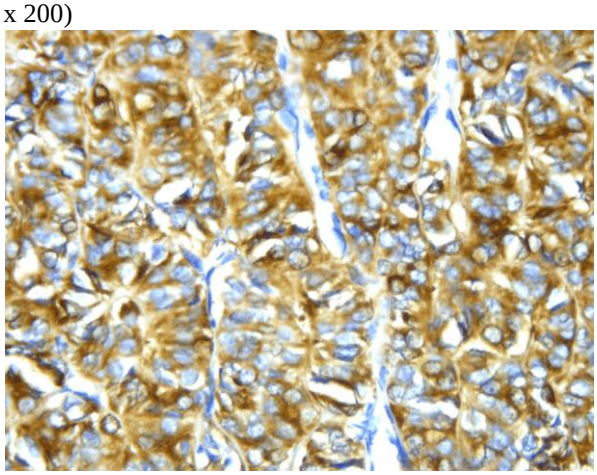


Figure 4.16. PTC, solid variant: membranous and cytoplasmic MOC-31 expression (IHC, anti-MOC-31, x 400)

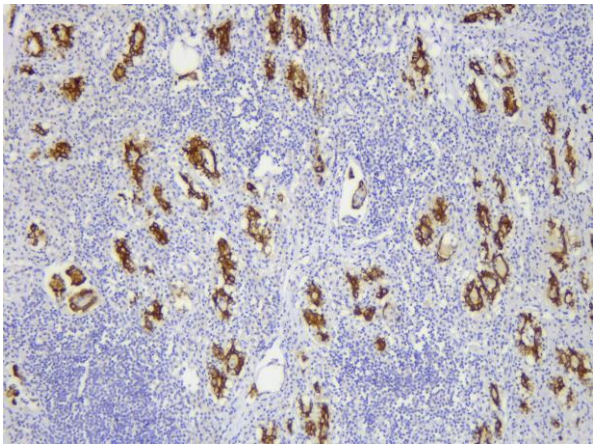


Figure 4.17. PTC, diffuse sclerosing variant: membra-nous and cytoplasmic MOC-31 expression (IHC, anti-MOC-31, x 100)

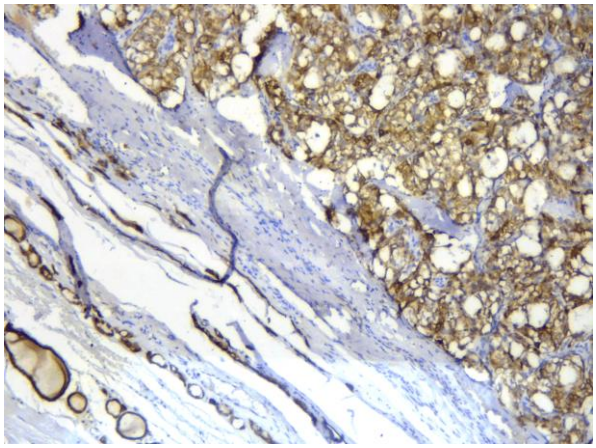


Figure 4.18. PTC, clear cell variant: membranous MOC-31 expression (IHC, anti-MOC-31, x 100)

4.3.3. β-tubulin 3 assessment in PTC

Clinicopathological features

The main clinicomorphological parameters that characterized G3 group are summarized in Table 4.7.

Table 4.7. The clinicomorphological parameters in G3 group

Variables	**Number #**	**Percent %**
Gender		
Female	53	75.72%
Male	17	24.28%
Age at time of diagnosis		
Mean age	49.27 ± 14.74 yo	
< 55 yo	44	62.86%
> 55 yo	26	37.14%
Mean tumoral diameter	32.32 ± 21.32 mm	
Histological variants		
Low risk group	**23**	**32.86%**
Conventional	16	22.86%
Follicular	7	10%
High risk group	**47**	**67.14%**
Columnar cell	3	4.28%
Tall cell	15	21.43%
Cribriform	6	8.57%
Hobnail	1	1.43%
Diffuse sclerosing	5	7.14%
Solid	6	8.57%
Follicular angioinvasive	7	10%
Conventional with dedifferentiation to squamous cell carcinoma	3	4.28%
Oncocytic with undifferentiated solid areas	1	1.43%
Extrathyroid extention		
Yes	55	78.57%
No	15	21.43%

Variables	Number #	Percent %
Focality		
Unifocal	36	51.43%
Multifocal	34	48.57%
Lymph-vascular invasion		
Yes	47	67.14%
No	23	32.86%
Lymph node metastases		
Yes	24	47.05%
N1a	13	18.57%
N1b	11	15.71%
No	46	52.95%
T stages		
T1	4	5.72%
T2	6	8.57%
T3	54	77.14%
T4	6	8.57%
Relaps		
Yes	6	8.57%
No	63	91.43%

TUBB3 (β-tubulin 3) expression

TUBB3 low expression was noted in 3 cases with low intensity of cytoplasmic staining and focal location in less than 50% of the tumor cells (Figure 4.19). A moderate expression was reported in 18 cases, where the cytoplasmic staining intensity was moderate in over 50% of tumor cells, present either focal or in a diffuse pattern in the specimens (Figure 4.20). The high expression was assessed in 49 cases; here, the immunoreaction had a strong and homogeneous intensity in almost 100% of the tumor cells (Figures 4.21 – 4.24).

In the adjacent benign thyroid tissue, TUBB3 was negative in all the cases with low and moderate TUBB3 expression in tumor cells, and in 43 out of 49 cases with high expression. Moreover, all relapse cases had negative TUBB3 expression.

Low and moderate expression of TUBB3 was observed in 11 (47.8%) cases of low-risk group and 10 (21.3%) cases of high-risk group. High expression of TUBB3 was observed in 12 (52.2%) cases from low-risk group and 37 (78.7%) cases of high-risk group.

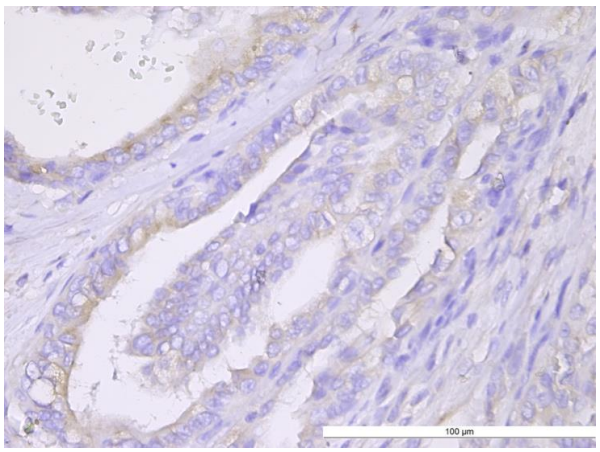


Figure 4.19. PTC, conventional subtype: focal cytoplasmic immunoreaction of low intensity (IHC, anti-TUBB3, x 400)

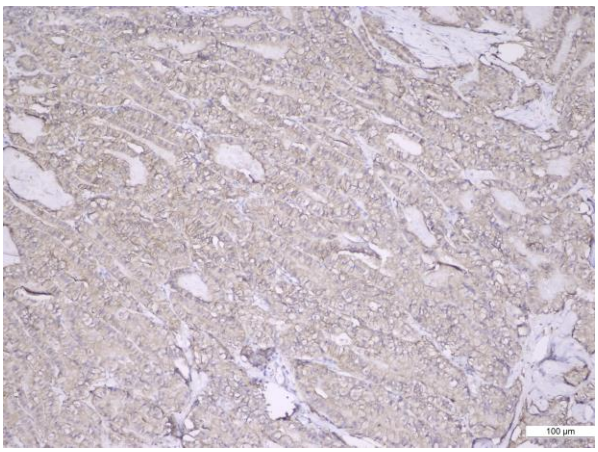


Figure 4.20. PTC, tallcell subtype: moderate cytoplasmic immunoreaction in over 50% of cells (IHC, anti-TUBB3, x 100)

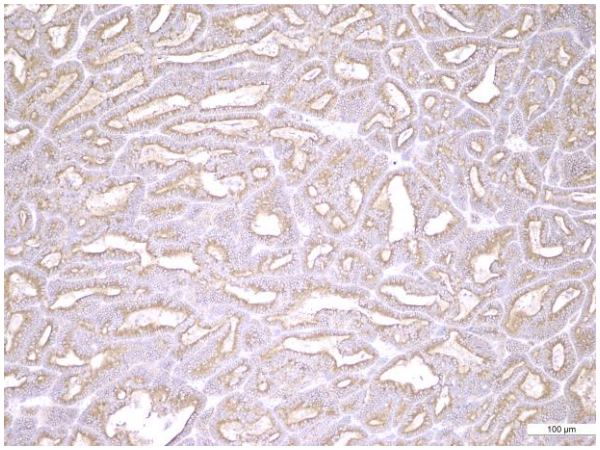


Figure 4.21. PTC follicular subtype: diffuse cytoplasmic immunoreaction of strong intensity (IHC, anti-TUBB3, x 100)

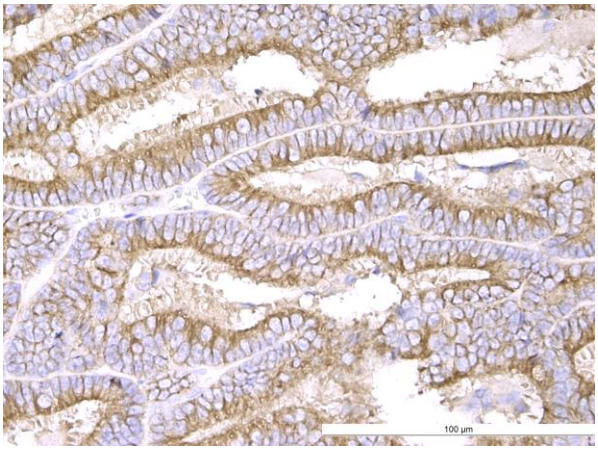


Figure 4.22. PTC follicular subtype: diffuse cytoplasmic immunoreaction of strong intensity (IHC, anti-TUBB3, x 400)

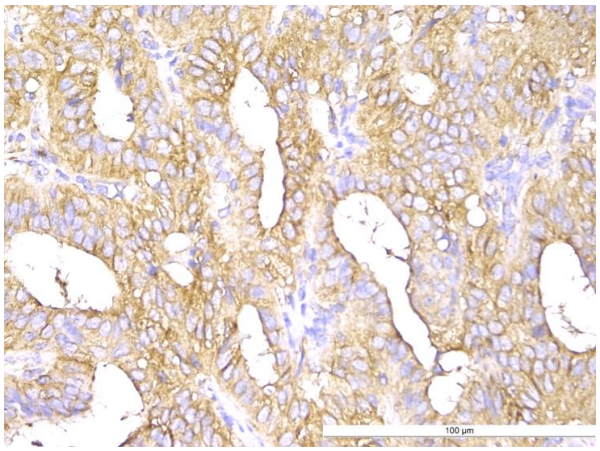


Figure 4.23. PTC follicular subtype: diffuse cytoplasmic immunoreaction of strong intensity (IHC, anti-TUBB3, x 200)

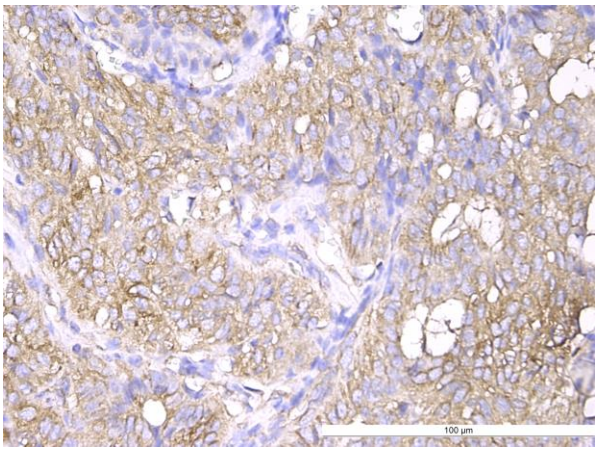


Figure 4.24. PTC follicular subtype: diffuse cytoplasmic immunoreaction of strong intensity (IHC, anti-TUBB3, x 400)

4.3.4. Periostin assessment in PTC

Clinicopathological features

The main clinicomorphological parameters that characterized G4 group are summarized in Table 4.8.

Table 4.8. The clinicomorphological parameters in G4 group

Variables	Number #	Percent %
Gender		
Female	41	82.0%
Male	9	18.0%
Age at time of diagnosis		

Variables	Number #	Percent %
Mean age	48.24±14.70 yo	
< 45 yo	21	42%
> 45 yo	29	58%
Mean tumoral diameter	21.8 ± 13.6 mm	
Histological variants		
Conventional	10	20%
Follicular	21	42%
Macro-follicular	7	14%
Oncocytic	8	16%
Tall Cell	4	8%
Extrathyroid extention		
Yes	23	46.0%
No	27	54.0%
Focality		
Unifocal	16	32%
Multifocal	34	68%
Lymph-vascular invasion		
Yes	14	28%
No	36	72%
Lymph node metastases		
Yes	7	14%
No	43	86%
Stages		
I	18	36%
II	6	12%
III	25	50%
IV	1	2%

PN expression

PN immunopositivity has been noticed both in tumor cells and intratumoral stroma. PN expression exhibited a predominantly cytoplasmic, perinuclear, finely granular pattern, in tumor cells. The distribution was predominantly homogenous; however some heterogenous areas were focally identified. The reaction intensity was predominantly moderate or strong.

The histological variants of PTC showed different patterns of PN immunoreaction.

The immunoexpression was diffusely cytoplasmic, with weak apical or basal polarization, in conventional (Figure 4.25), follicular, and macrofollicular (Figure 4.26) variants.

The tall cell variant was characterized by localized immunoexpression, preserving cell polarization, predominantly apical and also focally basal, infranuclear (Figure 4.27).

The immunoreaction was predominantly negative or very weak in oncocytic variant (Figure 4.28).

The intratumoral stromal PN expression was variable within the histological variants of PTC, from strong positivity in fibroblasts and collagen fibers up to lack of expression.

PN expression has been negative or weak, exhibiting a homogenous and diffuse cytoplasmic distribution in the follicular cells of non-neoplastic thyroid tissue.

Tumor cells PN expression has been evaluated as low score in 14 cases (28.0%) and with high score in 36 cases (72.0%).

Intratumoral stroma exhibited PN negativity or weak expression in 16 cases (32.0%), whereas the other 34 cases (68%) showed PN strong positivity.

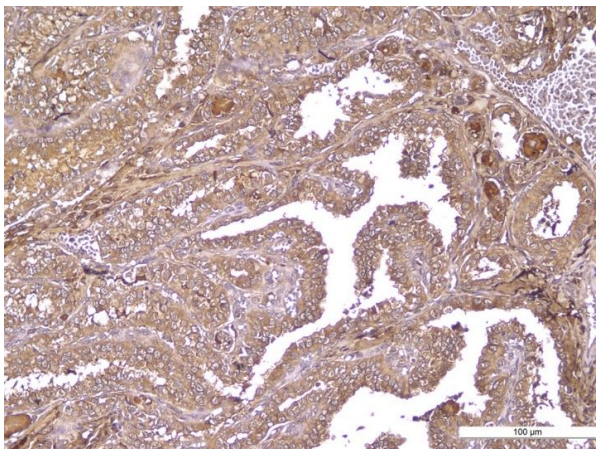


Figure 4.25. PTC, conventional subtype: positive PN staining in tumor cell with diffuse cytoplasmic positivity and moderate intensity and negative staining of PN in tumor stroma (IHC, anti-PN, x 200)

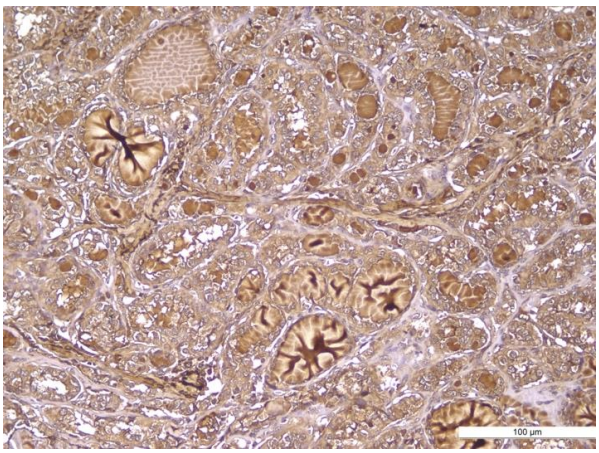


Figure 4.26. PTC, macrofollicular subtype: positive PN staining in tumor cells with diffuse cytoplasmic immunoreaction of moderate intensity, and negative staining of PN in tumor stroma (IHC, anti-PN, x 200)

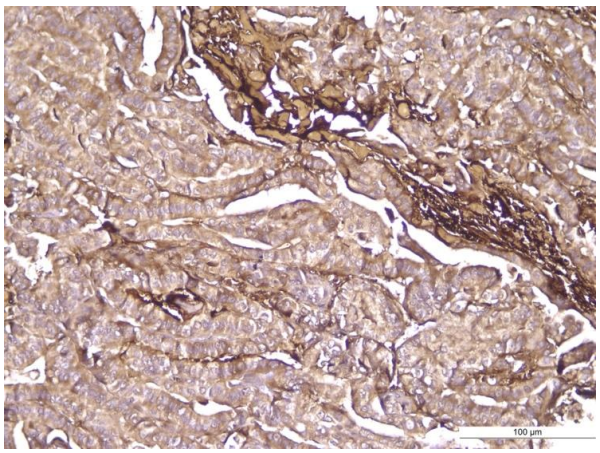


Figure 4.27. PTC, tall cell subtype: positive cytoplasmic PN, exhibiting focal apical and basal, moderate intensity immunoreaction, and positive PN in intratumoral stroma (IHC, anti-PN, x 200)

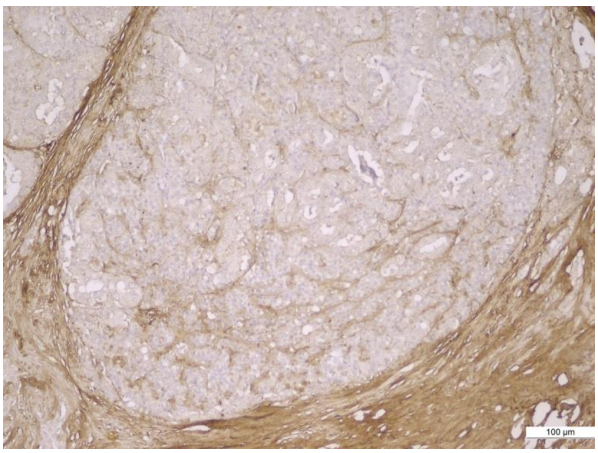


Figure 4.28. PTC, oncocytic subtype: negative PN expression in tumor epithelial cells and positive PN expression in tumor stroma (IHC, anti-PN, x 200)

4.3.5. Correlations between "candidate" biomarkers' expression and clinicopathological prognostic factors

The statistical analysis addressed the possible relationship between the immunohistochemical expression of the analyzed biomarkers and classical clinicopathological characteristics already defined for PTC.

HER-2/neu in G1 group

The statistical analysis revealed significant differences between HER-2/neu expression (positive *versus* negative) and histological subtypes, extrathyroidal extension, and tumor focality (Table 4.9). No significant correlation was found between HER-2/neu expression and age, gender, tumor size, lympho-vascular invasion, tumor stage, as well as lymph node metastases (Table 4.9).

Table 4.9. Relationship between HER-2/neu expression and clinicopathological characteristics in G1 group

Clinicopathological characteristics	HER-2/neu expression		*Chi*-square test
	Negative (n=95)	*Positive (n=25)*	
Age at diagnosis			
<55 years old	51 (85%)	9 (15%)	*p*=0.088
≥55 years old	44 (73.33%)	16 (26.66%)	
Gender			
males	18 (66.7%)	9 (33.33%)	*p*=0.064
females	77 (82.8%)	16 (17.2%)	
Tumor size (median)			
<22 mm	49 (79.03%)	13 (20.97%)	*p*=0.575
≥22 mm	46 (79.31%)	12 (20.69%)	
Histological subtype			
low-risk group	26 (61.9%)	16 (38.1%)	***p*=0.001***
high-risk group	69 (88.5%)	9 (11.5%)	
Lympho-vascular invasion			
absent	45 (77.59%)	13 (22.41%)	*p*=0.425
present	50 (80.65%)	12 (19.35%)	
Extrathyroidal extension			
absent	35 (92.1%)	3 (7.9%)	***p*=0.013***
present	60 (73.17%)	22 (26.83%)	
Focality of the tumor			
unifocal	61 (87.14%)	9 (12.86%)	***p*=0.011***
multifocal	34 (68%)	16 (32%)	
Tumor stage			
T1 + T2	26 (86.67%)	4 (13.33%)	*p*=0.183
T3 + T4	69 (76.67%)	21 (23.33%)	
Lymph node metastases			
N0	25 (69.44%)	11 (30.56%)	*p*=0.201
N1	26 (81.25%)	6 (18.75%)	

**p*-value <0.05 was considered to be statistically significant

E-cadherin and β-catenin in G2 group

The statistical analysis showed significant differences between the E-cadherin expression (low *versus* high) and tumor size (*p*=0.017), PTC risk groups (*p*=0.003), tumor stage (*p*=0), lymph node metastases (*p*=0.001), vascular invasion (*p*=0) and tumor relapse (*p*=0.005). No correlation of E-cadherin expression with age, gender, multifocality and extra-thyroidal invasion was found (Table 4.10).

Table 4.10. Relationship between E-cadherin expression and clinicopathological characteristics in G2 group

Clinicopathological characteristics	E-cadherin membranous expression		*Chi-square test*
	Low (n=42)	*High (n=28)*	
Age at diagnosis			
<55 years old	15 (35.7%)	27 (64.3%)	*p=1*
≥55 years old	10 (35.7%)	18 (64.3%)	
Gender			
Female	20 (36.4%)	35 (63.6%)	*p=0.828*
Male	5 (33.3%)	10 (66.7%)	
Tumor size (median)			
<30 mm	6 (20%)	24 (80%)	***p=0.017****
≥30 mm	19 (47.5%)	21 (52.5%)	
Histopathological subtype			
Low-risk group	11 (22.7%)	34 (77.3%)	***p=0.003****
High-risk group	15 (57.7%)	10 (42.3%)	
Focality of the tumor			

Unifocal	15 (33.3%)	30 (66.7%)	*p=0.577*
Multifocal	10 (40%)	15 (60%)	
Tumor stage			
T1 + T2	8 (17.8%)	37 (82.2%)	***p=0.001****
T3	17 (68%)	8 (32%)	
Lymph node metastases			
N0	3 (15.8%)	16 (84.2%)	***p=0.001****
N1	15 (68.2%)	7 (31.8%)	
Lympho-vascular invasion			
Absent	1 (4.2%)	23 (95.8%)	***p=0.001****
Present	24 (52.2%)	22 (47.8%)	
Extrathyroidal invasion			
Absent	5 (23.8%)	16 (76.2%)	*p=0.174*
Present	20 (40.8%)	29 (59.2%)	
Tumor relapse			
Absent	18 (29.5%)	43 (70.5%)	***p=0.005****
Present	7 (77.8%)	2 (22.2%)	

*p-value <0.05 was considered to be statistically significant

The membranous *β*-catenin expression (low *versus* high) was statistically significant correlated with PTC risk groups, tumor size and tumor stage (Table 4.11). No statistically significant differences were found between cytoplasmic *β*-catenin (low *versus* high) and the other clinicopathological characteristics (Table 4.11).

Table 4.11. Relationship between β-catenin expression and clinicopathological characteristics in G2 group

Clinicopathological characteristics	***β*-Catenin membranous expression**		***Chi-square test***	***β*-Catenin cytoplasmic expression**		***Chi-square test***
	Low (n=42)	***High (n=28)***		***Low (n=36)***	***High (n=34)***	
Age at diagnosis						
<55 years old	25 (59.5%)	17 (40.5%)	*p=0.921*	20 (47.6%)	22 (52.4%)	*p=0.473*
≥55 years old	17 (60.7%)	11 (39.3%)		16 (57.1%)	12 (42.9%)	
Gender						
Female	31 (56.4%)	24 (43.6%)	*p=0.234*	26 (47.3%)	29 (52.7%)	*p=0.247*
Male	11 (73.3%)	4 (26.7%)		10 (66.7%)	5 (33.3%)	
Tumor size (median)						
<30 mm	16 (47.1%)	18 (52.9%)	***p=0.032****	16 (47.1%)	18 (52.9%)	*p=0.633*
≥30 mm	26 (72.2%)	10 (27.8%)		20 (55.6%)	16 (44.4%)	
Histopathological subtype						
Low-risk group	23 (51.1%)	22 (48.9%)	***p=0.042****	26 (59.1%)	18 (40.9%)	*p=0.095*
High-risk group	19 (76%)	6 (24%)		10 (38.5%)	16 (61.5%)	
Focality of the tumor						
Unifocal	26 (54.2%)	22 (45.8%)	*p=0.141*	27 (56.3%)	21 (43.7%)	*p=0.305*
Multifocal	16 (72.7%)	6 (27.3%)		9 (40.9%)	13 (59.1%)	
Tumor stage						
T1 + T2	24 (51.1%)	23 (48.9%)	***p=0.029****	24 (51.1%)	23 (48.9%)	*p=0.93*
T3	18 (78.3%)	5 (21.7%)		12 (52.2%)	11 (48.8%)	
Lymph node metastases						
N0	14 (73.7%)	5 (26.3%)	*p=0.121*	12 (63.2%)	7 (36.8%)	*p=0.257*
N1	11 (50%)	11 (50%)		10 (45.5%)	12 (54.5%)	
Lympho-vascular invasion						
Absent	14 (51.9%)	13 (48.1%)	*P=0.27*	15 (55.6%)	12 (44.4%)	*p=0.63*
Present	28 (65.1%)	15 (34.9%)		21 (48.8%)	22 (51.2%)	
Extrathyroidal invasion						
Absent	12 (57.1%)	9 (42.9%)	*P=0.749*	9 (42.9%)	12 (57.1%)	*P=0.437*
Present	30 (61.2%)	19 (38.8%)		27 (55.1%)	22 (44.9%)	
Tumor relapse						

Clinicopathological characteristics	*β*-Catenin membranous expression		*Chi-square test*	*β*-Catenin cytoplasmic expression		*Chi-square test*
	Low (n=42)	*High (n=28)*		*Low (n=36)*	*High (n=34)*	
Absent	36 (59%)	25 (41%)	*P=0.662*	29 (47.5%)	32 (52.5%)	*P=0.152*
Present	6 (66.7%)	3 (33.3%)		7 (77.8%)	2 (22.2%)	

*p-value <0.05 was considered to be statistically significant

MOC-31 in G2 group

The statistical analysis showed significant differences between MOC-31 expression (low *versus* high) and tumor size (p=0.047), PTC risk group (p=0.002) and tumor relapse (p=0.02). No correlation of MOC-31 expression with age, gender, multifocality, tumor stage, lymph node metastases, vascular invasion and histopathological subtypes was found (Table 4.12).

Table 4.12. Relationship between MOC-31 expression and clinicopathological characteristics in G2 group

Clinicopathological characteristics	MOC-31 membranous/cytoplasmic expression		*Chi-square test*
	Low (n=23) 33%	*High (n=47) 67%*	
Age at diagnosis			
<55 years old	13 (30.9%)	29 (69.1%)	p=0.677
≥55 years old	10 (35.7%)	18 (64.3%)	
Gender			
Female	18 (32.7%)	37 (67.3%)	p=0.964
Male	5 (33.3%)	10 (66.7%)	
Tumor size (median)			
<30 mm	6 (20%)	24 (80%)	**p=0.047***
≥30 mm	17 (42.5%)	23 (57.5%)	
Risk-group			
Low-risk group	9 (20%)	36 (80%)	**p=0.002***
High-risk group	14 (56%)	11 (44%)	
Focality of the tumor			
Unifocal	15 (33.3%)	30 (66.7%)	p=0.909
Multifocal	8 (32%)	17 (68%)	
Tumor (T) stage			
T1 + T2	13 (28.8%)	32 (71.2%)	p=0.342
T3	10 (40%)	15 (60%)	
Lymph node (N) metastases			
N0	8 (42.1%)	11 (57.9%)	p=0.495
N1	7 (31.8%)	15 (68.2%)	
Lympho-vascular invasion			
Absent	6 (25%)	18 (75%)	p=0.312
Present	17 (36.9%)	29 (63.1%)	
Capsular invasion			
Absent	8 (38%)	13 (62%)	p=0.541
Present	15 (30.6%)	34 (69.4%)	
Tumor relapse			
Absent	17 (28%)	44 (72.2%)	**p=0.02***
Present	6 (67%)	3 (33.4%)	
Histopathological variants			
Conventional	3	13	p=0.171
Other variants	20	34	

*p-value <0.05 was considered to be statistically significant

TUBB3 (β-tubulin 3) in G3 group

The statistical analysis revealed significant differences between TUBB3 expression (low and moderate *versus* high) and histological subtypes defined as risk PTC subgroups, and tumor relapse (Table 4.13). Our results showed no correlation between TUBB3 expression and age, gender, tumor size, tumor focality, lympho-vascular invasion, extrathyroidal extension, lymph node metastases and tumor stage (Table 4.13).

Table 4.13. Relationship between TUBB3 expression and clinicopathological characteristics in G3 group

Clinicopathological characteristics	**TUBB-3 cytoplasmic expression**		***Chi-square test***
	low and moderate (n=21)	High (n=49)	
Age at diagnosis			
< 55 years old	12 (27.3%)	32 (72.7%)	*p=0.517*
≥ 55 years old	9 (34.6%)	17 (65.4%)	
Gender			
male	3 (17.6%)	14 (82.4%)	*p=0.201*
female	18 (34%)	35 (66%)	
Tumor size *(median)*			
< 25 mm	8 (25%)	24 (75%)	*p=0.402*
≥ 25 mm	13 (34.2%)	25 (65.8%)	
Histopathologic subtype			
low risk group	11 (47.8%)	12 (52.2%)	***p=0.023****
high risk group	10 (21.3%)	37 (78.7%)	
Focality of the tumor			
unifocal	8 (22.2%)	28 (77.8%)	*p=0.144*
multifocal	13 (38.2%)	21 (61.8%)	
Lympho-vascular invasion			
absent	4 (17.4%)	19 (82.6%)	*p=0.107*
present	17 (36.2%)	30 (63.8%)	
Extrathyroidal extension			
absent	3 (20%)	12 (80%)	*p=0.340*
present	18 (32.7%)	37 (67.3%)	
Tumor stage			
T1 + T2	1 (10%)	9 (90%)	*p=0.136*
T3 + T4	20 (33.3%)	40 (66.7%)	
Lymph node metastases			
N0	7 (41.2%)	10 (58.8%)	*p=0.158*
N1	5 (20.8%)	19 (79.2%)	
Tumor relapse			
absent	16 (25.8%)	46 (74.2%)	***p=0.033****
present	5 (62.5%)	3 (37.5%)	

*p-value <0.05 was considered to be statistically significant

PN in G4 group

Statistically significant differences were registered only between epithelial PN immunoreaction (low *versus* high) and PTC histological variants (p = 0.0002). A high PN score was more frequently noted in conventional subtype than in oncocytic subtype (OD = 105, CI 3.73-2948.28, p = 0.0062) (Table 4.14).

Our results show significant differences between stromal PN immunoreaction (negative *versus* positive) and tumor stage (p = 0.04), and extrathyroidal extension (p = 0.008). Moreover, a high PN score was more frequently observed in advanced tumor stage

(OR 0.28, 95%, CI 0.07-0.99; p=0.0491) and in the occurrence of extrathyroidal extension (OR 0.16, CI 0.03-0.67, p = 0.0124) (Table 4.15). We have also noted a very close value to the statistical significant p value for the lymph node metastasis.

Table 4.14. PN expression in tumor epithelial cells and clinicopathological characteristics of PTC in G4 group

Clinicopathologic characteristics	Case number		PN expression				Chi-square test	OR (95% CI)
			Low score		High score			
	#	%	#	%	#	%	p *value*	
Gender								
Female	41	82	12	29.27	29	70.73	p=0.6699	0.69 (0.12-3.81)
Male	9	18	2	22.22	7	77.78		
Age								
< 45	21	42	7	33.33	14	66.67	p=0.4748	0.63 (0.18-2.20)
> 45	29	58	7	24.14	22	75.86		
< 55	28	56	9	32.14	19	67.86	p=0.537	0.65 (0.45-5.75)
> 55	22	44	5	22.73	17	77.27		
Tumor stage								
STAGE I, II	24	48	7	29.17	17	70.83	p=0.8599	0.89 (0.26-3.07)
STAGE III, IV	26	52	7	26.92	19	73.08		
Histologic subtype								
Conventional	10	20	0	0	10	100	**p=0.0002**	10.86 (0.55-211.91)
Follicular	21	42	7	33.33	14	66.67		
Macrofollicular	7	14	0	0	7	100		1.4 (0.02-78.80)
Tall cells	4	8	0	0	4	100		1.00 (0.24-4.13)
Oncocytic	8	16	7	87.5	1	12.5		105.00 (3.73-2948.28)
Multifocality								
yes	34	68	10	29.41	24	70.59	p=0.7459	0.8 (0.20-3.08)
no	16	32	4	25	12	75		
Tumor size								
< 2.18 cm	35	70	10	28.57	25	71.43	p=0.8907	0.90 (0.23-3.53)
>2.18 cm	15	30	4	26.67	11	73.33		
Lympho-vascular invasion								
absent	36	72	12	33.33	24	66.67	p=0.1780	0.33 (0.06-1.73)
present	14	28	2	14.29	12	85.71		
Lymph node metastasis								
absent	43	86	12	27.91	31	72.09	p=0.9710	1.03 (0.17-6.06)
present	7	14	2	28.57	5	71.43		
Extrathyroidal invasion								
absent	27	54	8	29.63	19	70.37	p=0.7810	0.83 (0.24-2.90)
present	23	46	6	26.09	17	73.91		

χ^2: chi-square test; OR: odd ratio; CI: confidence interval

Table 4.15. PN expression in intratumor stroma and clinicopathological characteristics of PTC in G4 group.

Clinicopathologic features	Case number		PN expression				Chi-square test	OR (95% CI)
			Low score		High score			
	#	%	#	%	#	%	p *value*	
Gender								
Female	41	82	13	31.71	28	68.29	p=0.9246	1.07 (0.23-4.99)
Male	9	18	3	33.33	6	66.67		
Age								
< 45	21	42	5	23.81	16	76.19	p=0.2907	1.05 (0.55-6.84)
> 45	29	58	11	37.93	18	62.07		
< 55	28	56	7	25	21	75	p=0.536	0.62 (0.17-1.97)
> 55	22	44	9	40.91	13	59.09		
Tumor stage								
Stage I, II	24	48	11	45.83	13	54.17	**p=0.0439**	0.28 (0.07-0.99)
Stage III, IV	26	52	5	19.23	21	80.77		
Histologic subtype								
Conventional	10	20	5	50	5	50	p=0.7522	0.40 (0.08-1.90)
Folicular	21	42	6	28.57	15	71.43		
Macrofolicular	7	14	2	28.57	5	71.43		1.00 (0.15-6.64)
Tall cells	4	8	1	25	3	75		3.00 (0.22-39.60)
Oncocytic	8	16	2	25	6	75		1.20 (0.12-11.86)
Multifocality								
no	34	68	13	38.24	21	61.76	p=0.1683	0.37 (0.08-1.56)
yes	16	32	3	18.75	13	81.25		
Tumor size								
< 2.18 cm	35	70	9	25.71	26	74.29	p=0.1455	2.52 (0.71-8.96)
>2.18 cm	15	30	7	46.67	8	53.33		
Lympho-vascular invasion								
absent	36	72	13	36.11	23	63.89	p=0.3176	0.48 (0.11-2.04)
present	14	28	3	21.43	11	78.57		
Lymph node metastasis								
absent	43	86	16	37.21	27	62.79	p=0.0503	0.11 (0.006-2.07)
present	7	14	0	0	7	100		
Extrathyroidal invasion								
absent	27	54	13	48.15	14	51.85	**p=0.008**	0.16 (0.03-0.67)
present	23	46	3	13.04	20	86.96		

χ^2: chi-square test; OR: odd ratio; CI: confidence interval

4.4. DISCUSSIONS

Even though the prognostic assessment of PTC, according to WHO, relies on the standard clinicomorphological factors, nowadays the pathologists look on the "candidate" prognosis markers and try to validate more sensitive criteria for the assessment of neoplastic

evolution. These markers could be related to the carcinogenesis mechanism, their distinctive involvement controlling the subtle differences in prognostic. The endeavor of this approach could be the identification within the same histological phenotype of new subclasses of diagnosis (at present just predicted in an intuitive way, in relationship with the tumor behavior), characterized by molecular features.

Her2-neu in PTC

HER-2/neu as an essential marker in the molecular classification of breast cancer [Perou et al., 2000] is overexpressed in 15–30% of invasive forms [Burstein et al., 2005] and its role of prognostic and predictive factor is already accepted. Extensive researches demonstrated that HER-2/neu overexpression correlates with the disease stage, number of metastatic axillary lymph nodes, histological type, absence of estrogen and progesterone receptors and recurrence risk [Iqbal et al., 2014]. Consequently, the value of HER-2/neu as a therapeutic target reformed the breast cancer treatment, improving the clinical outcome [O'Sullivan et al., 2013; Kim et al., 2014].

Starting with the '90s, several studies analyzed the HER-2/neu expression in ovary [Berchuck et al., 1990], gastric, colonic and esophageal [Reichelt et al., 2007; Cervera et al., 2011; Ieni et al. 2013; Ieni et al., 2014; Ieni et al., 2015], endometrial [Buza et al., 2014], lung [Heinmöller et al., 2003; Stephens et al., 2004], and bladder [Coogan et al., 2004] tumors. These studies sustain that HER-2/neu overexpression is associated with a more aggressive disease, incomplete response to primary therapy and worse overall survival – particularly in ovarian cancer [English et al., 2013].

The mechanism underlying the specific role of HER-2/neu in the thyroid carcinogenesis is still unknown. A large heterogeneity in HER-2/neu expression, varying between 0% and 79%, is reported [Lemoine et al., 1990; Haugen et al., 1992; Utrilla et al., 1999; Kremser et al., 2003; Mondi et al., 2003; Sugishita et al., 2013; Mdah et al., 2014; Rakha et al., 2014; Ruggeri et al., 2016; Siraj et al., 2017].

The most studied histological type is the PTC, followed by follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC), and anaplastic thyroid carcinoma (ATC), with contradictory and confusing results. The following three examples are relevant in support of this remark. Ruggeri et al. show a significantly higher HER-2/neu expression rate in the FTC compared to PTC [Ruggeri et al., 2016], whereas Utrilla et al. [Utrilla et al., 1999] and Mdah et al. [Mdah et al., 2014] report HER-2/neu positivity in 52%, respectively 6.9% of the analyzed PTC cases, but no expression in FTC. These differences are coming from the great variability of the size and general characteristics of the studied groups, and from the discrepancies in the methods used for the HER-2/neu scoring, including the subjectivity of evaluation [Lemoine et al., 1990; Haugen et al., 1992; Sugg et al., 1998; Utrilla et al., 1999; Kremser et al., 2003; Mondi et al., 2003; Elliott et al., 2008; Qin et al., 2012; Sugishita et al., 2013; Wu et al., 2013; Mdah et al., 2014; Ruggeri et al., 2016]. There is no agreement for HER-2/neu scoring in thyroid [Siraj et al., 2017]. An important aspect is the cytoplasmic staining, frequently considered as positive immunoreaction in the HER-2/neu assessment [Lemoine et al., 1990; Sugg et al., 1998; Utrilla et al., 1999; Kremser et al., 2003; Wu et al., 2013]. However, the cytoplasmic immunoreactivity of HER-2/neu, possible due to the presence of a distinct protein in the mitochondrial cristae [De Potter et al., 1989], is not yet explained. Therefore, other studies use the updated ASCO–CAP (*American Society of Clinical Oncology*/*College of American Pathologists*) scoring system applied in breast cancer [Rakha et al., 2014; Ruggeri et al., 2016].

The controversial results lead to the lack of consensus regarding the HER-2/neu involvement, as prognostic and predictive factor and also as therapeutic target, in TC in general and PTC in particular [Mdah et al., 2014; Ruggeri et al., 2016; Siraj et al., 2017].

However, published data show a relationship between HER-2/neu and BRAFV600E mutation in familial PTC with aggressive behavior [Caria et al., 2016], and sustain the HER-2/neu overexpression even in the absence of the gene amplification [Siraj et al., 2017].

The present work focused on the HER-2/neu expression in different histological subtypes of PTC and its relationship with classical clinicopathological prognostic factors. In our series, we obtained HER-2/neu positivity in 20.8%, similar to the results reported in other previous papers – namely 18% [Ruggeri et al., 2016], 19.7% [Siraj et al., 2017], 23% [Sugishita et al., 2013], and 28% [Mohapatra et al., 2019].

To the best of our knowledge, from today there are just a few researches in the field about the particular aspects of HER-2/neu expression in rare variants of PTC associated with an aggressive course, except for tall cell [Siraj et al., 2017; Mohapatra et al., 2019]. Therefore, it is worth to mention that in our study HER-2/neu overexpression was found mainly in non-aggressive histological subtypes of PTC (20 of the total number of 25 cases) in comparison with the aggressive ones. This observation complements the present data on HER-2/neu expression in PTC, and sustains the association between HER-2/neu and histological subtypes with good prognosis. Our results overlap with those founded by Mohapatra et al. who showed a majority HER-2/neu positivity (3+) to low-risk PTC group [Mohapatra et al., 2019].

The role of HER-2/neu as prognostic factor falls in the same framework of controversies. Several studies report no HER-2/neu association with age, gender, tumor size [Mdah et al., 2014; Ruggeri et al., 2016; Kim et al., 2018; Mohapatra et al., 2019], tumor stage [Kremser et al., 2003; Mondi et al., 2003; Balta et al., 2012; Sugishita et al., 2013; Siraj et al., 2017], lymph node metastasis [Mondi et al., 2003; Balta et al., 2012; Sugishita et al., 2013; Mdah et al., 2014; Ruggeri et al., 2016; Siraj et al., 2017; Kim et al., 2018; Mohapatra et al., 2019], histological type [Kremser et al., 2003; Mondi et al., 2003; Balta et al., 2012; Sugishita et al., 2013, Mdah et al., 2014], extrathyroidal extension [Mondi et al., 2003; Sugishita et al., 2013], and patient survival [Siraj et al., 2017]. However, there are some positive results that sustain the association between the HER-2/neu in PTC and FTC and the distant metastasis [Kremser et al., 2003], extrathyroidal invasion and tumor recurrences [Akslen et al., 1993; Ruggeri et al., 2016].

Most of our results are similar with the negative findings of the above mentioned studies. We found no relationship between HER-2/neu expression and age, gender, tumor size, lympho-vascular invasion, tumor stage, and lymph node metastases. On the other hand, we demonstrate that HER-2/neu overexpression correlates with the histological subtypes with a better clinical course, with extrathyroidal extension, and tumor focality. Although statistical data do not confirm the usefulness of specific Herceptin therapy due to statistically significant results only for the low-risk PTC subgroup, mostly clinically indolent, association with multicentric tumors or possible local aggressive tumors with rapid extrathyroidian extension determines the opening of new perspectives of adapted therapies in these tumoral categories.

E-cadherin and β-catenin in PTC

The molecular structure of E-cadherin and its function are intimately related to *β*-catenin, their interactions ensuring the normal cell morphology and stability [Ozawa et al., 1989; McCrea et al., 1991; Lilien et al., 2005]. Alterations in E-cadherin lead to the increase of cytoplasmic *β*-catenin expression and, subsequently, to the amplification of transcription [Heuberger et al., 2010]. This phenomenon, regarded as a sequence of the carcinogenic mechanisms, facilitates the tumor growth and spreading. Starting from the ’90, several studies focus on E-cadherin changes in PTC and FTC and show a significant loss of its membranous expression in poorly and undifferentiated forms, respectively [Brabant et al.,

1993; Soares et al., 1997; von Wasielewski et al., 1997; Walgenbach et al., 1998; Naito et al., 2001; Kato et al., 2002; Brecelj et al., 2005; Choi et al., 2005]. Conversely, in benign thyroid lesions, a high expression of E-cadherin in thyroid cells is reported [Liu et al., 2015]. In PTC, the low E-cadherin expression is associated with tumor size, multifocality, capsular invasion, extrathyroidal extension, local recurrence and lymph nodes metastases [Rocha et al., 2003; Wiseman et al., 2006; Erdem et al., 2011; Sethi et al., 2011; Calangiu et al., 2014; Ceyran et al., 2015; Ivanova et al., 2017]. Consequently, the absence of E-cadherin could be regarded as a negative prognostic factor.

However, few papers analyze the E-cadherin profile in different histological subtypes of PTC, with limited results regarding conventional, follicular, tall cell and diffuse sclerosing variant, respectively [Rocha et al., 2001; Ito et al., 2008, Slowinska-Klencka et al., 2012; Calangiu et al., 2014; Ceyran et al., 2015]. These results indicate that the PTC variants associated with poor outcome present lower level of E-cadherin in comparison with the conventional and follicular subtypes, and also with minimally invasive FTC.

Until 2018, to the best of our knowledge, the differences in E-cadherin expression in all histological subtypes of PTC are still not established. Thus, our research brings new data on this topic, based on the particularities of the study group that includes a large variability of histological subtypes of PTC, divided in low- and high-risk groups. Our data show that the E-cadherin expression (low *versus* high) is significantly correlated with the histological risk groups. In our opinion, this assumption sustains the impact of the cellular pattern of PTC on the tumor behavior and the potential prognostic value of the histological variants. Moreover, the present study adds supplementary proofs that support the relationship between E-cadherin expression and tumor aggressiveness, reflected by tumor size, tumor stage, lymph node metastases, vascular invasion and tumor relapse, in concordance with the previous reported papers.

In TC, *β*-catenin is less studied than E-cadherin. A limited number of reports show a strong membranous *β*-catenin pattern in normal thyroid tissue or benign lesions, whereas in PTC, FTC and ATC the staining is heterogeneously positive in the plasma membrane, cytoplasm or nuclei [Böhm et al., 2000; Garcia-Rostan et al., 2001; Rocha et al., 2001; Ralhan et al., 2010]. The main types of TC present a significantly lower membranous *β*-catenin expression in comparison with the normal or benign thyroid, correlated with the tumor stage, extra-thyroidal extension and distant metastasis [Böhm et al., 2000; Garcia-Rostan et al., 2001]. In PDTC and ATC, nuclear *β*-catenin appears concomitantly with the loss of membranous expression [Garcia-Rostan et al., 2001; Ralhan et al., 2010; Lam et al., 2017]. This fact represents the hallmark for the activation of Wnt/*β*-catenin signaling pathway, the nuclear *β*-catenin operating as a transcriptional activator [Shang et al., 2017]. Aberrant *β*-catenin expression or localization is also associated with a worse course in papillary thyroid micro-carcinomas [Lantsov et al., 2005]. Thus, the changes in *β*-catenin expression indicate a progressive loss of tumor differentiation with results in a poor prognostic [Garcia-Rostan et al., 2001; Lantsov et al., 2005; Ralhan et al., 2010; Lam et al., 2017].

Strictly referring to PTC, *β*-catenin was previously analyzed in conventional, follicular, tall cell and diffuse sclerosing variants [Rocha et al., 2001; Min et al., 2013], showing a predominant membranous expression in all subtypes and an infrequent dot-like cytoplasmic (paranuclear) expression in tall cell subtype; the nuclear expression, completely absent in these subtypes [Min et al., 2013], is reported only in cribriform-morular variant [Jung et al., 2009; Lam et al., 2017]. The novelty of our study consists in the twofold analysis of *β*-catenin (membranous- and cytoplasmic-oriented) in different histological variants of PTC, classified as high- and low-risk, and the comparison of these expressions by referring to the clinicopathological factors. Our data reveal significant statistically differences between

the membranous β-catenin expression in the two risk groups. Moreover, our results sustain the value of the membranous β-catenin, assessed as high and low, in relationship with tumor size and tumor stage. Similar results prove, in PTC, the association of low membranous β-catenin pattern with an increased tumor size and distant metastases [Böhm et al., 2000; Căruntu et al, 2018]. The absence of significant statistically differences between the β-catenin cytoplasmic expression and clinicopathological factors could be explained through an early stage of Wnt pathway activation, not necessarily reflected by the cytoplasmic (or nuclear) translocation of β-catenin.

MOC-31 in PTC

Due to the development of numerous antibodies directed against it, a wide range of synonyms is used for EpCAM – the most commonly being: MOC-31, tumor-associated calcium signal transducer 1 (TACSTD1), CD326, 17-1A, BerEp4 [Baeuerle et al., 2007].

Although identified since the early 1970s among the first tumor-associated antigens, the EpCAM contribution in carcinogenesis has long been unexplored. The first evidence referring to the carcinogenic role of EpCAM is described in the 2000s, and supports the involvement of this molecule in cell adhesion, cell proliferation and differentiation, migration and metastasis [Armstrong et al., 2003; Ang et al., 2017]. Moreover, the value of potential prognostic marker and target in immunotherapeutic strategies is being discussed [Ang et al., 2017].

The EpCAM expression indicates its activation by the cleavage of the intracellular domain and represents a key stage in neoplastic transformation [Fong et al., 2014]. Several studies demonstrate the EpCAM role in digestive [Balzar et al., 1999; Went et al., 2006; Stoecklein et al., 2006; Kuhn et al., 2007; Patriarca et al., 2012; Fong et al., 2014; Kim et al., 2016; Dai et al., 2017; Han et al., 2017], pancreatic [Fong et al., 2008; Fong et al., 2014], liver [Zhou et al., 2018], renal and prostate [Seligson et al., 2004; Went et al., 2004; Went et al., 2006; Fong et al., 2014; Campos et al., 2016; Hu et al., 2019], lung [Went et al., 2006; Fong et al., 2014], endometrial [Fong et al., 2014], ovarian [Spizzo et al., 2004; Fong et al., 2014; Battista et al., 2014], breast [Gastl et al., 2000; Schmidt et al., 2008; Pai et al., 2009; Schmidt et al., 2010; Alberti et al., 2012; Soysal et al, 2013; Fong et al., 2014;], and head and neck [Murakami et al., 2019] carcinomas. The EpCAM expression shows a large variability in different types of malignancies. Some reports reveal low immunostaining in the gastric neoplastic epithelium, while marked in colorectal carcinoma [Balzar et al., 1999; Kim et al., 2016; Dai et al., 2017; Han et al., 2017]. Significant differences in EpCAM expression are recorded in renal carcinomas variants, and the increase of EpCAM expression occurs together with the development of androgen resistance in prostate cancer [Went et al., 2004; Hu et al., 2019]. EpCAM overexpression in primary and metastatic breast cancer is associated with nodal metastasis, overall survival and disease-free survival [Schmidt et al., 2008]. In contrast, the loss of membrane expression of EpCAM significantly correlates with the presence of nodal metastasis and the infiltration of tumor margins in colorectal carcinomas, as well as with advanced staging and reduced survival [Patriarca et al., 2012; Kim et al., 2016; Han et al., 2017]. The relationship between EpCAM and prognosis is thus controversial. The negative prognosis is associated either with EpCAM overexpression in esophagus squamous cell carcinoma (SCC) [Stoecklein et al., 2006] and breast cancer [Gastl et al., 2000; Spizzo et al., 2004; Schmidt et al., 2010], or with loss of expression in colorectal [Han et al., 2017] and renal [Seligson et al., 2004] carcinomas. Contrary, EpCAM overexpression is correlated with favorable course of ovarian carcinoma [Battista et al., 2014].

However, few results refer to EpCAM expression in TC [Ralhan et al., 2010; Kunavisarut et al., 2012], focusing on its relationship with the aggressive behavior. One study presents a comparative analysis of MOC-31 expression in different types of TC (19 ATC, 4

PDTC, 25 PTC, 4 FTC, and 4 SCC) [Ralhan et al., 2010]. The authors proposed an index of tumor aggressiveness, based on the degree of nuclear and cytoplasmic accumulation of EpCAM, and the loss of membranous EpCAM [Ralhan et al., 2010]. Their results showed: loss of the membranous expression in ATC, PDTC, FTC and SCC; nuclear expression in ATC (strong), and PDTC (moderate to inconstant); cytoplasmic expression in PTC, FTC, and PDTC (with different intensity); and membranous expression in PTC, and PDTC. These results sustain that an increased cytoplasmic and nuclear EpCAM expression, in parallel with the loss of membranous one is associated with an unfavorable prognosis and a reduced overall survival. The loss of the EpCAM extracellular component at the membrane level, together with the accumulation of intracellular component in cytoplasm and nucleus, acts as an oncogenic signal transducer. This phenomenon is possible by activating the Wnt pathway, within which β-catenin initiates the rapid tumor growth and, consequently, aggravates the prognosis [Ralhan et al., 2010]. Based on these results, MOC-31 can be associated with tumor aggressiveness of some histological types of TC [Ralhan et al., 2010]. The same group of researchers has analyzed the expression of EpCAM on 36 PTMC, in relation to the presence or absence of metastases. The obtained data show that the subcellular localization may represent a new prognostic marker for the metastatic PTMC [Kunavisarut et al., 2012].

To the best of our knowledge, until 2019, there are no reports regarding MOC-31 in the most common type of PTC, namely conventional PTC, and PTC histological variants.

In our study, MOC-31 was expressed circumferential membranous in all 70 PTC cases, in association with a cytoplasmic expression in 65 (93%) of the cases. On the other hand, in the normal and benign thyroid tissue adjacent to the tumor areas, MOC-31 had a limited basolateral membranous location. Thus, our study confirms the changes in the distribution of MOC-31 in the thyroid tumor cells, which certainly reflect instability in the mechanism of cell adhesion. These changes determine the extension from the restricted location in the adherens junctions to the entire cell membrane and to cytoplasm, with repercussions on cell adhesion through interactions with other molecules involved in this process – mainly cadherin and β-catenin. Thus, we consider that the distinctiveness of PTC, characterized in most cases by well-differentiated forms and non-aggressive biological behavior, lies in the fact that the membrane expression of MOC-31 is not lost, but is reallocated at the membranous and cytoplasmic level.

As to be able to analyze the differences in MOC-31 profile, we have semi-quantitatively evaluated the MOC-31 expression through a scoring system, classifying it into two categories: low and high. The assessment of the characteristics of the cases in every score category has been refined by reference to PTC stratification into high-risk or low-risk groups [Sherman et al., 2003; Kakudo et al., 2004; LiVolsi et al., 2011; Lloyd et al., 2011], according to the latest *WHO* Classification [Lloyd et al., 2017]. Our data indicate the predominance of cases with MOC-31 high score, which represents two-third of the entire study group (67%), while cases with MOC-31 low score represent one-third (33%). It is worth to mention that the expression of MOC-31 evaluated as high score was entirely circumferential membranous and cytoplasmic. On the other hand, MOC-31 evaluated as low score was mainly circumferential membranous and cytoplasmic, but also exclusively membranous. We emphasize the fact that each category of score has included both histological variants of the low-risk and high-risk groups. A first significant element in this association is that all five cases of PTMC defined as well-differentiated, low-risk variant have presented high score, as an argument which supports favorable evolution and excellent prognosis of PTMC. A second significant element is represented by the fact that more than a half of high-risk cases showed MOC-31 low score, close to the reported profile of MOC-31 in poorly-differentiated PTC [Ralhan et al., 2010]. These facts suggest that the reduced MOC-31 expression may be useful in identifying a particular subpopulation of patients with

TC, characterized by a weak differentiation, advanced tumor stage and increased invasiveness.

All these observations are supported by statistical analysis, which confirmed significant differences (p=0.002) between MOC-31 expression (low *versus* high) and risk groups. These differences open solid perspectives for understanding the aggressive behavior present in only some cases of PTC, regarded through the involvement of cell-adhesion molecules.

Our results supplement the existing data, according to which the modification of the EpCAM expression is frequently associated with malignancies of epithelial origin, being considered an indicator for carcinogenesis [Went et al., 2006; Fong et al., 2014]. Our work brings evidences that sustain the association of MOC-31 overexpression with the early stages of carcinogenesis in well-differentiated tumors with mild biological behavior (that correspond to the low-risk group), whereas loss of membranous MOC-31 correlates with more aggressive tumors.

Our study reveals statistically significant differences between low *versus* high MOC-31 expression and tumor size (p=0.047), and tumor relapse (p=0.02), respectively. Firstly, the correlation of the MOC-31 expression with tumor size can be considered a valuable indicator for the way in which the transition from overexpression to low expression influences tumor growth, reflecting the amplified growing and proliferation that could be related during the tumor progression with a less differentiated phenotype. The second important result was the correlation with tumor relapse. Taking into account the favorable prognosis of PTC, tumor relapse is an unusual phenomenon reported in that limited number of cases which do not respect the pattern of the non-aggressive behavior.

Tubulin in PTC

The interest in the study of TUBB3 is justified by the part it plays in cell division, an important event in carcinogenesis [Levallet et al., 2012; Karki et al., 2013; Raspaglio et al., 2014; Tsourlakis et al., 2014; McCarroll et al, 2015]. Particularities of TUBB3 expression and distribution are reported in small-cell and non-small cell lung cancer [Seve et al., 2010; Powell et al., 2014; Ohashi et al., 2015], ovarian [Ohishi et al., 2007], gastrointestinal [Carles et al., 1994; Jirasek et al., 2002; Zhao et al., 2016], pancreatic, kidney, prostate, breast [Leandro‐García et al., 2010; Tsourlakis et al., 2014; Quaas et al., 2015; Lebok et al., 2016;], and head and neck [Koh et al., 2009] tumors. In breast carcinoma, TUBB3 assessment indicates differential expression, according to the histological type, ER and PR presence, and HER2/neu status [Wang et al., 2013]. TUBB3 overexpression is related to poorly differentiated high-grade [Wang et al., 2013], triple-negative hormonal status, higher HER2 [Lebok et al., 2016]. In renal carcinoma, TUBB3 expression is more frequent in papillary and chromophobe subtypes and oncocytoma compared to clear-cell subtype, where its overexpression correlates to high Fuhrman grade, advanced stage, lymph node and hematogeneous metastases, and shortened overall survival; on the contrary, in the papillary subtype, strong TUBB3 expression is associated to early tumor stage and overall survival [Quaas et al., 2015]. These variances can be explained by the differences in VHL function involved in the carcinogenesis of clear-cell and papillary subtype, respectively, because VHL has a similar role to TUBB3, interfering in microtubule stabilization. In bladder carcinoma, some studies analyze different tubulin isotypes [Cheng et al., 2011; Cheng et al., 2014; Massari et al., 2015] following advanced stage patients' stratification, to customize the treatment; another paper indicated the association between the overexpression of β-tubulin 1, β-tubulin 2, β-tubulin 3 and tumor degree and stage, as well as shorter disease free survival [Choi et al., 2014]. TUBB3 analysis in a series of malignant melanoma showed high

expression in 80% cases, but no correlation to classical clinical and pathological variables [Shimizu et al., 2016]; surprisingly enough, TUBB3 decreased expression was associated to survival variables (overall survival and progression-free survival) [Shimizu et al., 2016], indicating an unfavorable prognosis. Overexpression of TUBB3 in colorectal carcinoma, identified in tumor invasion margins [Giarnieri et al., 2005; Jirásek et al., 2009; Portyanko et al., 2009], is correlated to the degree of tumor differentiation, lymphatic metastasis [Zhao et al., 2016], poorer prognostic and lower overall survival [Mariani et al., 2012]. Not in the least, TUBB3 expression, 4.1 times higher in the uterine serous carcinomas compared to the ovarian serous ones, pleads for its prognostic value [Ferrandina et al., 2006; Roque et al., 2013].

Due to its functions, tubulin became a target for the development of new therapeutic agents, used in adjuvant chemotherapy. Taxanes (Paclitaxel, Docetaxel, Cabazitaxel) are a class of drugs that serve the function of stabilizing the microtubules by interfering with spindle microtubule dynamics. Consequently, cell cycle is arrested, apoptosis initiated and tumor progression can be stopped [Nowak et al., 2004; McGrogan et al., 2008].

Several researches on the prognostic role of tubulin sustain its value for predicting the response to neo-adjuvant chemotherapy with taxane that improves the prognosis of patients with various solid cancers, compared to other therapies. The most relevant example is the breast carcinoma [Paradiso et al., 2005; Chen et al., 2012], an increased complete remission rate being related to lower TUBB3 expression [Chen et al., 2012]. Additionally, in vitro and in vivo studies showed that TUBB3 can predict paclitaxel chemosensitivity in gastric cancer [Yu et al., 2014]. A strong TUBB3 expression is correlated to a favorable response to neo-adjuvant chemotherapy in non-small-cell lung carcinoma [Sève et al., 2007], clear-cell ovarian carcinoma [Ferrandina et al., 2006; Aoki et al., 2009], ER negative breast carcinoma [Wang et al., 2013], HER2 positive breast carcinoma [Jung et al., 2012], advanced locally and metastatic breast cancer [Galmarini et al., 2008].

On the other hand, several reports showed that TUBB3 overexpression is directly involved in the resistance to taxanes. Reports on this matter demonstrate an altered expression of specific β-tubulin genes in taxol-resistant ovarian tumors [Kavallaris et al, 1997; Umezu et al., 2008; Roque et al., 2014]. Supplementary evidence for the relation TUBB3 – chemoresistance is supported by experimental works, using cancer cell lines [Kavallaris et al, 1997; Ranganathan et al., 1998; Burkhart et al., 2001; Liu et al., 2001], and clinical studies. The association between the TUBB3 overexpression, chemoresistance to taxanes and poor prognostic is documented in several malignancies, including breast [Hasegawa et al., 2003; Paradiso et al., 2005; Tommasi et al., 2007; Chen et al., 2012], gastric [Urano et al., 2006], pancreatic [Lee et al., 2007], colonic [Mariani et al., 2012] and prostatic [Ploussard et al., 2010] carcinomas, clear-cell ovarian carcinoma [Roque et al., 2013], serous uterine carcinoma [Roque et al., 2013], uterine carcinosarcoma [Carrara et al., 2012], thymic carcinoma [Kaira et al., 2011; Okuda et al., 2017], non-small and small-cell lung carcinoma [Rosell et al., 2003; Dumontet et al., 2005; Powell et al., 2014], locally advanced head and neck squamous-cell carcinoma [Koh et al., 2009], as well as in metastatic carcinomas of unknown primary site [Hari et al., 2003; Paradiso et al., 2005; Magnani et al, 2006; Ferrandina et al., 2006; Seve et al, 2007].

This different tumor behavior reflects various biological characteristics of tubulin, including changes in the β-tubulin isotype composition in tumor sub-clones which influences tumor dynamics and the responsiveness of the chemical compounds that interfere with microtubules [Katsetos et al., 2003], in relation to tumor stage [Du et al., 2015]. The variability of these results could be explained by the differences occurring in the design of various studies, namely the divergences in the group size, tumor stage, histological type and eligible patients [Jung et al., 2012]. Moreover, the immunohistochemical assessment of

TUBB3 uses variable scores and cut-offs, being sometimes doubled by qRT-PCR [Du et al., 2015].

TUBB3 study in thyroid tumors is limited to ATC (the most aggressive and rare solid thyroid tumor, with lethal evolution) in strict relation with the taxane therapy [Ain et al., 2004; Gómez Sáeza et al., 2015]. A research [Gómez Sáeza et al., 2015] carried out under the coordination of the Working Group of Thyroid Cancer of the Spanish Society of Endocrinology and Nutrition and GETHI of the Spanish Society of Oncology established that for the operable ATC, adjuvant chemotherapy must include taxanes [Shinohara et al., 2009; Sosa et al., 2014; Gómez Sáeza et al., 2015], associated to doxorubicin, and cisplatin.

Published data referring to TUBB3 expression in PTC are practically inexistent until 2011. There are only two oral reports on TUBB3 immunohistochemical assessment [Colato et al., 2011; Colato et al., 2012] in PTC, follicular adenoma, nodular hyperplasia and normal thyroid tissue. The authors demonstrated the absence of TUBB3 in the normal follicular epithelium, nodular goiter and follicular adenoma, and a heterogeneous expression in PTC. They noted a negative TUBB3 reaction in the conventional, follicular or encapsulated variant of PTC, and a strong cytoplasmic TUBB3 expression in PTC widely infiltrative associated with fibrous stroma, particularly at the tumor invasive front, and in PTC moderately differentiated, with loss of cell polarity/cohesivity [Colato et al., 2011; Colato et al., 2012]. Thus, the TUBB3 overexpression in PTC with "aggressive" histological features supports its potential role in invasion and metastasis.

Our study demonstrates low and moderate TUBB3 expression in 21 cases and high expression in 49 cases, and also the correlation with the histological risk subgroups, and tumor relapse. This fact suggests a relationship between TUBB3 and those histological characteristics of PTC that can explain the invasion and metastasis in the cases with poor prognosis. Starting from this idea, the taxanes' efficacy should be proved in those PTC cases that come out from the classic pattern of favorable prognostic, and need a personalized therapy.

Periostin in PTC

Currently, eight PN isoforms are known, only five of them being sequenced and identified in different tissues: isoform 1 or (a) in osteosarcoma, isoform 2 or (b) in human placenta, isoform 3 or (c) in ovarian carcinoma, and 2 (b), 4 (d), and 5 (e) in either normal or tumoral urinary bladder [Takeshita et al., 1993; Gillan et al., 2002; Litvin et al., 2005; Kim et al., 2008; Bai et al., 2010]. Different PN isoforms may variably influence ECM fibrillogenesis [Hoersch et al., 2010] but it is still unknown if their effect on ECM increases the invasiveness or metastatic potential [Morra et al., 2011; Kudo et al., 2011; Conway et al., 2014].

During the last 20 years, several papers provided evidences that support PN involvement in different malignancies. According to these studies, stromal PN expression is a negative prognostic factor for patients' survival [Tischler et al., 2010; Dahinden et al., 2010; Riener et al., 2010; Morra et al., 2011; Jang et al., 2016] and, in association with epithelial PN, is significantly correlated with different clinicopathological prognostic factors [Soltermann et al., 2008; Zhang et al., 2010; Schramm et al., 2010; Morra et al., 2011; Zhang et al., 2012; Utispan et al., 2012; Lv et al., 2013; Nuzzo et al., 2014;]. PN involvement in the epithelial-mesenchymal transition (EMT) has been also a matter of research interest, due to its potential therapeutic target value [Gillan et al., 2002; Bao, et al., 2004; Li et al., 2004; Yan et al., 2006; Kim et al., 2011; Morra et al., 2011]. Therefore, PN expression was analyzed in correlation to EMT (vimentin, elastin, and collagen) and angiogenesis specific markers, demonstrating its involvement as a promoter of this process [Bao, et al., 2004; Siriwardena et al., 2006; Zhu et al., 2010; Kudo et al., 2012; Lv et al., 2013].

Few papers addressed PN in thyroid tumors, predominantly using techniques of molecular biology (cDNA microarrays and real-time PCR) [Fluge et al., 2006; Puppin et al., 2008; Bai et al., 2009; Bai et al., 2010]. Eight h-periostin isoforms have been identified in both TC and in corresponding non-neoplastic tissues, all of them being related to thyroid carcinogenesis, invasion or lymph node metastasis, regardless of differences between their expression patterns [Bai et al., 2010]. A high PN gene expression is associated with aggressive, poorly differentiated PTCs [Fluge et al., 2006] and is correlated with specific morphological cellular features (loss of polarization and cohesiveness) registered in the invasive front of the tumor [Bai et al., 2009]. Only one of the four studies from literature has also analyzed PN immunoexpression, within a rather limited number of cases (10 normal thyroids, 10 follicular adenomas, 10 FTCs, and 10 PTCs samples, respectively) [Puppin et al., 2008]. No PN staining has been noticed in normal thyroid tissue, in follicular adenoma, and in follicular TC, and only 4 cases from a total of 10 PTCs showed a diffuse cytoplasmic immunoreaction [Puppin et al., 2008].

Within this context, our work provides new data regarding the specific PN immunoexpression in epithelial tumor cells and intratumoral stroma, in different histological subtypes of PTC.

To the best of our knowledge, this is the first report of qualitative differences in epithelial and stromal PN expression between conventional, follicular, macrofollicular, tall cell, and oncocytic subtypes. Thus, the idea that PN may be tissue-specific [Nuzzo et al., 2014] is strengthened by supplementary evidences of its heterogeneity, reported in different histological subtypes of a specific tumor, such as clear cell, papillary, and chromophobe renal cell carcinoma types [Morra et al., 2011], and conventional and non-conventional osteosarcoma subtypes [Hu et al., 2014].

The pivotal role of PN synthesis in different malignancies is under scrutiny, by comparing the involvement of tumor epithelial cells with that of the tumor stromal component. As a consequence, it has been hypothesized that PN acts in a cell-type-dependent manner related to its expression in stromal *versus* epithelial cells, as a result of the activity of different PN terminal regions [Morra et al., 2011].

This hypothesis has been the starting point of our work which has individually quantified PN immunoexpression in tumor cells and in tumor stroma. We have additionally refined the reported scores already used for PN assessment [Choi et al., 2011; Jia et al., 2016; Sung et al., 2016], considering both the percentage of positive cells and the reaction intensity, using a threshold to label the investigated cases into low and high score categories. This modality of semiquantitative evaluation, based on a specific algorithm, has not been yet applied in thyroid tumor pathology.

Our study showed a heterogeneity of PN stromal immunoexpression, comparable to other malignancies reporting either PN positivity [Li et al., 2004; Puglisi et al., 2008; Contie et al., 2011; Tian et al., 2014; Nuzzo et al., 2016], either PN negativity [Fukushima et al., 2008]. Most papers have reported that stromal PN has a more aggressive potential than the epithelial PN. This aggressiveness can be attributed to the capacity of the PN produced by the stromal components to act not only by intracellular signaling pathways but also by its fibrillogenic potential within ECM, its C-terminal region interacting with ECM molecules [Takayama et al., 2006; Morris et al., 2007].

Our results support the dominant pro-tumorigenic role of stromal PN, while epithelial PN action is less evident. We found that the high stromal PN expression is significantly associated with an advanced tumor stage and extrathyroidal extension [Giuşcă et al., 2017]. Similar results are also reported in renal cell carcinoma [Castronovo et al., 2006; Morra et al., 2011], prostate [Tsunoda et al., 2009; Tischler et al., 2010; Morra et al., 2011], penile [Gunia

et al., 2013], and breast cancer [Zhang et al., 2010; Nuzzo et al., 2016]. There are no available literature data about the stromal PN profile in thyroid tumors.

On the other hand, PN overexpression in tumor epithelial cells was correlated to specific histological PTC variants, the highest risk being recorded in the conventional subtype in comparison to the oncocytic one. Our data are supplementing other results in the mainstream publications. Strictly referring to the thyroid pathology, the single published paper on PN immunoexpression in PTC [Puppin et al., 2008] reports a correlation between PN overexpression and clinicopathological features (i.e. extrathyroidal invasion, distant metastasis, and higher grade staging). Despite the small number of cases, the authors outline the correlation between PN, ETM, and an aggressive tumor behavior [Puppin et al., 2008]. Moreover, they consider that PN could be a stronger negative prognostic marker than B-RAF, regardless of B-RAF mutation [Puppin et al., 2008]. In other types of malignancies, comparable relationships are demonstrated in renal cell carcinoma (mainly for clear cell subtype) where a greater tumor epithelial PN expression is significantly associated with sarcomatoid differentiation, higher tumor stage, lymph node metastases, and poor overall survival [Dahinden et al., 2010; Morra et al., 2011], and also in hepatocellular carcinoma, where PN correlates with microvascular invasion, multiple tumors, and advanced tumor stage [Riener et al., 2010; Jang et al., 2016].

4.5. FINAL REMARKS

Our study sustains large heterogenity of the molecular markers' expression in PTC histological subtypes, classified in low and high-risk group. The aggressive behavior of the high-risk histological variants of PTC is associated with low expression of Her-2neu, E-cadherin, β-catenin and MOC-31 in tumoral cells, overexpression of TUBB3 in tumoral cells and PN in stromal tumor.

CHAPTER 5.
PAPILLARY THYROID CARCINOMA ASSOCIATED WITH PARATHYROID PATHOLOGY

5.1. INTRODUCTION

Hyperparathyroidism (HPT) is due to increased activity of the parathyroid glands, both from an intrinsic abnormal change altering excretion of parathyroid hormone (primary HPT – PHPT or tertiary HPT – THPT) or from an extrinsic abnormal change affecting calcium homoeostasis stimulating production of parathyroid hormone (secondary HPT – SHPT) [Fraser, 2009; Walker, Silverberg, 2018].

PHPT is the third most common endocrine disorder, with the highest incidence in postmenopausal women, with a prevalence of 0.1 to 0.4% in the general population, and more frequently diagnosed in the fifth decade of life, in female patients [Gopinath et al., 2011; Arrangoiz et al., 2016; Walker, Silverberg, 2018]. Asymptomatic disease is common and severe disease with renal stones and metabolic bone disease arises less frequently now than it did 20–30 years ago [Fraser, 2009; Walker, Silverberg, 2018]. PHPT can be cured by surgical removal of an adenoma, increasingly by minimally invasive parathyroidectomy. Medical management of mild disease is possible with bisphosphonates, hormone replacement therapy, and calcimimetics [Fraser, 2009; Walker, Silverberg, 2018].

SHPT is the result of failure of one or more components of the calcium homoeostatic mechanisms [Messa, Alfieri, 2019]. When plasma ionized calcium decreases, the calcium-sensing receptor responds by increasing secretion of parathyroid hormone (PTH), resulting in a compensatory mechanism to restore normal function [Mizobuchi et al., 2019]. Vitamin D deficiency is a common cause of SHPT, particularly in elderly people [Mizobuchi et al., 2019]. However, the biochemical definition of vitamin D deficiency and its treatment are subject to much debate [Jesudason et al., 2002; Vieth et al., 2003; Fraser, 2009; Messa, Alfieri, 2019; Mizobuchi et al., 2019]. SHPT is also a common complication of end-stage renal disease, and might develop finally in nearly all patients with chronic kidney disease (CKD) with consequences in the progress of bone disease and vascular calcification [Ho et al., 2017; Sun et al., 2018; Messa, Alfieri, 2019; Rodríguez-Ortiz, Rodríguez, 2020]. Some SHPT patients at early stage can be treated with drugs such as Lanthanum carbonate and Cinacalcet, while others need surgical intervention because of drugs ineffectiveness or resistance [Aaseth et al., 2018; Fukagawa et al., 2018].

The surgical cure for HPT is the removal of the parathyroid glands. The standard operation is a full neck exploration with identification of all glands, recognizing that 15–25% of PHPT can have multiple adenomas [Fraser, 2009]. Local anaesthesia and minimally invasive parathyroidectomy are increasingly used. The value of minimally invasive parathyroidectomy is questioned, but it can have advantages in an elderly population who stand at risk from a general anaesthetic and full neck exploration. Minimally invasive parathyroidectomy requires preoperative localization studies with identification of one adenoma, and might benefit from intraoperative determination of PTH confirming adenoma removal. Localization techniques include ultrasound, MRI, and computerised axial tomography. Several papers, published starting from the first decade of 2000s, address the

efficiency of imaging methods in the preparation of surgical treatment. One of the greatest reported success implies the use of ^{99}technetium labelled sestamibi-single photon emission CT [Gayed et al., 2005]. On the other hand, imaging techniques are less successful for investigation of patients with mild hypercalcaemia and in identification of multiple glands [Katz et al., 2003]. In minimally-invasive radio-guided parathyroidectomy (MI-RP) a handheld gamma probe is used to facilitate intraoperative localization, identification and dissection of the pathologic gland(s), and to confirm removal of all hyperfunctioning parathyroid tissue [Johnson et al., 2001; Desiato et al., 2016]. This approach requires intravenous injection of technetium-99m sestamibi 2-4 hours prior to surgery. Obviously, a prerequisite for this approach is the precise coordination between the operating room, nuclear medicine department, the surgeon and the nuclear medicine radiologist so that everything is timed correctly. The neck of the patients is scanned on the operating table and the site with highest counts is explored. PTH assays with short incubation times have established intraoperative measurements of this hormone as a method to determine successful removal of an adenoma, which can affect intraoperative decisions when localization techniques are equivocal. A 50% decrease in PTH from baseline 5–10 min after excision of an adenoma is evidence of successful parathyroidectomy [Johnson et al., 2001; Desiato et al., 2016].

Concomitant PHPT and thyroid pathology have been reported in several studies since the first cases were reported by Kissin and Ogburn [Kissin et al., 1947; Ogburn et al., 1956]. In PHPT patients, several studies show the prevalence of papillary thyroid carcinoma (PTC) to range from 2% to 15% [Pickard et al., 2002; Nilsson et al., 2007; Goswami et al., 2012; Yazici et al., 2015; Palmieri et al., 2017]. Patients with SHPT and THPT (parathyroid gland autonomization) were also described to associate thyroid nodular disease and cancer [Linos et al., 1982; Miki et al., 1992; Kaptein et al., 1996; Klyachkin et al., 2001; Tarrass et al., 2005].

Today, the number of experts proposing routine bilateral neck exploration has decreased and the paradigm has recently removed to minimally invasive parathyroidectomy [Smith et al., 2000; Desiato et al., 2016], offering a more limited exposure of the thyroid gland, making the detection of concomitant thyroid malignancy difficult during surgery. The presence of concomitant PTC can change the evaluation and surgical management of patients with HPT. In the coexistence of these two diseases, thyroid PTC must be identified prior to surgical management to minimize complications from additional surgical procedures, patient discomfort, and costs [Wright et al., 2017].

Aim

The aim of our study was to analyze the clinico-morphological characteristics of the thyroid cancer in patients surgically treated in a single center for PHPT or SHPT, in which simultaneous excision of the parathyroid glands and thyroid tissue was performed.

5.2. MATERIAL AND METHODS

Patients

The study included an initial number of 224 patients treated for PHPT or SHPT between 2010-2017 at “Sf. Spiridon” Clinical Emergency County Hospital Iasi. PHPT cases were diagnosed in the Endocrinology Department of “Sf. Spiridon” Clinical Emergency County Hospital Iasi, whereas SHPT cases in the context of advanced CKD were addressed by the Department of Nephrology, “C.I. Parhon” University Hospital Iasi. The research was been approved by the Ethics Committee of the “St. Spiridon” Clinical Emergency County Hospital Iasi, pursuant to the ethical standards of Helsinki declaration regarding the patients’ informed consent for the use of their medical information for scientific purpose.

Patients' clinical data and pathological results were documented from the hospital's electronic registry. Seven patients were diagnosed as HPT in syndromic conditions: 6 cases of multiple endocrine neoplasia syndromes (MEN), 1 case of hypeparathyroidism - jaw tumor syndrome (HPT-JT), and were excluded from the study.

Thus, the study group comprised 217 patients, as follows: 140 patients diagnosed with PHPT (64.5%) and 77 patients diagnosed with SHPT in CKD (35.5%).

Surgical treatment and pathological examination

Fine needle aspiration biopsy (FNAB) was performed from large thyroid nodules in 28 patients with PHPT and 9 patients with SHPT, but no elements of suspicion for malignancy were found before surgery. The cytological samples revealed abundant, watery amorphous basophil, easily identifiable colloid, honeycomb and ordered sheets and macrofollicles of small follicular cells, with small uniform nuclei, with clear to granular cytoplasm, and rare hemosiderin-laden histiocytes or mature lymphocytes.

Surgery was performed by a team specialized in endocrine pathology.

Parathyroidectomy was accompanied by thyroidectomy in 75 out of the 140 patients with PHPT and 31 out of the 77 patients with SHPT. Surgical intervention on thyroid varied from adenomectomy and/or hemithyroidectomy to subtotal or total thyroidectomy, according to the dimensions and spreading of the observed lesions. The main reason for thyroidectomy was the coexistence of solitary or multiple thyroid nodules, discovered before surgery and accompanied by compressive or esthetical complaints, followed by the incidental intraoperative findings.

MI-RP was made for one case of 56 yo obese male with persistent SHPT following an extensive cervical exploration and removal of three parathyroid (PT) glands, as the right superior PT could not be found. A reintervention was scheduled and in order to secure the location of the PT, Tc-99m MIBI was injected intravenously one hour prior to surgery.

The surgical specimens of all 217 patients were histopathologically analyzed, and PTC was diagnosed in 28 cases (Fig. 5.1).

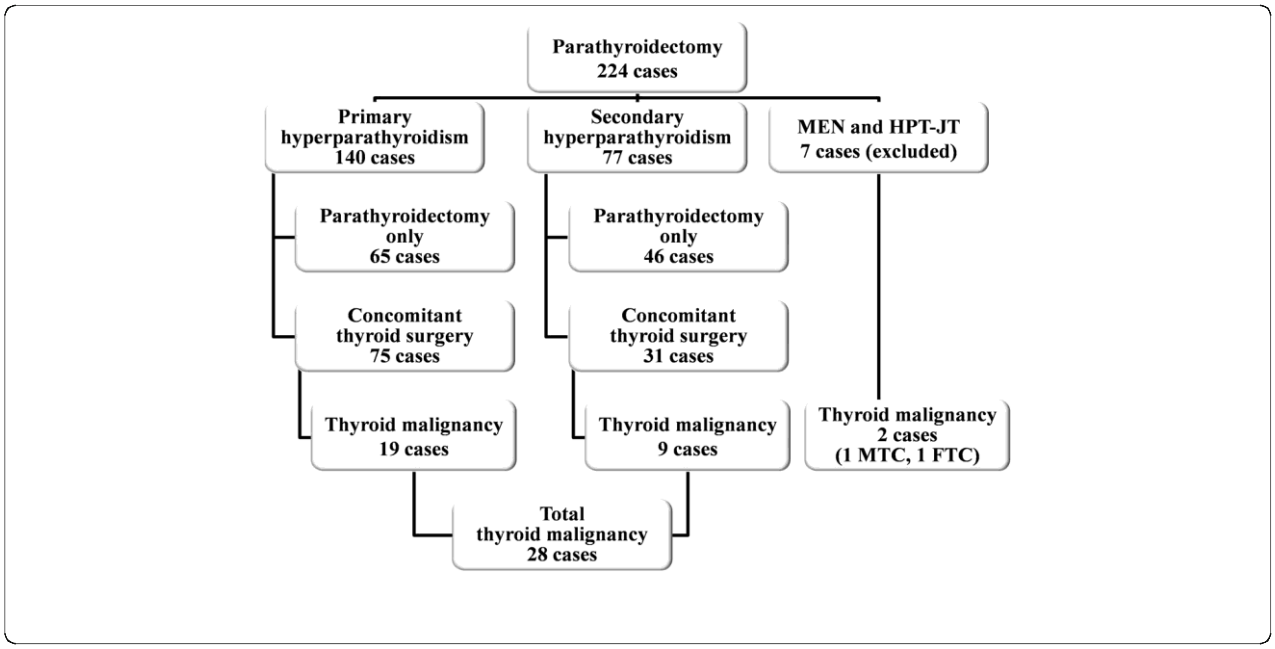


Figure 5.1. From the study group selection to pathological exam

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Descriptive statistics for continuous variables was expressed as mean ± standard deviation. Bivariate analysis was conducted with independent sample t-test, comparing means. Categorical variables were expressed in number and percent (%), and Fisher's chi-square test was used to assess the differences between groups with regard to the categorical variables.

5.3. RESULTS

5.3.1. General characteristics of the study groups

The demographic characteristics of patients from the PHPT and SHPT groups, together with the particularities of the surgical treatment and histological aspects, are depicted in Table 5.1.

Table 5.1. Comparison of parathyroid and concomitant thyroid disease in patients with PHPT *vs.* SHPT.

Clinicopathological characteristics		**PHPT**		**SHPT**		**p-value**
		No. cases	(%)	No. cases	(%)	
General data	Number of patients	140	(64.5)	77	(35.5)	
	Mean age	54.6 ± 13		48.8 ± 12		0.001
	Gender					< 0.001
	Male	15		39		
	Female	125		38		
	Female male ratio	8:1		~ 1:1		
	Preoperative PTH level [pg/ml]	357.51± 38.11		1020 ± 161.38		< 0.001
Parathyroid disease	**Parathyroid surgery***					< 0.001
	Single gland parathyroidectomy	116	(82.8)	7	(9.1)	
	Right superior gland	*17*	*(14.7)*	*0*	*(0)*	
	Right inferior gland	*11*	*(9.5)*	*1*	*(14.3)*	
	Left superior gland	*47*	*(40.5)*	*1*	*(14.3)*	
	Left inferior gland	*38*	*(32.8)*	*2*	*(28.6)*	
	Ectopic	*3*	*(2.5)*	*1*	*(0)*	
	Unspecified	*0*	*(0)*	*3*	*(42.8)*	
	Two glands parathyroidectomy	8	(5.7)	2	(2.6)	
	Subtotal parathyroidectomy	10	(7.2)	66	(85.7)	
	Multiple parathyroid surgeries	6	(4.3)	2	(2.6)	
	Parathyroid histopathology results*					**< 0.001**
	Parathyroid carcinoma	1	(0.7)	–	–	
	Parathyroid adenoma	92	(65.7)	3	(3.9)	
	Adenomatous hyperplasia	47	(33.6)	74	(96.1)	
	Nodular	*27*	*(57.4)*	*59*	*(79.7)*	
	Diffuse	*7*	*(14.9)*	*3*	*(4.1)*	
	Both nodular and diffuse	*8*	*(17.1)*	*9*	*(12.1)*	
	Unspecified	*5*	*(10.6)*	*3*	*(4.1)*	

Clinicopathological characteristics		PHPT		SHPT		p-value
		No. cases	(%)	No. cases	(%)	
Thyroid disease	**Thyroid surgery**	75	(53.6*)	31	(40.3*)	0.061
	Adenomectomy	4	(5.3**)	5	(16.1**)	
	Lobectomy/Lobisthmectomy	21	(28**)	11	(35.5**)	
	Right	*7*	*(33.3**)*	*6*	*(63.6**)*	
	Left	*14*	*(66.7**)*	*4*	*(36.4**)*	
	Subtotal thyroidectomy	1	(0.7**)	–	–	
	Total thyroidectomy	48	(34.3**)	14	(45.6**)	
	Multiple thyroid surgeries	1	(0.7**)	2	(2.6**)	
	Thyroid histopathology results					
	Papillary thyroid carcinoma (PTC)	19	(13.6*)	9	(11.7*)	0.692
			(25.3**)		(29**)	0.694
	Benign lesions Thyroid adenoma	9	(12**)	–	–	
	Adenomatous/colloid goiter	14	(18.7**)	14	(45.2**)	
	Adenomatous/colloid goiter with adenomas	40	(53.3**)	10	(32.3**)	
	Hashimoto thyroiditis with nodularization	12	(16**)	6	(19.4**)	
	Graves Basedow disease	–	–	1	(3.2**)	

PHPH – primary hyperparathyroidism, SHPH – secondary hyperparathyroidism; * statistical significance;**percentage of concomitant disease cases.

Statistical analysis revealed significant age ($p = 0.001$) and gender distribution ($p < 0.001$) differences, as well as type of parathyroid surgery, with more frequent minimally invasive surgery in PHPT (where the disease frequently involves only one parathyroid gland) and more frequent open neck surgery in SHPT, usually accompanied by hyperplasia of all parathyroid glands ($p < 0.001$, Table 5.1). There were no significant differences between the type of thyroid surgery ($p = 0.109$) performed, as the most frequent procedure was total thyroidectomy in both PHPT and SHPT. Mean values of presurgical parathormone (PTH) levels were higher in patients with SHPT than with PHPT (357.5 ± 38.1 pg/ml in PHPT and 1020 ± 161.4 pg/ml in SHPT, $p < 0.001$, Table 5.1).

5.3.2. Pathological findings in primary hyperparathyroidism

The pathological examination of the parathyroid glands confirmed the diagnosis of parathyroid adenoma in 92 cases (65.7%) and of glandular hyperplasia in 47 cases (33.6%). One patient (0.7%) in this group was diagnosed with parathyroid carcinoma.

The pathological exam of the thyroid specimens allowed the identification of PTC in 19 cases of PHPT, which accounted for 13.6% of all PHPT cases and 25.3% of patients who underwent both thyroid and parathyroid surgery. Micropapillary thyroid carcinomas (PTMC) were detected in 12 cases (63.2%) whereas in the other 7 cases PTC had a diameter above 1 cm. Lymph node invasion was documented in one case (5.3%) and multifocal tumors were present in 4 patients (21.1%). The predominant histological variant was the classical, conventional PTC in 14 cases (73.6%), followed by the follicular variant of PTC in 4 cases (21.1%) and the oncocytic PTC variant in 1 case (5.3%, Table 5.2).

Parathyroid adenomas, on gross level, appeared as a solid mass with cystic areas, brown due to hemosiderin deposits. The benign nodule was surrounded by a fibrous capsule. On microscopic level, a rim of normal parathyroid tissue was often present at the periphery of adenomas, although this feature in some case was absent (Figures 5.2 – 5.3). The component

cells were arranged in cords, nests, sheets or acinar disposition, and were organized around blood vessels (Figures 5.4 – 5.5). Commonly round and densely stained nuclei were larger than those present in the non-neoplastic parathyroid tissue. Isolated nuclei were hyperchromatic and pleomorphic, with the so-called "endocrine atypia" (Figures 5.6 – 5.7). Most adenomas were composed of chief cells, oxyphilic cells, and large clear cells or contain admixed parathyroid cells with adipose tissue constituting "lipoadenomas".

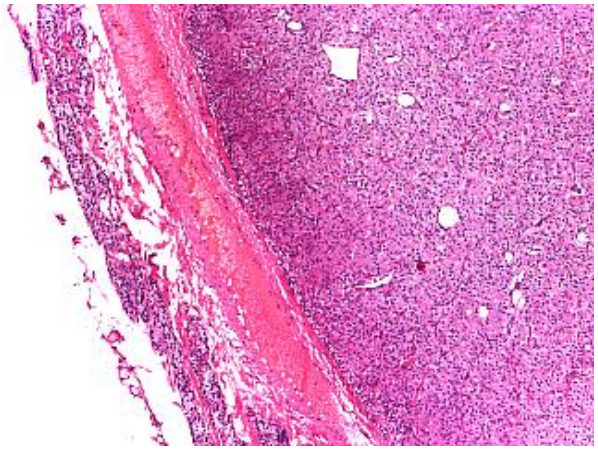


Figure 5.2. A rim of normal parathyroid tissue present at the periphery of parathyroid adenoma (HE, x 40)

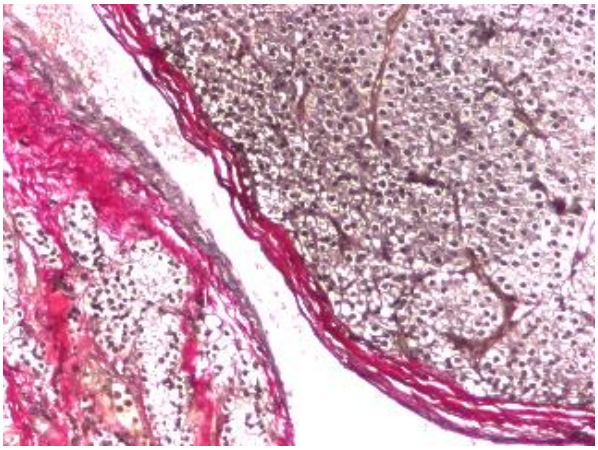


Figure 5.3. Parathyroid adenoma, fibrous capsule (VG, x 100)

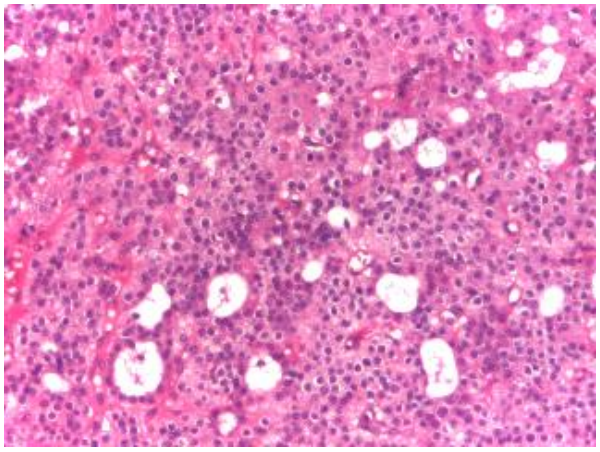


Figure 5.4. Solid and acinar disposition in PT adenoma (HE, x 100)

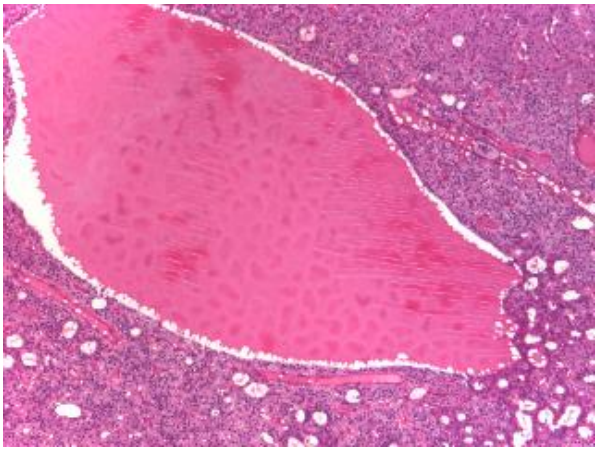


Figure 5.5. Cystic degeneration, solid and acinar architecture in PT adenoma (HE, x 40)

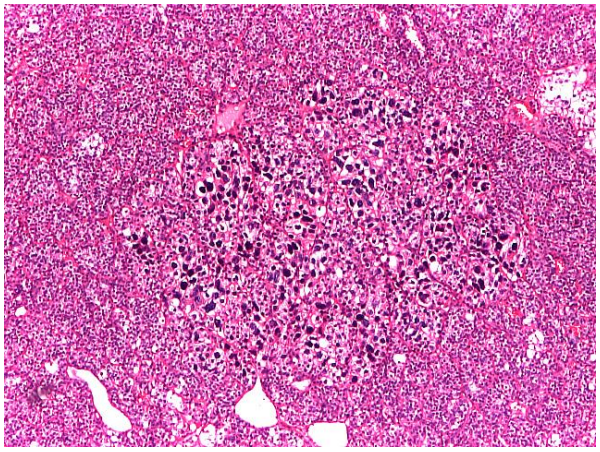


Figure 5.6. Focal pleomorphic nuclei in PT adenoma (HE, x 40)

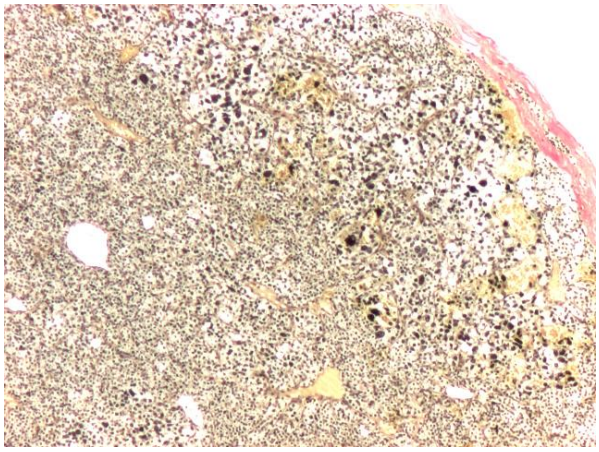


Figure 5.7. Focal "endocrine atypia" in PT adenoma (VG, x 40)

In glandular parathyroid hyperplasia the glands were enlarged, hypercellular and functioning with decreased or absent intracellular fat (Figure 5.8).

The glands could exhibit diffuse proliferation of parenchymal cells, with little or no extracellular fat or nodular development consisting mainly in chief cells, although foci of oxyphilic and clear cells may be admixed (Figures 5.9 – 5.11).

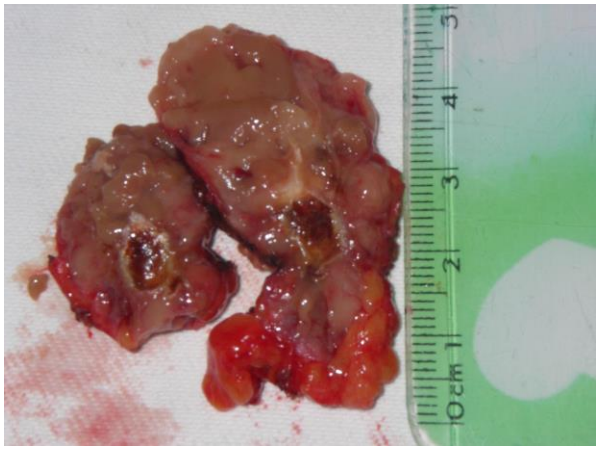


Figure 5.8. Diffuse and nodular HPT with cystic degeneration and lobular aspect

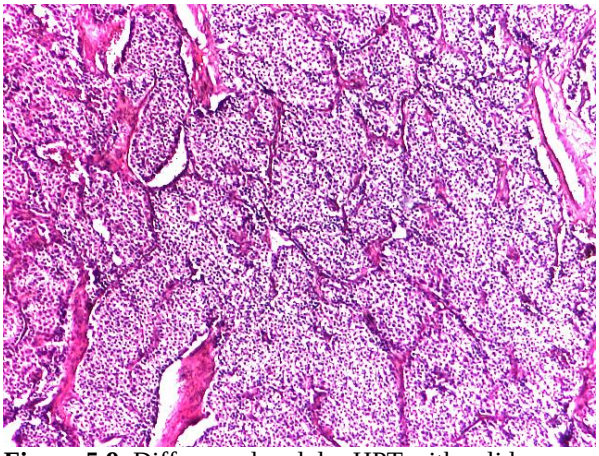


Figure 5.9. Diffuse and nodular HPT with solid architecture (HE, x 40)

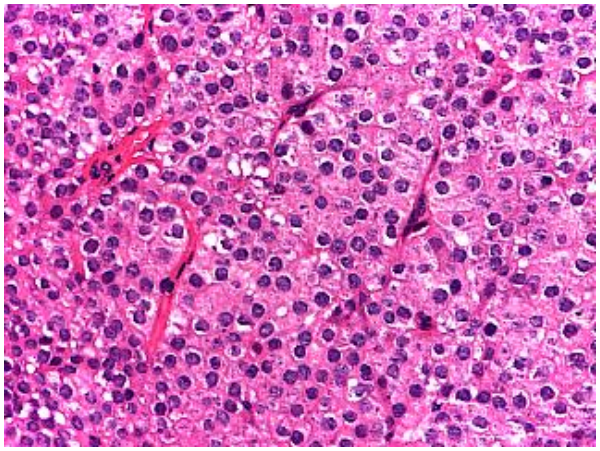


Figure 5.10. Solid architecture in HPT with oxyphilic and chief cells (HE, x 200)

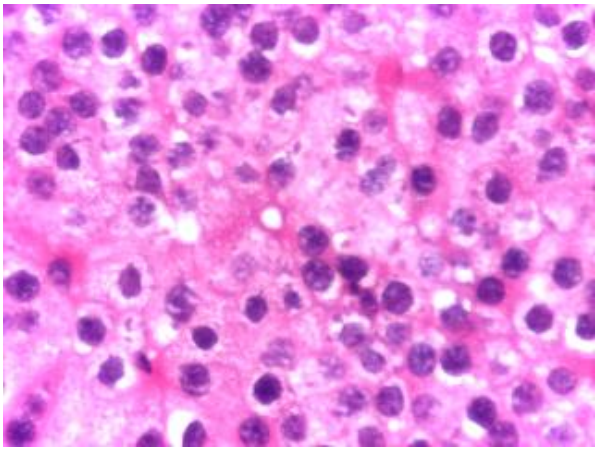


Figure 5.11. Cellular monomorphism, in chief cells (HE, x 400)

In the case with parathyroid carcinoma the following criteria were fulfilled in order to confirm the diagnosis: presence of vascular invasion (in the capsule or adjacent tissues), capsular invasion with extension to adjacent tissues and/or presence of metastases (Figures 5.12 – 5.14). Abnormal mitotic activity was inconsistent. The growth pattern was solid, with tumor cells arranged in diffuse masses, small nests or trabeculae. There was no variation in nuclear size and shape, and this aspect may be an indistinguishable feature from adenomas. Isolated areas presented focal pleomorphism with coarse chromatin and macronucleoli, a feature that had to be distinguished from the so-called “endocrine atypia” encountered in parathyroid adenomas and other benign endocrine tumors (Figure 5.15).

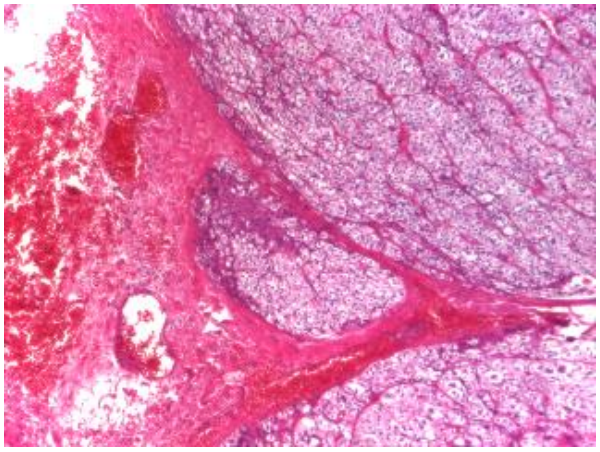


Figure 5.12. Capsular infiltration in PT carcinoma (HE, x 40)

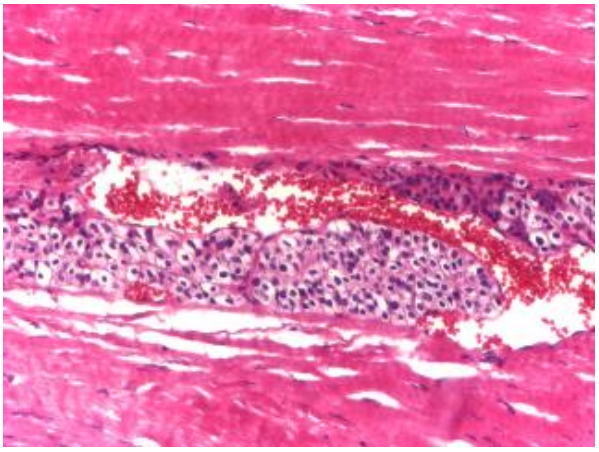


Figure 5.13. Vascular invasion in PT carcinoma (HE, x 100)

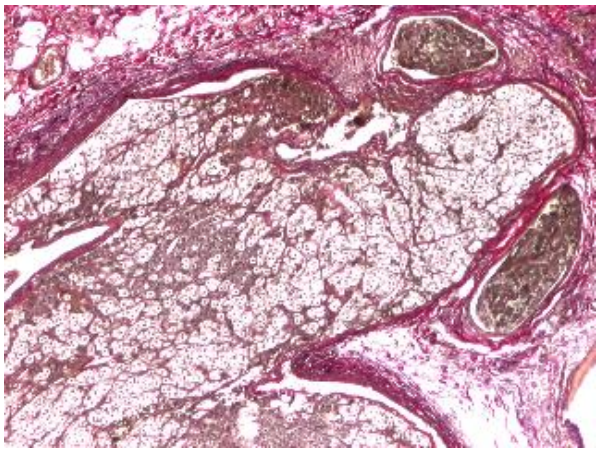


Figure 5.14. Capsular infiltration and vascular invasion in PT carcinoma (VG, x 40)

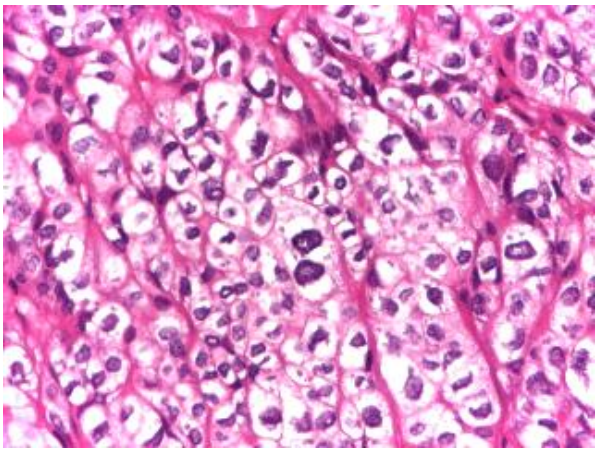


Figure 5.15. Discrete cell pleomorphism (HE, x 200)

The gross appearance of PTC was quite variable. The lesions were localized anywhere within the gland, and had variable sizes mostly under a centimeter. The lesions were firm and usually white in color with an invasive appearance. Pathological calcifications were a common feature. Owing to the extensive sclerosis, the lesion could grossly resemble with a scar (Figures 5.16 – 5.17).

Microscopically, PTC presented certain characteristic aspects. In classic, conventional type of PTC the neoplastic papillae contained a central core of fibrovascular tissue lined by one or occasionally several layers of cells, with crowded clear oval nuclei, or intranuclear inclusions and nuclear grooves (Figures 5.18 – 5.19).

Other histologic findings that were present included: psammoma bodies, prominent cystic degeneration or dystrophic calcification.

In the follicular variant of PTC neoplastic cells were arranged as macro or microfollicles, with central dense colloid, same characteristic nuclear features, and non-circumscribed and infiltrative growth pattern. PTMC had the same microscopic appearance as PTC, while the maximum tumor size was 10 mm (Figures 5.20 – 5.21).

In oncocytic variant the cells had an abundant eosinophilic cytoplasm and the same criteria for PTC (Figures 5.22 – 5.23).

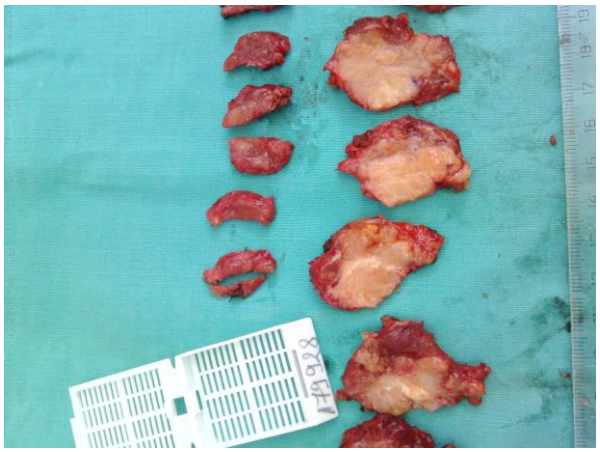


Figure 5.16. Large white PTC with an invasive appearance

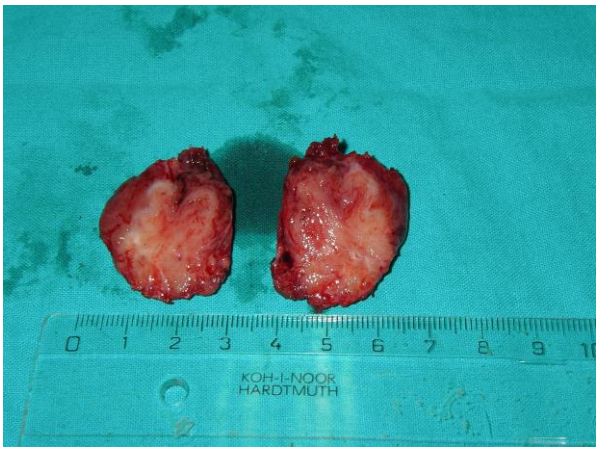


Figure 5.17. Large PTC with central scar and infiltrative margins

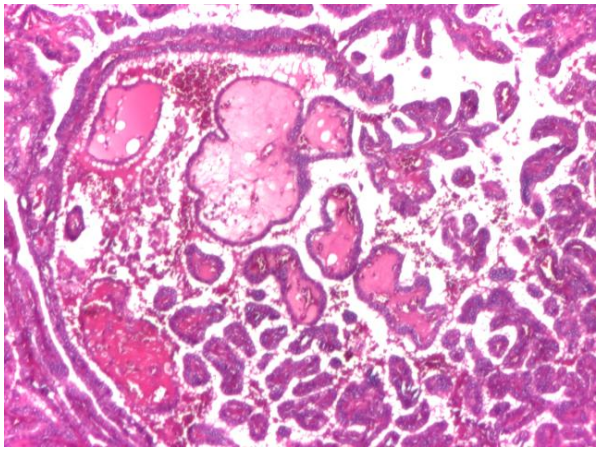


Figure 5.18. PTC with central fibrovascular core (HE, x 40)

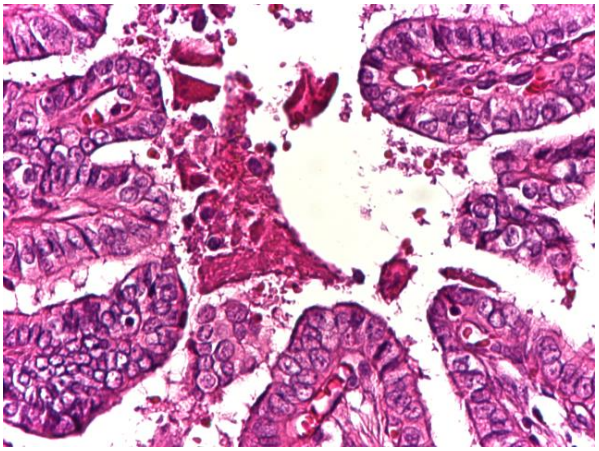


Figure 5.19. Cells with crowded clear oval nuclei, or intranuclear inclusions and nuclear grooves (HE, x 200)

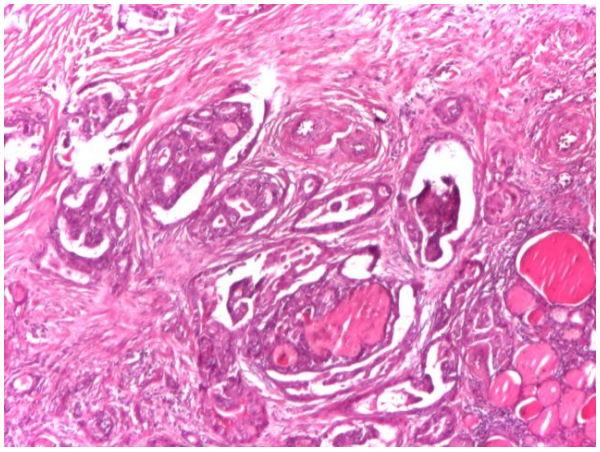


Figure 5.20. PTMC with follicular and papillary architecture (HE, x 40)

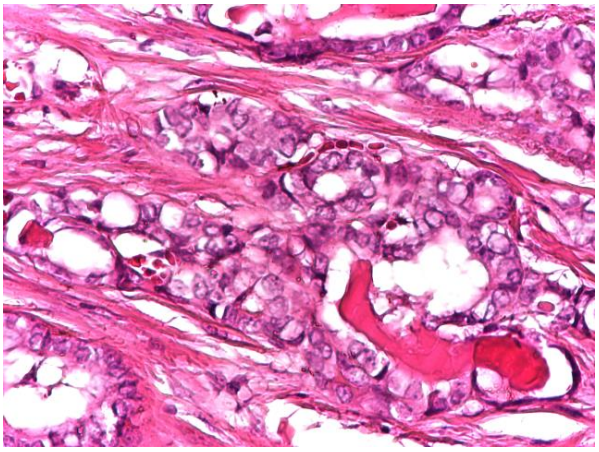


Figure 5.21. PTMC with follicular disposition (HE, x 200)

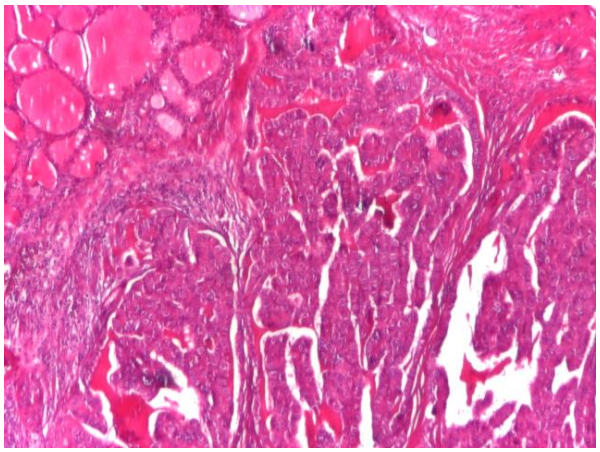


Figure 5.22. Oncocytic variant of PTC (HE, x 40)

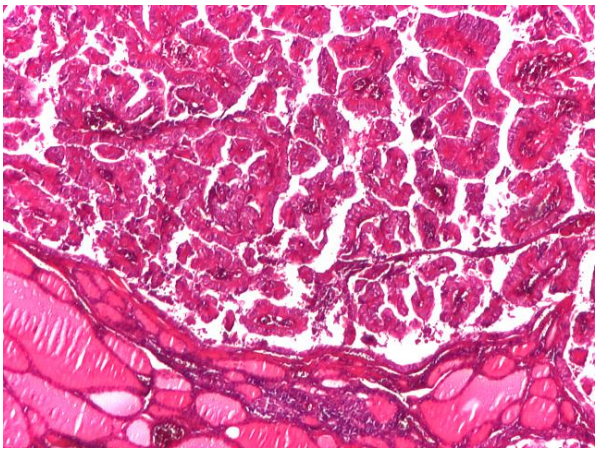


Figure 5.23. Oncocytic variant of PTC with rare psammoma bodies (HE, x 40)

5.3.3. Pathological findings in secondary hyperparathyroidism

Thirty-one (40.3%) patients underwent both thyroid and parathyroid surgery leading to the diagnosis of 9 cases with PTC (8 cases of classic variant and 1 case of follicular variant of PTC), which accounted for 11.7% of all patients diagnosed with SHPT and of 25.7% of patients who underwent both thyroid and parathyroid surgery. All tumors were PTMC, and in 8 out of 9 cases the lesions were unifocal. Similarly with PHPT patients, the most frequent thyroid pathology was the adenomatous/colloid goiter with and without nodularization in 16 cases (51.7%, Table 5.2). The elected procedure in SHPT was subtotal parathyroidectomy performed in 66 cases (84.4%), as parathyroid hyperplasia was the most frequent diagnosed pathology (74 cases, 96.1%).

The 56 yo patient surgically treated for SHPT, had a persistent HPT due to an ectopic parathyroid. After first surgical therapeutically intervention, PTH decreased from 3400 pg/ml (preoperatively) to 1900 pg/ml postoperatively. Subsequently a diagnostic work-up consisting of Tc-99m MIBI scintigraphy (Figure 5.24) and computer tomography (Figure 5.25) revealed an ectopic upper posterior mediastinum PT gland.

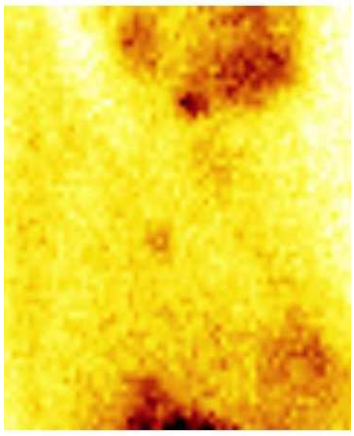


Figure 5.24. Tc-99m MIBI scintigraphy after initial surgery

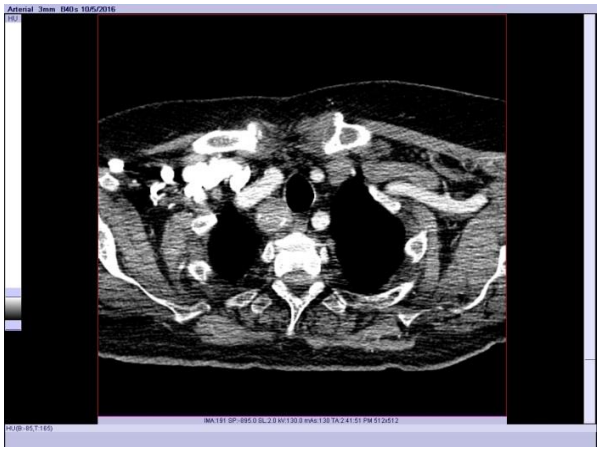


Figure 5.25. Computer tomography after initial surgery

Intraoperatively was identified the ectopic right upper PT with portable gamma probe (Figures 5.26 – 5.27) and it was removed, followed by reimplantation of PT fragments into the sternocleidomastoid muscle. A significant decrease of PTH levels was recorded both intraoperatively (iqPTH = 864 > 50 % decrease) and 48 hours postoperatively (12 pg/ml). Both frozen section and paraffin histology confirmed the adenomatous hyperplasia of removed PT (Figures 5.28 – 5.29).

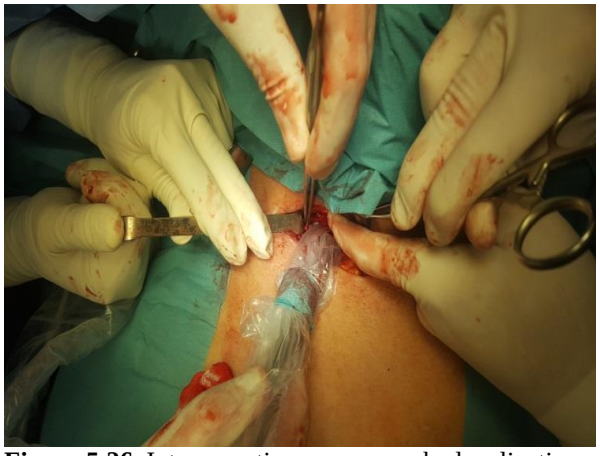


Figure 5.26. Intraoperative gamma probe localization

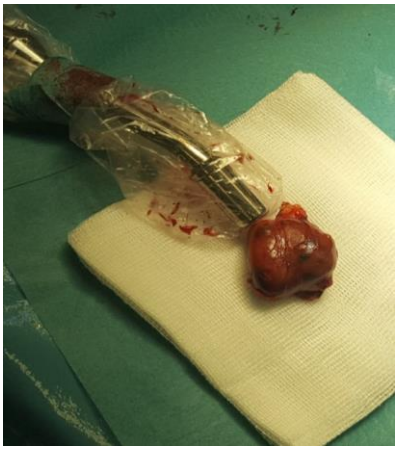


Figure 5.27. Removed PT and ex vivo radiation count

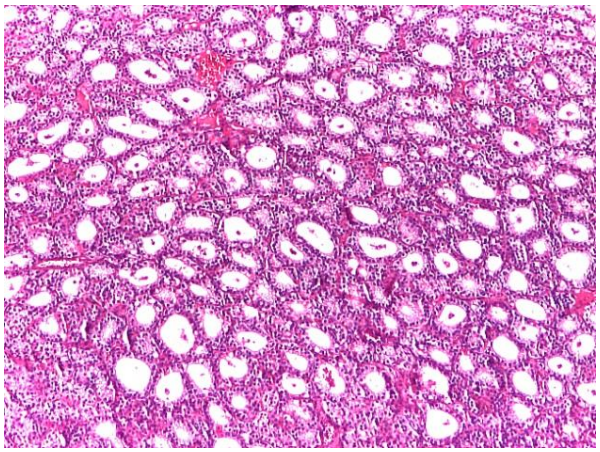


Figure 5.28. Acinar architecture in removed PT (HE, x 40)

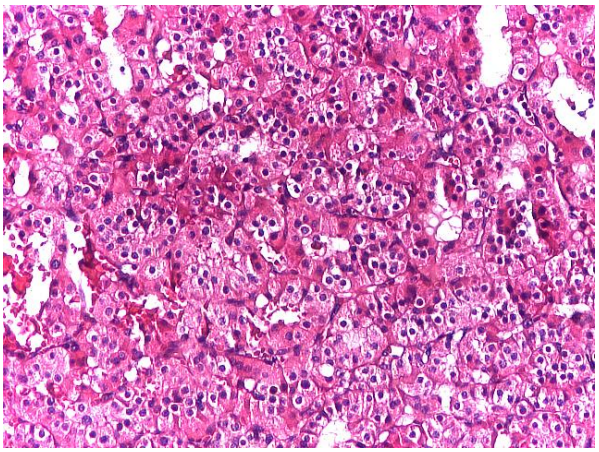


Figure 5.29. Chief and oxyphil cells in removed PT (HE, x 100)

5.3.4. Similarities and differences between the pathological findings in primary and secondary HPT

Pathological examination revealed more frequent solitary parathyroid adenoma in PHPT and more frequent multiglandular hyperplasia in SHPT ($p < 0.001$). The incidence of PTC was not different in patients with PHPT and SHPT ($p = 0.694$, Table 5.1). We did not find significant differences between the two groups with respect to the histological particularities of PTC ($p = 0.517$), TNM classification (lymph node status: $p = 0.253$; no distant metastases involved), tumor diameter ($p = 0.571$) and multifocality ($p = 0.507$, Table 5.2). The mean tumor size of PTCs under PHPT and SHPT categories revealed a significant difference ($p \leq 0.005$). PTC with a diameter larger than 1 cm were found only in 6 patients with PHPT but at none of the patients with SHPT (Table 5.2).

Table 5.2. Particularities of papillary thyroid carcinoma associated with PHPT or SHPT

Pathological characteristics	PHPT		SHPT		p value
	No. cases	(%)	No. cases	(%)	
Papillary thyroid carcinoma (PTC)	**19**		**9**		0.517
Classic variant of PTC	14	(73.6)	8	(88.9)	
Follicular variant of PTC	4	(21.1)	1	(11.1)	
Oncocytic variant of PTC	1	(5.3)	-	-	
T (tumor) classification					
T1 (a and b)	13	(68.4)	9	(100)	
T2	-	-	-	-	
T3 (a and b)	6	(31.6)	-	-	
T4	-	-	-	-	
N (lymph node) classification					0.466
Nx	13	(68.4)	4	(55.6)	
N0	5	(26.3)	5	(44.4)	
N1	1	(5.3)	-	-	
Maximum tumor diameter					
< 10 mm (microcarcinoma)	12	(68.4)	9	(100)	
≥ 10 and < 20 mm	3	(15.8)	-	-	
≥ 20 and < 40 mm	1	(5.3)	-	-	
≥ 40 cm	3	(15.8)	-	-	
Mean tumor size [mm]	13.6 ± 18.4		2.7 ± 2.1		0.005
Multifocality	4	(21.1)	1	(11.1)	0.507

PHPH – primary hyperparathyroidism, SHPH – secondary hyperparathyroidism;

5.4. DISCUSSION

PHPT is considered nowadays a frequent disease, mainly due to the high incidence of mild sporadic forms appearing more often in women between 50 and 60 years of age [Arrangoiz et al., 2016; Walker, Silverberg, 2018]. On the other hand, SHPT is commonly associated with CKD, as a reactive condition which appears at an earlier age than PHPT [Pitt et al., 2009; Messa, Alfieri, 2019; Rodríguez-Ortiz, Rodríguez, 2020]. Due to deleterious effects of very high PTH levels on bone (renal osteodystrophy), selected patients with SHPT need parathyroid gland removal as an ultimate attempt to arrest this severe complication [Pitt et al., 2009; Messa, Alfieri, 2019; Rodríguez-Ortiz, Rodríguez, 2020].

Several studies focuses on the association between parathyroid and thyroid surgery only in cases of PHPT [Ellenberg et al., 1962; Feind et al., 1964; Trout et al., 1972; Krause et al., 1991; Attie et al., 1993; Burmeister et al., 1996; Sidhu et al., 2001; Bentrem et al., 2002; Kösem et al., 2004; Beus et al., 2004; Masatsugu et al., 2005; Zheng et al., 2007; Monroe et al., 2008; Gates et al., 2009; Kutluturk et al., 2014] or SHPT [Kaptein et al., 1996; Miyauchi et al., 2017; Walker, Silverberg, 2018] alone, and just a few dealt with this association in both PHPT and SHPT operated cases taken together [Burmeister et al., 1996; Jovanovic et al., 2017].

Our study specifically evaluated the association of thyroid pathology in parathyroidectomized patients. We included 140 patients diagnosed with PHPT and 77 cases with advanced CKD and SHPT requiring parathyroidectomy. Seventy-five patients with PHPT and 31 patients with SHPT (53.6 and 40.3% respectively) underwent also thyroid surgery, mainly decided for nodular thyroid enlargement.

Patients with PHPT had a more advanced diagnostic age, predominance of females and of solitary parathyroid adenomas. Solitary parathyroid disease implied a more frequent

use of minimally invasive parathyroidectomy at PHPT patients, whereas open neck surgery was the elective technique used in patients with SHPT, where patients are more at risk for parathyroid hyperplasia.

Contrary to other reports, in our patients with PHPT we found parathyroid adenomas mainly on the left side (in 73.3% cases). The right location, identified in the previous study were made more than four decades ago, when the only surgical technique used was open neck surgery [Krementz et al., 1971; Fraser, 2009], or in recently one but on a geographically distinct population [Cuhaci et al., 2017]. Therefore, it seems that the substrate of different adenoma locations may be the genetic variations. In our study, ectopic glands were found in 3 cases (2.58%) of PHPT and one with SHPT, in which was performed MI-RP for identification of the rare mediastinal location. Theoretical ectopic glandular position can be present on 4–16% of cases, and could be within the mediastinum (often associated with the thymus), around the oesophagus, within the thyroid, or up to the jaw angle [Phitayakorn et al., 2006; Fraser et al., 2009].

The review of the literature shows that the most frequent type of thyroid surgery performed concomitantly in HPT is either total/subtotal thyroidectomy [Kutluturk et al., 2014; Emirikci et al., 2015] or unilateral lobisthmectomy [Jovanovic et al., 2017]. Total thyroidectomy was commonly used in our study, justified by the high frequency of nodular disease in a region previously known for iodine deficient disorders [Coculescu et al., 2001; Buzduga et al., 2011]. The most frequent histological findings described in the literature are nodular goiter (8.4–47% of HPT cases), followed by thyroiditis (1.4–17.6%) and solitary thyroid adenoma (3.8–6.4%) [Sidhu et al., 2001; Bentrem et al., 2002; Kösem et al., 2004; Masatsugu et al., 2005; Zheng et al., 2007]. Graves' disease remains an infrequent association (1.8%) [Masatsugu et al., 2005]. Our findings were in accordance with these previous results with a high percent on colloid or nodular goiter with or without adenomatous nodules (72.0%), Hashimoto thyroiditis (16%) and thyroid adenomas (12%).

Parathyroid carcinoma was diagnosed in only one case of PHPT, and it did not associate any thyroid disease. Other studies also described an incidence of parathyroid carcinoma in less than 1% of PHPT cases [Ruda et al., 2005]. The association of parathyroid cancer and differentiated TC was rarely described, suggesting coincidence rather than causality [Walgenbach et al., 2000; Savli et al., 2001; Schoretsanitis et al., 2002; Lin et al., 2005; Vargas-Ortega et al., 2018; Podlasek et al., 2018; Jeong et al., 2020].

Non-medullary TC, who originated from the follicular cells of the thyroid gland, and included mostly PTC, is reported with an incidence varying from 0.9 to 18.2% (mean value of 4%) of surgically treated PHPT patients [Palmieri et al., 2017]. Autopsy controlled studies show that TC occurs more frequently in patients with PHPT, fact not observed for autoimmune or thyroid nodular disease [Kaplan et al., 1971; Lever et al., 1983]. PHPT patients seem to have an increased overall cancer risk and parathyroidectomy is not a risk-reducing, but rather a delaying factor in the occurrence of cancer [Nilsson et al., 2007].

A synopsis of the studies that analyzes the presence of TC in patients surgically treated for PHPT is presented in Table 5.3. We found PTC in 19 (13.6%) of our PHPT cases - fourth highest rate of occurrence reported in the mainstream (Table 5.3).

In accordance with previous reports [Fujiwara et al., 1994; Boehm et al., 2011; Kutluturk et al., 2014; Jovanovic et al., 2017; Kaminskyi et al., 2017], twelve out of 19 cases (63.2%) were PTMC and did not involve any lymph node or distant metastases. Low risk PTMC could be managed with lobectomy instead of total thyroidectomy [Conzo et al., 2014; Calò et al., 2017; Song et al., 2019], improving the quality of life. Rare metastatic PTMC cases were however described [Morita et al., 2008].

Table 5.3. Frequency of thyroid cancer in patients undergoing parathyroidectomy for PHPT

Study	Country	PHPT patients	Concomitant thyroid cancer	
		No.	*No.*	*%*
Vargas-Ortega et al., 2018	Mexico	59	12	20.3
Masatsugu et al., 2005	Japan	110	20	18.2
Kösem M et al., 2004	Turkey	51	9	17.6
Current study, 2019	**Romania**	**140**	**19**	**13.6**
Attie et al., 1993	USA	242	31	12.8
Gates JD et al., 2009	USA	24	3	12.5
Kutlutürk K et al., 2014	Turkey	46	5	10.9
Trout et al., 1972	USA	30	3	10.0
Jeong et al., 2020	Korea	154	14	9.1
Ellenberget al., 1962	USA	93	7	7.5
Wright MC et al., 2017	USA	103	7	6.8
LiVolsi et al., 1976	USA	471	31	6.6
Kaplan et al., 1971	USA	62	4	6.5
Krementz et al., 1971	USA	100	6	6.0
Morita et al., 2008	USA	200	12	6.0
Petro et al., 1974	USA	104	5	4.8
Monroe et al., 2008	USA	194	9	4.6
Sindhu et al., 2001	Australia	65	3	4.6
Prinz et al., 1982	USA	351	15	4.3
Podlasek et al., 2018	Poland	95	4	4.2%
Krause et al., 1991	Germany	163	6	3.7
Feind et al., 1964	USA	119	4	3.4
Nishiyama et al., 1979	Japan	420	13	3.1
Beus et al. , 2003	USA	101	3	3.0
Burmeister et al., 1996	USA	700	18	2.6
Yazici et al., 2015	Turkey	228	6	2.6
Jovanovic et al., 2017	Serbia	849	21	2.5
Linos et al., 1982	USA	2058	51	2.5
Bentrem et al., 2002	USA	580	12	2.1
Zheng et al., 2007	China	52	1	1.9
Ogburn et al., 1956	USA	230	4	1.7
Emirikçi et al., 2015	Turkey	550	5	0.9
Total		**8744**	**363**	**4.15**

Parathyroidectomy decreases mortality in patients with CKD and severe SHPT [Apetrii et al., 2017]. Subtotal parathyroid excision is frequently needed, since all parathyroid glands are involved. Several authors described a more frequent association of CKD [Ito et al., 2009; Ito et al., 2014; Miyauchi et al., 2017], chronic dialysis [Eigelberger et al., 2000; Prager et al., 2003], SHPT [Kaptein et al., 1996; Lee et al., 2017] or kidney transplant [Linos et al., 1982; Dideban et al., 2016, Walker, Silverberg, 2018] with TC than in the general population. Although all these studies suggested that CKD is accompanied by an increased risk of malignancy, including PTC, they did not, however, systematically evaluate the patients with SHPT submitted to both parathyroidectomy and thyroidectomy. These patients are different than other patients with CKD, in terms of higher severity of parathyroid and bone disease.

We confirmed 9 cases of PTC out of the 31 SHPT patients also operated for goiter, all of them having infracentrimetric dimensions (PTMC) while only one had multifocal lesions. Similarly, other studies confirmed a predominance of PTMC, being attributed to closer thyroid surveillance in the context of HPT [Burmeister et al., 1996; Hatada et al., 1998; Ma et al., 2021]. A previous study in our center including 43 patients with SHPT who underwent parathyroidectomy between 1994 and 2004 and, when indicated, concomitant thyroid surgery (in 17 cases, 39.5% of SHPT), revealed a lower incidence of PTMC of only 4.7% (2 out of 17 cases) [Diaconescu et al., 2011]. The participants were however fewer and PTMC incidence was not significantly different from the present study (9 out of 31 cases, $p = 0.125$). After reviewed the publications with the association SHPT and PTC, excluding case reports, the reporting data are summarized in Table 5.4.

Table 5.4. Frequency of TC in patients undergoing parathyroidectomy for SHPT

Study	Country	SHPT patients	Concomitant thyroid cancer	
		No.	*No.*	*%*
Hatada et al., 1998	Japan	19	4	21
Current study, 2019	**Romania**	**77**	**9**	**11.7**
Jeong et al., 2020	Korea	154	9	7.2
Ma et al., 2021	China	541	34	6.4
Diaconescu et al., 2011	Romania	43	2	4.7
Total		**834**	**58**	**6.96**

PHPT and SHPT are different etiopathogenic entities, and literature data regarding the concomitant investigation between these and TC are scarce [Miki et al., 1992; Burmeister et al., 1996; Klyachkin et al., 2001; Jovanovic et al., 2017]. Our study demonstrated for the first time that the incidence of PTC was high and similar in both PHPT and SHPT (13.6 and 11.7% respectively, $p = 0.517$) in patients operated in the same surgical center. Likewise, Burmeister et al. reported similar, albeit lower frequencies for both PHPT (2.6%) and SHPT (3.2%, $p = 0.550$) [Burmeister et al., 1996].

The opinions on the association of HPT with non-medullary TC or thyroid nodularization shifted over time from mere coincidental [Ogburn et al., 1956; Burmeister et al., 1996; Bentrem et al., 2002; Seehofer et al., 2005] to considering them causally related [Morita et al., 2008; Ryan et al., 2014; Kutluturk et al., 2014; Cinamon et al., 2015; Wright et al., 2017]. Although PHPT and concomitant MTC are well described in MEN2A syndrome, no obvious genetic link between PTC and PHPT has been yet demonstrated. Most cases of PHPT are sporadic and may associate a germline mutation in MEN1, CDC73, CASR, CDKIs or PTH genes, especially in patients under 45 [Thakker et al., 2016]. PTC is the most common thyroid malignancy and mutations in RET proto-oncogene, BRAF and Ras may be involved in its development [Cappola et al., 2013], especially in younger patients and after radiation exposure [Bounacer et al., 1997].

Some studies investigated several predisposing factors for PTC in PHPT, such as the tumor promoting effect of PTH [Seehofer et al., 2005], the goitrogenic effect and increased mitotic activity induced by hypercalcemia [LiVolsi et al., 1976; Prinz et al., 1982; Nilsson et al., 2007] and neck irradiation [Nishiyama et al., 1979; Prinz et al., 1982; Nilsson et al., 2007]. A presumed role for PTH excess in triggering the onset of PTC remains controversial [Burmeister et al., 1996]. One of the criteria for choosing parathyroidectomy in SHPT due to CKD is that of high PTH levels (over 800 pg/ml) [Pitt et al., 2009], whereas even milder forms of PHPT with modestly elevated PTH actually represent an indication for surgery [Walker, Silverberg, 2018]. Not surprisingly, our patients with SHPT had therefore much higher preoperatory PTH levels when compared to PHPT patients. Despite this clear

quantitative difference, the incidence of PTC was similar in the two groups (25.3% in PHPT and 29% in SHPT, $p = 0.694$), suggesting that higher PTH may not increase PTC incidence further, and that PTH may therefore even not be involved at all in the onset of PTC. Moreover, the incidence of PTC drastically increased recently. This increase is exclusively due to the over-diagnosis of PTMC frequently found with more detailed histological investigation, more often thyroid ultrasound investigation and ultrasound-guided FNAB [Ito et al., 2014; Dideban et al., 2016; Kaliszewski et al., 2016; Miyauchi et al., 2017; Takano et al., 2017; Bradley et al., 2017; Uhliarova et al., 2018]. The evolution of PTMC is usually indolent and their radical therapy did not contribute to a decrease of mortality, therefore certain authors even recommend conservative therapy and follow-up in these cases [Ito et al., 2009; Dideban et al., 2016; Miyauchi et al., 2017]. The presence of PTMC was also described in up to one third of thyroid gland autopsies of persons deceased for other reasons [Kaliszewski et al., 2016; Lee et al., 2017; Bradley et al., 2017; Uhliarova et al., 2018].

Since PTMC incidence is so high in the general population, it is not unexpected to observe the presence of PTMC also in patients operated for both thyroid and parathyroid glands, irrespective of parathyroid pathology. Six patients with PHPT, but no patients with SHPT had, however, PTC with a diameter larger than 1 cm, and of over 4 cm in 3 cases. These results suggest that larger forms of PTC with presumed poorer prognosis may be occasionally found into the thyroids of patients with PHPT, but less frequently in patients with SHPT. The coexistence of a nodular lesion in the thyroid of patients with PHPT, which is usually detectable before surgery during ultrasound investigation of the cervical anterior region should therefore not be neglected, FNAB being strongly advisable in order to rule out malignancy.

It is not clear how PHPT may influence the evolution of differentiated thyroid malignancy. As stated above, PTH levels seem not to be important since larger PTC were found exclusively in patients with PHPT, despite their much lower PTH compared to SHPT patients. A presumed common genetic background, although not yet defined, may thus predispose certain patients to both PHPT and PTC with larger dimensions.

5.5. FINAL REMARKS

PTCs are frequently diagnosed in association with HPT, especially in regions with endemic goiter. Undiagnosed concomitant thyroid nodules represent the main hazard to minimally invasive procedures for parathyroid adenomas, since they may veil malignancy.

Our study is the first which compares the incidence and histology of TC in patients with surgical treatment for PHPT or SHPT caused by CKD. The limitations of this study consist, however, in its retrospective nature, as well as the absence of a control group submitted to thyroid surgery, but without coexistent parathyroid pathology.

In our study, the incidence of PTC was similar in patients with PHPT and SHPT, even if PTH levels were significantly higher in patients operated for SHPT, suggesting that PTH may not be directly involved in the PTC pathogenesis.

Although PTC found in patients with SHPT were all PTMC, seven out of the 19 patients with PHPT and PTC had thyroid tumors with a diameter above 1 cm, and even above 4 cm in three cases. This observation raises the following question that needs to be answered appropriately: why larger PTC are present in an important number of patients with PHPT, but not SHPT.

Based on the solid expertise in endocrinology, endocrine surgery and nephrology provided by the multi-disciplinary team involved in this research, the study will be further developed for a deeper understanding of the simultaneous changes in thyroid and parathyroid glands, in patients with PHPT and SHPT.

CHAPTER 6.
MEDULLARY THYROID CARCINOMA: FROM DIAGNOSTIC TO HISTOLOGIC SUBTYPES

6.1. INTRODUCTION

Medullary thyroid carcinoma (MTC), C-cell carcinoma, solid carcinoma with amyloid stroma, or parafollicular cell carcinoma is a malignant epithelial tumor that accounts for only 2–5% of all thyroid malignancies [DeLellis et al., 2017]. It was not recognized as a distinct pathologic type of thyroid carcinoma (TC) until late 70s [Hazard, 1977]. The correct classification of this thyroid neoplasm followed earlier histopathologic studies that identified a separate population of parafollicular or C-cells, with an embryologic origin from the neural crest, responsible for the production of the peptide hormone calcitonin [Zabel et al., 1997]. The unique features of MTC include its intermediate level of biologic aggressiveness within the spectrum of TC, the production of calcitonin – the specific hormone that can serve as a sensitive tumor marker for occult or recurrent diseases, and its lack of susceptibility to radioiodine, making complete surgical removal of all cancer and lymphatic metastases the primary treatment goal [Hazard, 1977; Bhattacharyya et al., 2003; Wells et al., 2015].

The deciphering of MTC molecular pathogenesis advanced with the identification of germline-activating mutations in the RET proto-oncogene, that are responsible for its development in association with the multiple endocrine neoplasia type 2 (MEN2) syndromes, as well as similar somatic mutations that underlie a significant proportion of sporadic tumors [Wells et al., 2013]. Direct genetic testing allows detection of patients who have inherited a disease-associated RET mutation and have 100% risk of developing TC during their lifetime. This ability to make a definitive genetic diagnosis prior to the recognition of clinical, sonographic, and/or biochemical evidence of neoplasia allows the distinctive opportunity to perform an early thyroidectomy and to remove the organ before an invasive malignancy develops. Early prophylactic thyroidectomy for patients with MEN2 was one of the first and is perhaps still one of the best examples of a surgical intervention based on genetic testing that is intended to completely prevent subsequent cancer occurrence in patients with inherited cancer susceptibility [Wells et al., 2015].

6.2. SPORADIC *VERSUS* HEREDITARY FORMS

Approximately 75–80% of MTCs occur sporadically, while the inherited forms of MTC are responsible for the rest of the cases [Farreira at al., 2013; Wells et al., 2013; Wells et al., 2016]. The central roles in the pathogenesis of both hereditary and sporadic forms of MTC are mutations in the rearrangements of rearranged during transfection (*RET*) proto-oncogene, which cause an early oncogenic event that leads to tumorigenesis [Plazza-Menacho et al., 2014]. The most frequent variants are usually located in exons 10, 11, and 13 through 16 of the *RET* gene [Paragliola et al., 2018]. *RET* mutations occur in approximately 50% of patients with sporadic MTC and are associated with poor prognosis compared with the absence of such a mutation [Malgalhaes et al., 2003; Taccaliti et al., 2011; Wells et al., 2015].

Based on a specific germline mutation in the *RET* proto-oncogene [Roy et al., 2013], heritable MTC can be a component of three syndromes: multiple endocrine neoplasia 2A (MEN2A) syndrome, multiple endocrine neoplasia 2B (MEN2B) syndrome, and familial medullary thyroid carcinoma (FMTC) syndrome. All three syndromes are autosomal dominant and have variable phenotypic expression and penetrance [Chernock et al., 2015]. The sporadic MTC is usually diagnosed around the age of 60, but the heritable cases are diagnosed at a younger age [Kaserer et al., 2001]. The most common subtype of MEN2 is MEN2A, which is responsible for about 95% of all MEN2 cases [Wells et al., 2013]. Both MEN2A and MEN2B are associated with other endocrine abnormalities and clinical features (Table 6.1).

Patients with MEN2A syndrome will develop an MTC, often in the second or third decades of life, and will be diagnosed during their life with pheochromocytoma (50% of all MEN2A cases), and with parathyroid hyperplasia (only 25–35% of cases). Mutations associated with MEN2A are primarily located in the extracellular cysteine-rich domain of the RET proto-oncogene, usually in exon 10 (codons 609, 611, 618 or 620) or exon 11 (codon 634) but can also be found in the intracellular tyrosine kinase domain in exon 13 (codons 768 or 790), exon 14 (codon 804), or exon 15 (codon 891) [Eng et al., 1996; Kouvaraki et al., 2005; Wells et al., 2015]. Whereas in past decades, mutations in codon 634 (exon 11) accounted for the vast majority of MEN2A cases, more recently the prevalence of other mutations has increased [Grubbs, Gagel, 2015]. Individuals with a RET codon 634 mutation represent the prototypical MEN2A patient and have the greatest risk of developing early MTC followed by those with mutations in codons 609, 611, 618, 620, or 630, while mutations in codons 768, 790, 804, or 891 impart the lowest risk for clinically aggressive MTC [Wells et al., 2015].

Patients with MEN2B will have Marfanoid habitus, and will develop heritable MTC at the youngest age, often under the age of 10. Half of them will be also diagnosed with pheochromocytoma, and eventually will express intestinal ganglioneuromas, mucosal neuromas, ocular abnormalities and musculoskeletal manifestations [Sippel et al., 2008; Wells et al., 2013]. MEN2B is due to a de novo *RET* mutation in over 90 % of cases [Brauckhoff et al., 2014; Bottici et al., 2015], and the M918T *RET* mutation is identified in over 95 % of MEN2B patients. Rarer MEN2B mutations include double *RET* mutations involving codon 804 and *A883F* mutation, which may be associated with a less aggressive MTC phenotype [Jasim et al., 2011].

Familial MTC (FMTC) is characterized by the absence of any extrathyroidal endocrine tumors, but will develop a MTC during their adult life [Wells et al., 2015]. FMTC was historically regarded as a separate syndrome from MEN2A. FMTC was only considered when four or more family members across a wide range of ages had isolated MTC, with no other features of MEN2A.

Current guidelines include the performance of prophylactic thyroidectomy at approximately age 5 for patients with MEN2A and within the first years of life for patients with the more aggressive MEN2B syndrome [Lairmore et al., 2015]. For patients with mutations in one of the more infrequent, lower risk *RET* codons, thyroidectomy may be recommended at a later age [Lairmore et al., 2015].

Patients with hereditary MTC may be diagnosed in the first or second decade of life based on a program of biochemical screening in individuals at known risk, with an earlier distribution than for sporadic tumors. MTC incidence, however, generally increases steadily with age and then tapers off somewhat in the extreme elderly [Aschebrook-Kilfoy et al., 2011]. Regardless of the type of MTC, sporadic or heritable, female and white persons are affected more frequently [Roman et al., 2006].

The major prognostic factors of survival in MTC are age and tumoral stage at

diagnosis [Pillarisetty et al., 2009; Wells et al., 2016]. The 10-year survival rates range from 70% to 90% and 56% to 87% at five years [Rosai et al., 1990]. Patients younger than 40 with tumors limited to the thyroid gland have a better prognosis than older patients [Rosai et al., 1990; Pillarisetty et al., 2009]. The 10-year survival rate is 75% for patients with regional spread, decreasing to 40% for those with distant metastases [Thyroid Cancer Survivors' Association (ThyCa), 2014].

Table 6.1. Clinical manifestations in hereditary MTC syndromes
[Chernock et al., 2015, Links et al, 2015]

Clinical manifestations in MEN syndromes	Prevalence (%)		
	MEN2A	FMTC	MEN2B
MTC	100	100	100
Pheochromocytoma	10–60	0	50
Parathyroid hyperplasia/ parathyroid adenoma	10–30	0	0
Cutaneous lichen amyloidosis	10	0	0
Marfanoid habitus	0	0	100
Intestinal ganglioneuromatosis	0	0	60–100
Mucosal neuromas (tongue, subconjunctivas)	0	0	70–100

FMTC: Familial medullary thyroid carcinoma; MEN: Multiple endocrine neoplasia; MTC: Medullary thyroid carcinoma.

6.3. CLINICAL, BIOCHEMICAL AND IMAGING CHARACTERIZATION

Usually, upon admission to an Endocrinological Unit, patients present a painless, palpable thyroid nodule during physical examination, often accompanied by cervical adenopathies. The thyroid nodule is associated with clinical symptoms, such as dysphagia, hoarseness, dyspnea, and coughing. Moreover, some paraneoplastic symptoms may be present because MTC can release a wide range of ectopic hormones and other substances as well, in various amounts (Table 6.2).

Challenges regarding the implementation of routine serum calcitonin screening for patients with thyroid nodules in the USA include setting uniform threshold values, gender-specific thresholds, and applicability of stimulatory testing. Current European consensus guidelines recommend routine measurement of serum calcitonin in the initial diagnostic evaluation of all patients with thyroid nodules with concurrent evaluation for comorbid conditions which may cause false positive elevation of serum calcitonin levels: renal failure, ectopic calcitonin production from non-thyroidal neuroendocrine tumors, hypergastrinemia, Hashimoto's thyroiditis [Pacini et al., 2006]. Ultrasonographic findings in MTC are variable, with 66–72% demonstrating characteristic suspicious features including height greater than width, spikes, hypoechogenicity, calcifications, extrathyroidal extension, lymphadenopathy, or extranodal extension of the tumor mass [Lee et al., 2010]. While not universally present, suspicious ultrasound features confer a 450% increased risk of advanced stage MTC, especially for extrathyroidal extension or metastatic lymphadenopathy [Trimboli et al., 2014].

All the secretory products may cause metabolic disorders and clinical manifestations, such as diarrhea, painful bone metastasis, flushing, or Cushing's syndrome. The biochemical activity of MTC also includes the production of carcinoembryonic antigen (CEA) that, along with calcitonin, are sensitive tumor biomarkers that facilitate the diagnosis of MTC, and their postoperative detection correlates well with tumor relapse or progression being useful in assessing treatment effectiveness [Roy et al., 2013]. However, calcitonin-doubling times along with large tumor sizes, node metastases, and extrathyroid extension have been identified as prognostic factors for MTC [Ito et al., 2016].

Table 6.2. Secretory products of MTC
[Roy et al., 2013, Orlandi et al., 2001, Magalhaes et al., 2003]

Hormones and pro-hormones	Enzymes	Others
Calcitonin	NSE	CEA
ACTH	Histaminase	CgA
β-Endorphin	DOPA-decarboxylase	NGF
β-Melanocyte stimulating hormone	Kinin–kallikrein system	Syn
Somatostatin	–	Neurotensin
Neurotensin	–	Prostaglandin
Cathecolamine	–	Histamine
Substance P	–	Serotonin
Corticotrophin releasing hormone	–	–
Vasoactive intestinal peptide	–	–
Bombesin	–	–
Gastric-releasing peptide	–	–

ACTH: Adrenocorticotropic hormone; CEA: Carcinoembryonic antigen; CgA: Chromogranin A; DOPA: 3,4-Dioxyphenylalanine; MTC: Medullary thyroid carcinoma; NGF: Nerve growth factor; NSE: Neuron-specific enolase; Syn: Synaptophysin.

6.4. PITFALLS IN THE CYTOLOGICAL ASSESSMENT OF FNAB

Fine-needle aspiration biopsy (FNAB) is a useful and safe procedure for the preoperative diagnosis of MTC. It is considered a first line diagnostic test for evaluating a thyroid lesion, but it is not definitive, as the diagnostic accuracy of this method is reduced (76%) –probably because this type of cancer is rare and exhibits a wide range of cytological features. Indian researchers obtained a definite cytological diagnosis of medullary carcinoma in 87.1% of cases based on cytomorphology alone and in 12.9% of cases based on immunocytochemistry for calcitonin [Kaushal et al., 2011]. Moreover, in order to establish a definite diagnosis of MTC on FNAB, some Japanese authors proposed calcitonin measurement using needle washout fluid and immunocytochemical staining using anti-calcitonin antibody for diagnosing MTC on FNAB [Suzuki et al., 2017].

Cytological smears should be processed and stained by routine Papanicolaou and May-Grünwald–Giemsa (MGG) techniques.

FNAB samples of MTCs represent an adequate cellular aspirate when a dispersed cell pattern could be obtained on the smear. Usually, MTC exhibits a monomorphic cell pattern, or a slight to moderate pleomorphism. The tumor cells have a polygonal, round, plasmacytoid, and/or spindle-shaped, singly or in clusters (Figures 6.1 - 6.4). Cytoplasm is basophilic, granular, variable in quantity (moderate to abundant), and, in some cases, long cytoplasmic cell processes can be seen (Figures 6.5 – 6.6). Tumor nuclei are round, eccentrically placed, with “salt and pepper” chromatin (which represent neuroendocrine nuclear features) (Figure 6.6). Occasionally, intranuclear pseudoinclusions (indistinguishable from those seen in papillary carcinoma) can be noticed (Figure 6.7). Binucleated and multinucleated cells are usually seen (Figure 6.8). Nucleoli are usually discreet. Amyloid deposits may be found in more than half of MTCs. It appears as a dense, amorphous material, in the smear background similar with colloid [Kini et al., 2011] that stained in blue with Papanicolaou staining [Maruta et al., 2019] or variable shades of magenta with MGG (Figures 6.9 - 6.10). Congo Red staining helps to differentiate amyloid from colloid or hyaline fragments, being diagnostic for MTC [Vibhuti et al., 2013; Maruta et al., 2019].

Cytological examination can also highlight the morphological variants of MTCs. Kaushal *et al.* (2011) identified a follicular arrangement in 14.1% of their 78 cases, the melanin production variant, which was a rare event (2/78 cases), the giant cell variant (1/78 cases), with large pleomorphic nuclei and numerous bizarre tumor giant cells, the small cell variant, paraganglioma-like variant and papillary variant (each of them representing only one case from all 78 cases) [Kaushal et al., 2011].

On FNAB smears, MTC is one of the great mimickers of PTC; nuclear elongation, intranuclear pseudoinclusion, groove-like formation, and nuclear membrane irregularity are one of those cytomorphologic features that can be seen in both malignancies. However, predominantly dispersed cell population, neuroendocrine-type chromatin, some binucleation, eccentric nuclei, and the presence of both plasmacytoid and spindle cells help to identify MTC [Pitman et al., 2010]. If in doubt, immunohistochemical studies and/or serum calcitonin analysis will give the final diagnosis. Other thyroid lesions that must be excluded were Hürthle cell neoplasm, ATC, hyalinizing trabecular tumor (HTT), plasmacytoma, and metastatic tumors (particularly melanoma) [Pitman et al., 2010].

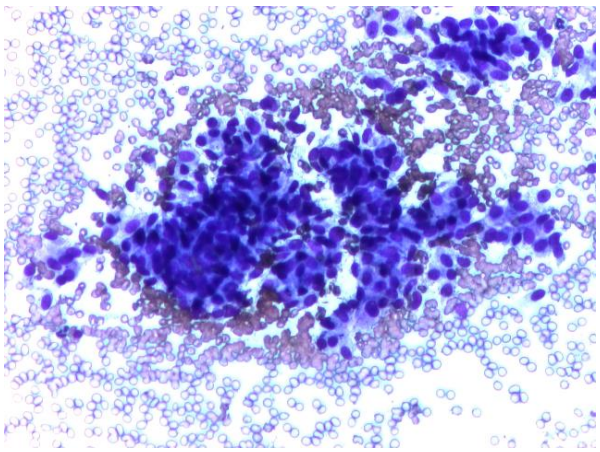


Figure 6.1. FNAB smear: richly cellular smear with three-dimensional cellular aggregations (MGG, x 100)

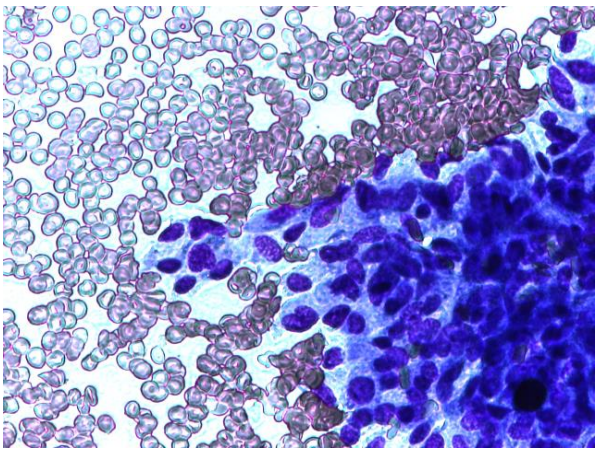


Figure 6.2. FNAB smear: round and fusiform cells with moderate pleomorphic aspect (MGG, x 200)

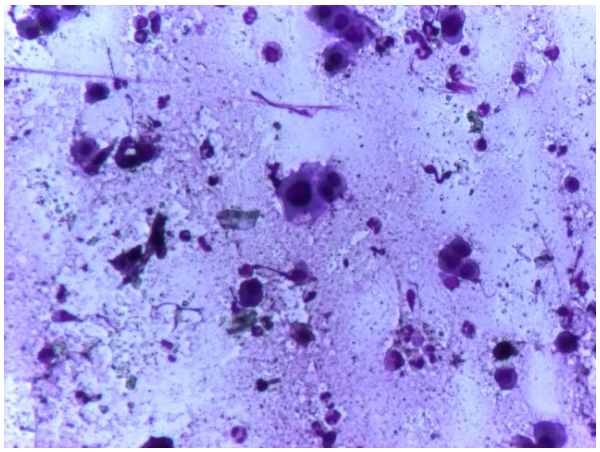


Figure 6.3. FNAB smear: isolated cells with oxyphilic aspect (MGG, x 100)

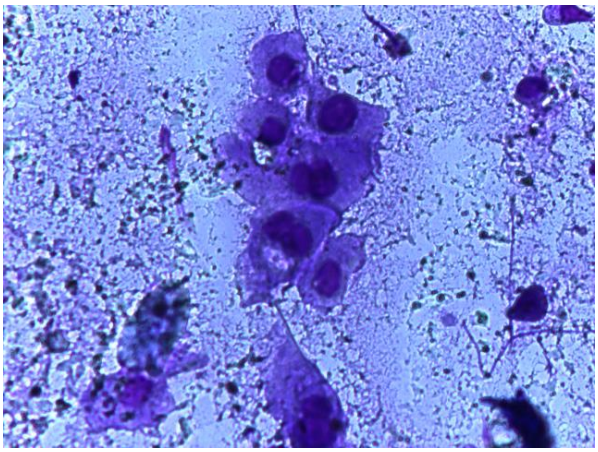


Figure 6.4. FNAB smear: details for isolated cells with oxyphilic aspect (MGG, x 200)

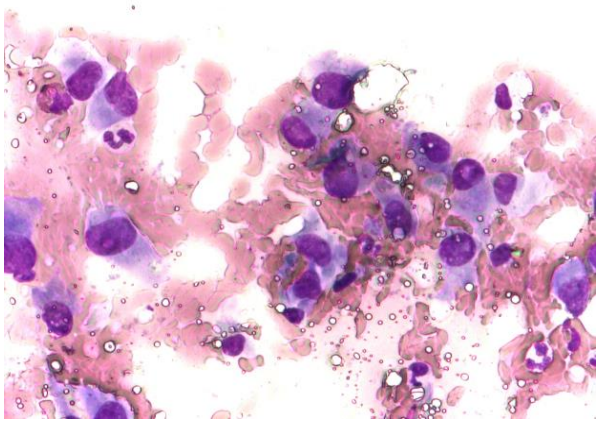


Figure 6.5. FNAB smear: dispersed cellular pattern with isolated cells and moderate pleomorphism (MGG, x 200)

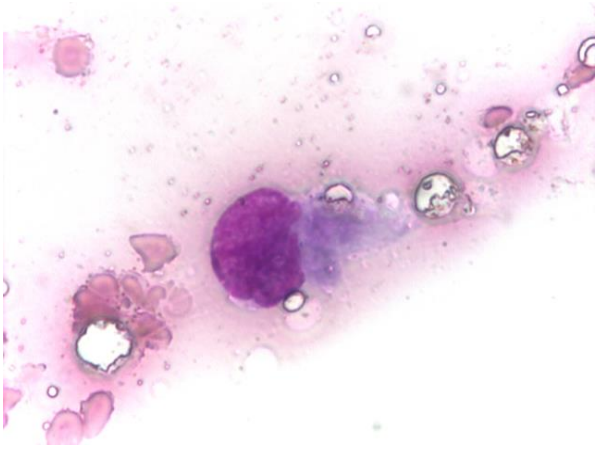


Figure 6.6. FNAB smears: eccentrically nucleus with "salt and pepper" chromatin and long cytoplasmatic processes (MGG, x 200)

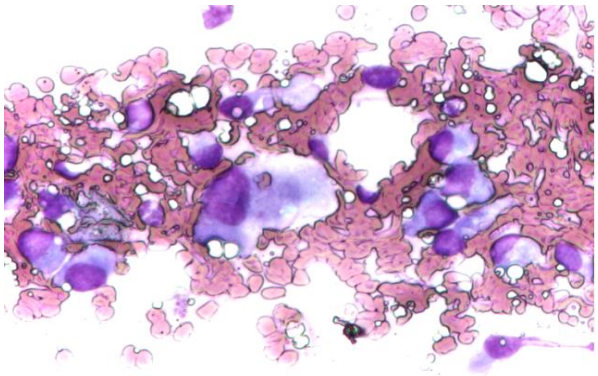


Figure 6.7. FNAB smears: nuclear pseudoinclusions (MGG, x 200)

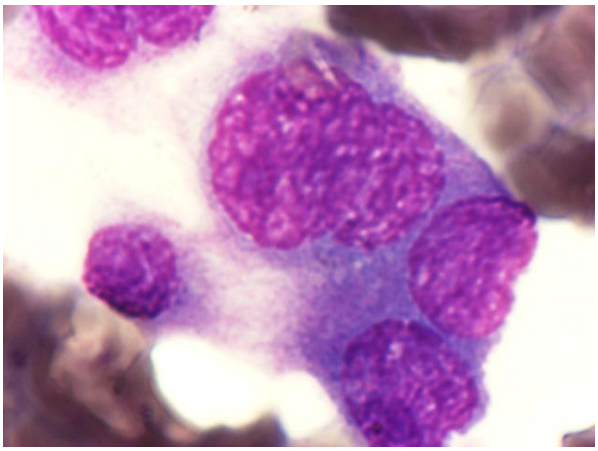


Figure 6.8. FNAB smears: uni- and bi-nucleated cells with granular chromatin (MGG, x 1000)

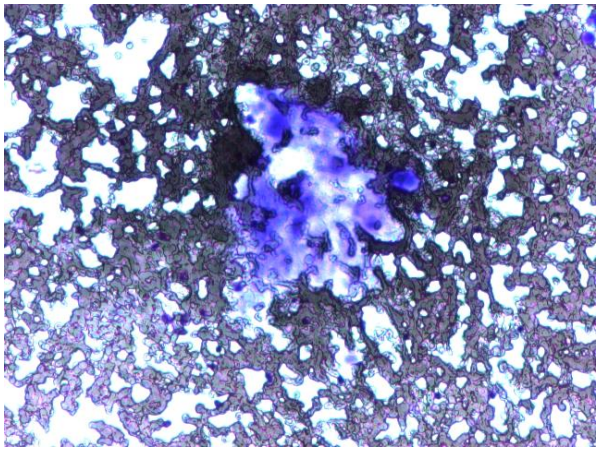


Figure 6.9. FNAB smear: amyloid deposit (MGG, x 100)

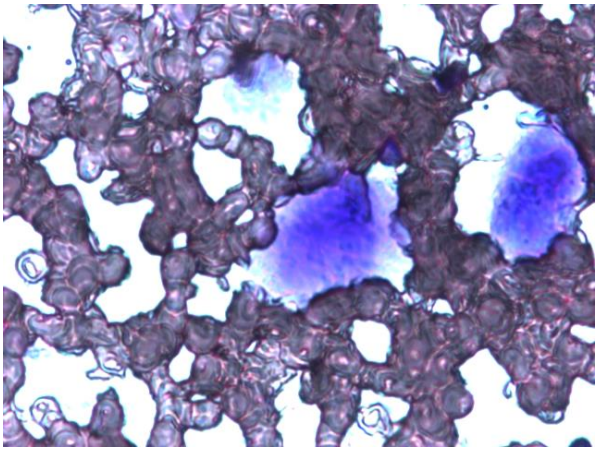


Figure 6.10. FNAB smear: detail of amyloid deposit (MGG, x 400)

6.5. DIFFERENTIAL DIAGNOSIS

The most important component of making the diagnosis of MTC is to consider it in the differential diagnosis. Meanwhile in many places the preoperative measurement of serum calcitonin is not routinely performed in the evaluation of thyroid nodules, this biochemical marker is not used to predict the likelihood of this disorder in patients with thyroid nodules, and often, the pathologist is the first to raise the possibility of the diagnosis of MTC and differentiate it from other endocrine pathologies [Lloyd et al., 2017; Verma et al., 2017]. Since MTC cells surround and trap thyroid follicles, the resulting pseudofollicular appearance can mimic a follicular lesion [Chan et al., 2013]. In addition, some rare tumors form true fibrovascular cores (papillary variant of MTC), or tumor dehiscence and fixation artifact can create pseudopapillae (pseudopapillary variant of MTC), and cystic changes (cystic variant of MTC); these variants can be mistaken for PTC [Chan et al., 2013; DeLellis et al., 2017]. Intranuclear pseudoinclusions and nuclear grooves, although extremely rare, can further complicate this distinction. The formation of glandular structures raises the possibility of an adenocarcinoma, and occasional mucinous variants have been described [Mardi et al., 2013; DeLellis et al., 2017].

MTC is usually located at the junction of the upper and medium thirds of the thyroid lobes, which corresponds to areas where C-cells are commonly placed [Gambardella et al., 2019]. A macroscopic examination of the thyroid gland may point to the type of MTC. Hereditary MTCs are more often multifocal and bilateral, being located in the upper to middle parts of the thyroid lobes [Moo-Young et al., 2009] (Figure 6.11). On the other hand, sporadic MTC develops as a unilateral, single, solid, sharply circumscribed, but non-encapsulated tumor, with a dense consistency, and a white-gray to tan color [Roy et al., 2013; DeLellis et al., 2017]. MTCs can have variable sizes ranging from 0.1 cm in diameter to those that replace the entire thyroid lobe (Figure 6.12). The presence of cystic degeneration or area of necrosis is extremely rare.

It was suggested that the term medullary thyroid "microcarcinoma" must be applied only for those tumors measuring less than 0.5 mm with a complete absence of metastatic disease or elevated post-operative calcitonin levels [Pillarisetty et al., 2009]. The recent WHO Classification (2017) defined this pathological entity as a tumor measuring less than 1 cm in diameter [DeLellis et al., 2017].

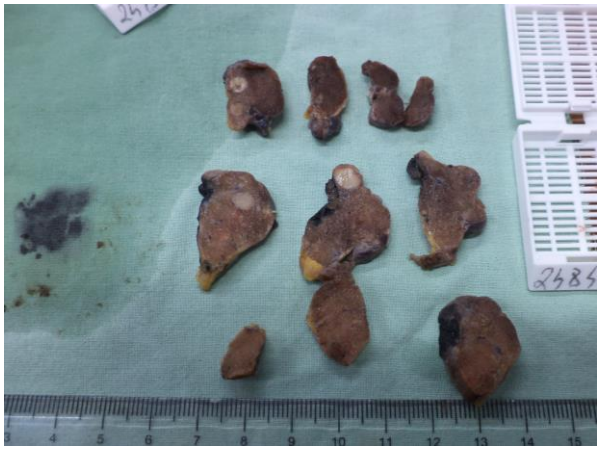


Figure 6.11. Gross specimens of hereditary MTC: multicentric lobulated, solid, and firm grayish masses

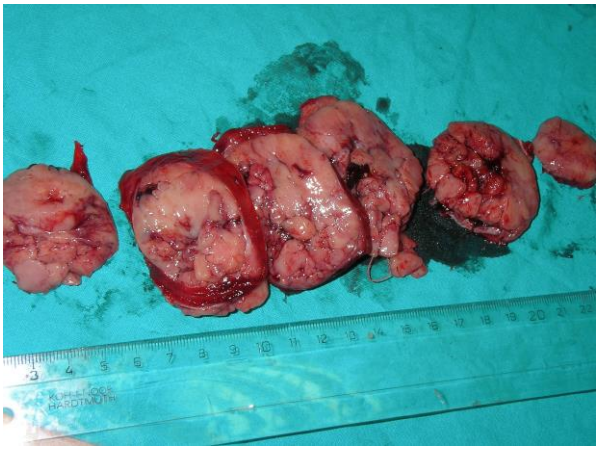


Figure 6.12. Gross specimens of MTC: a grayish tumor replaced the entire thyroid lobe and measured 3x6 cm

Usually, MTCs are identified on histological sections stained with hematoxylin–eosin (HE). At microscopic examination, most sporadic and heritable MTCs appear as well circumscribed but non-encapsulated tumors [Etit et al., 2008; DeLellis et al., 2017], exhibiting a solid pattern of growth with a wide morphological variety that can mimic any other thyroid malignancy.

The classical MTC shows a lobular, trabecular, insular or sheet-like growth arrangement [DeLellis et al., 2017]. Although most of the tumors appear sharply circumscribed at the gross level, microscopic examination often reveals extension of the tumor into the adjacent thyroid tissue [DeLellis et al., 2017].

The prototype cell of MTC is round, polygonal, plasmacytoid, or spindle in shape, with common admixtures of these cell types [Rosai et al., 1990; Venkataramana et al., 2018] (Figures 6.13 – 6.16). Usually, tumor cells have eosinophilic to amphophilic granular cytoplasm, due to secretory granules [Zaatari et al., 1983]. Regarding the nuclei morphology, most of the MTCs present round to oval pattern, with coarsely clumped ("salt and pepper") chromatin, indistinct nucleoli, and occasional nuclear pseudoinclusions. Bi- or multinucleated giant tumoral cells may be identified. In MTCs exhibiting spindle cell morphology, the nuclei are elongated, but the chromatin pattern is still the same [Etit et al., 2008]. Commonly, these tumors present only mild nuclear pleomorphism, and mitotic activity is low (Figures 6.17 – 6.18). In small MTCs foci of necrosis, hemorrhage, and mitotic activity are uncommon, while in larger tumors they are frequent [Desai et al., 2005; Mian et al., 2011; Venkataramana et al., 2018].

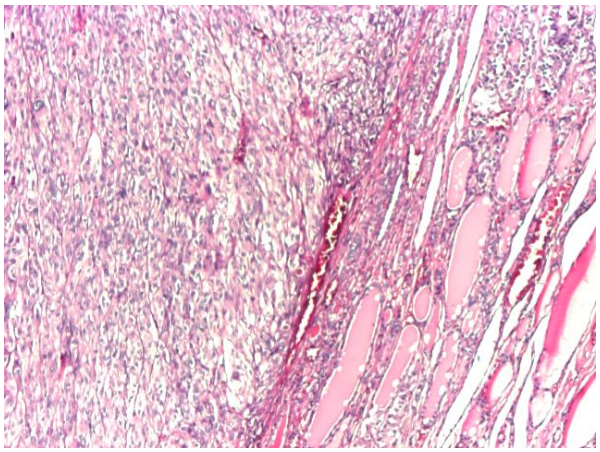


Figure 6.13. MTC with fusiform cells (HE, x 40)

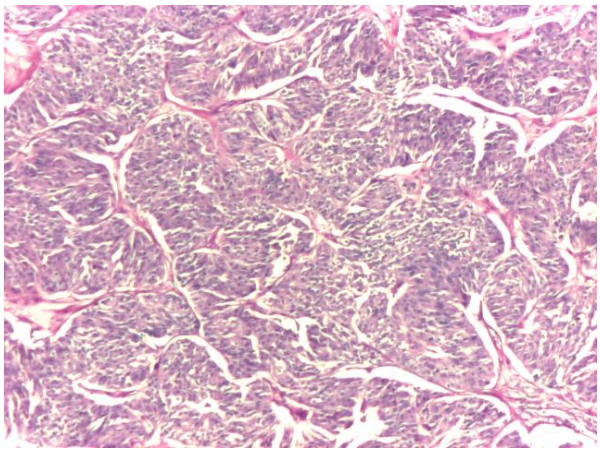


Figure 6.14. MTC with insular pattern (HE, x 40)

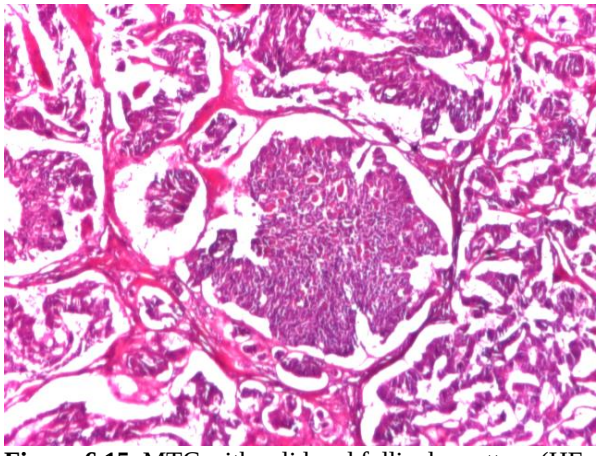


Figure 6.15. MTC with solid and follicular pattern (HE, x 40)

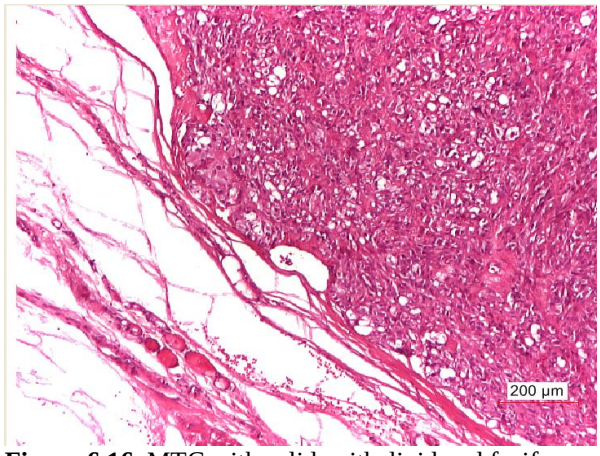


Figure 6.16. MTC with solid epithelioid and fusiform cells (HE, x 40)

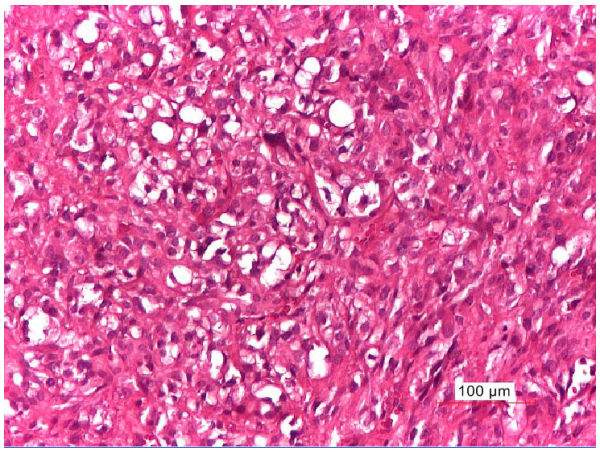


Figure 6.17. Moderate cell pleomorphism, and low mitotic activity (HE, x 100)

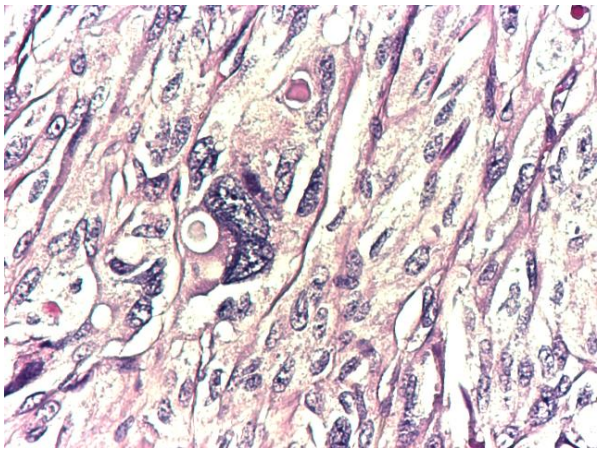


Figure 6.18. Marked cell pleomorphism (HE, x 200)

In up to 90% of MTC cases, stroma contains amyloid deposits (Figure 6.19), having procalcitonin and calcitonin as major constituents. Being unevenly distributed throughout the tumor, amyloid appears as a Congo Red-positive material (Figure 6.20), with typical "apple-green" birefringence (Figures 6.21 – 6.22) when examined in polarized light [Costache et al., 2017].

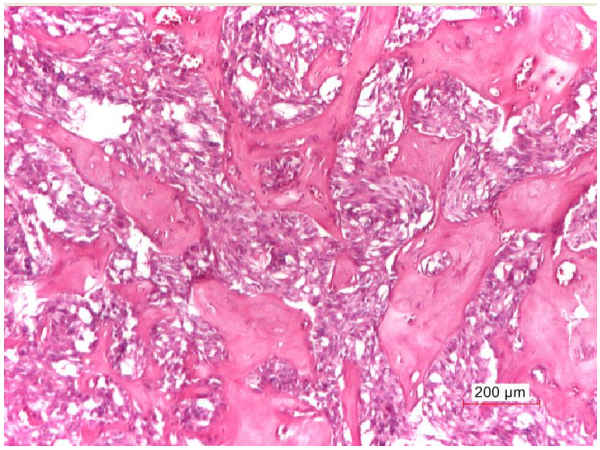


Figure 6.19. Tumor stroma contains strands of acellular, eosinophilic material (amyloid) (HE, x 40)

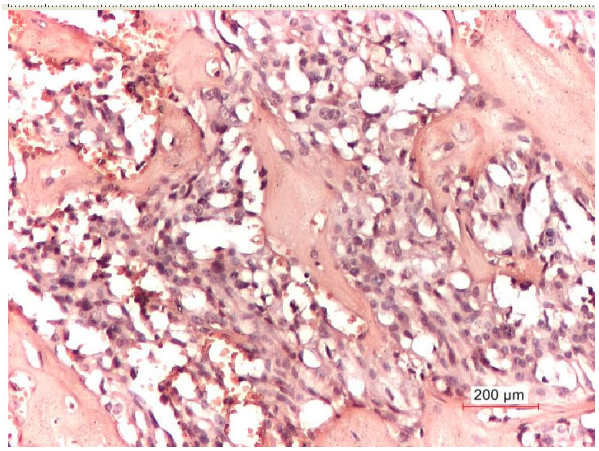


Figure 6.20. Unevenly distributed, Congo Red-positive material in tumor stroma (Congo Red, x 100)

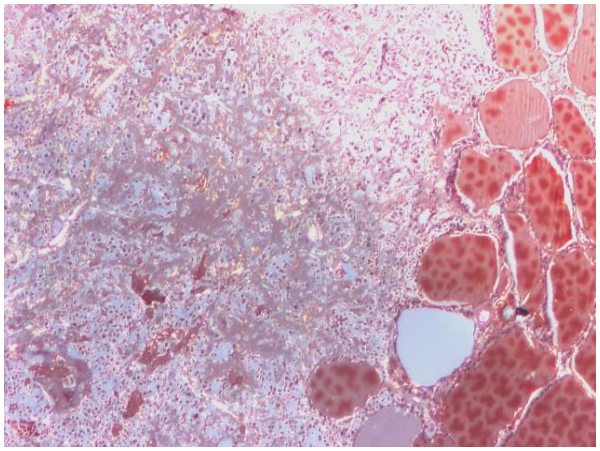


Figure 6.21. Amyloid deposits with typical birefringence (Polarized light microscopy, x 40)

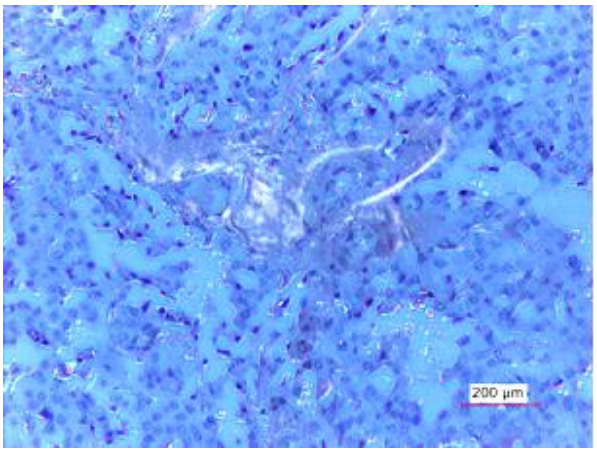


Figure 6.22. Amyloid deposits with typical "apple-green" birefringence (Polarized light microscopy, x 100)

In histological sections stained with Crystal Violet, the amyloid deposits are typically metachromatic [DeLellis et al., 2017]. Massive coarse deposition of amyloid form interlacing trabeculae between the tumor cells, separating them into irregular sheets. Thin amyloid deposits can lead to pseudopapillary pattern as tumor cells arrange around these fiber-like structures of eosinophilic material [DeLellis et al., 2017].

In addition to amyloid, stroma can also contain variable amounts of collagen and a prominent vascularity with glomeruloid configuration or long cords of vessels (Figure 6.23), coarse calcifications (Figure 6.24), and psammoma-like bodies [Ganeshan et al., 2013; DeLellis et al., 2017]. From a histological point of view, heritable MTCs are virtually indistinguishable from those exhibited by sporadic tumors, but there are researchers which found some differences. Kaserer *et al.* (2001) reported that hereditary MTCs present desmoplastic stroma and are prone to metastasizing into the regional lymph nodes [Kaserer et al., 2001]. Diaz-Cano *et al.* (2001) highlighted the fact that heritable MTCs are accompanied by C-cell hyperplasia [Diaz-Cano et al., 2001]; however, this histological feature is not an absolute diagnostic criterion for MTC. Taking all MTCs together, irrespective of the fact that the tumor is sporadic or heritable, Desai *et al.* (2005) reported that the thyroid gland adjacent to a MTC can exhibit a normal appearance in more than half of the cases, but in the rest of the cases a chronic autoimmune thyroiditis, or even a second tumor, *i.e.*, a PTC, could be found [Desai et al., 2005].

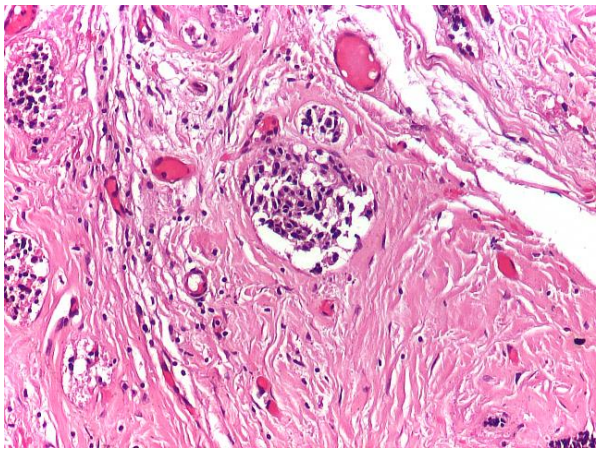


Figure 6.23. Abundant stroma and tumoral cells with glomeruloid configuration (HE, x 100)

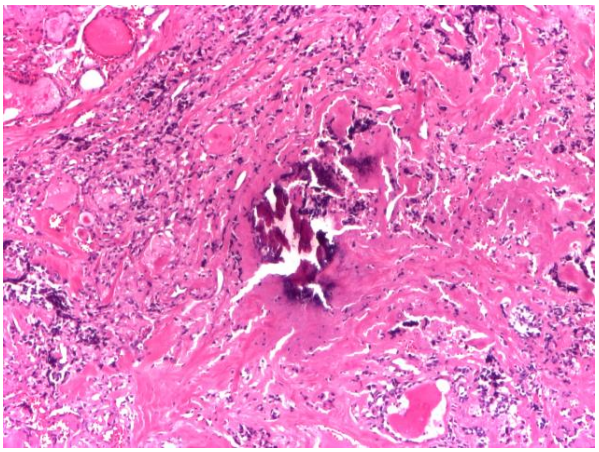


Figure 6.24. Stroma with variable amounts of collagen and calcifications (HE, x 40)

In the last three decades, the spectrum of morphological features observed in MTC enlarged with many new entities. The pathologists should be aware of all these variants in order to establish the correct diagnosis. The quantitative criteria used to define these subtypes in heterogeneous tumors have yet to be established [DeLellis et al., 2017]. Facing a rare histological variant of MTC, immunohistochemical (IHC) stainings (especially positivity for calcitonin and CEA to confirm an MTC), but also the presence of amyloids is essential.
MTCs include many histological variants under the same "umbrella", such as: encapsulated, follicular, pseudopapillary, oncocytic, squamous, with rosette formation, with small cells, with clear cells, with melanin pigmentation, with giant cells, amphicrine and paraganglioma-like type [Kakudo et al., 1978; Eng et al., 1989; Rosai et al., 1990; Tse et al., 2009; Mondal et al., 2012; Mardi et al., 2013; Chan et al., 2013; Rampioni Vinciguerra et al., 2016; Lichiardopol et al., 2016; DeLellis et al., 2017; Verma et al., 2017; Wang et al., 2018; Srinivas et al., 2019].

Tumors of the encapsulated variant are surrounded by a complete fibrous capsule. In some series, these tumors have been referred to as "C-cell adenomas"; however, until more is

known about their natural history, it is best to classify them as encapsulated MTCs [DeLellis et al., 2017]. The follicular/glandular subtype is made up of tumor cells that form follicular or glandular structures containing eosinophilic secretion in the lumens (Figure 6.25). The endoluminal cytoplasm of tumor cells is often more deeply eosinophilic and granular because of the accumulation of neurosecretion granules at this site, especially chromogranin A (CgA) [Chan et al., 2013]. The oncocytic/oxyphilic subtype exhibits tumor cells with abundant eosinophilic granular cytoplasm that are arranged in nests or in a trabecular fashion, in a focal or a diffuse pattern (Figure 6.26). Neoplastic oncocytic foci are immunoreactive to calcitonin, galectin-3 and thyroid transcription factor 1 (TTF1). This subtype develops at an older age (almost 64 years) and has a stronger predominance in women [Chan et al., 2013; Rampioni Vinciguerra et al., 2016; DeLellis et al., 2017].

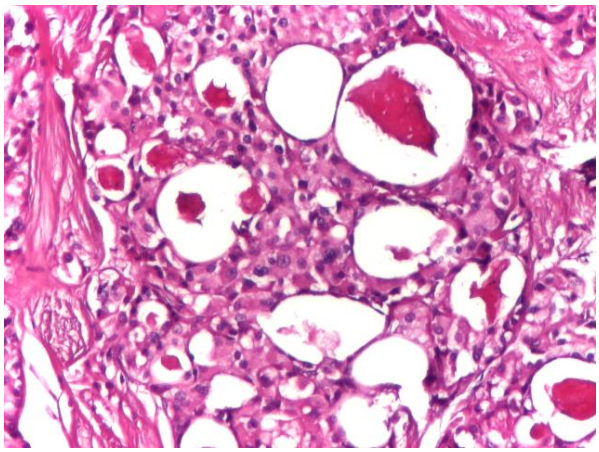


Figure 6.25. MTC follicular subtype (HE, x 100)

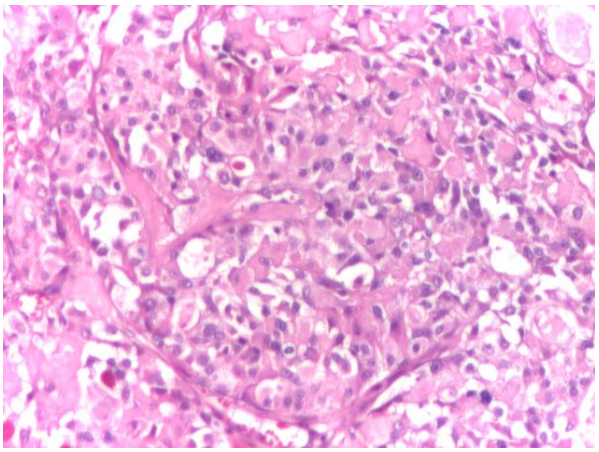


Figure 6.26. MTC oncocytic subtype (HE, x 100)

The pseudopapillary subtype defines a tumor made up of pseudopapillae formed by tissue fragmentation, but rarely even true papillae can occur [Chan et al., 2013; DeLellis et al., 2017] (Figures 6.27 – 6.28). Small cells subtype of MTC is a rare variant and an aggressive form, made up of diffusely infiltrating small blue round cells with scanty cytoplasm and inconspicuous nucleoli. The diagnosis is based on the identification of the amyloid deposits, using VG and Congo Red stainings (Figures 6.29 – 6.32). The tumor cells are strongly positive for CKAE1/AE3 and CEA, focally for CK7, synaptophysin (Syn), CD56, and CD99 (Ewing's sarcoma marker – MIC2), while negative for calcitonin, p63, p40, and CgA [Verma et al., 2017].

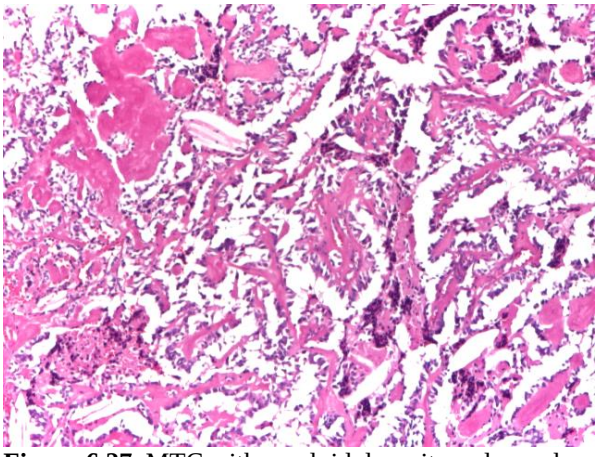


Figure 6.27. MTC with amyloid deposits and pseudo-papillary pattern (HE, x 40)

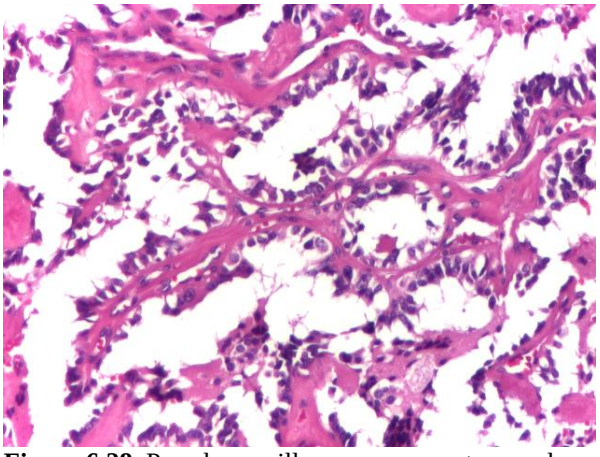


Figure 6.28. Pseudo- papillary arrangement around the amyloidotic "core" (HE, x 100)

Squamous variant of MTC presents focal squamous differentiation, but this is an exceptional finding [Lichiardopol et al., 2016; DeLellis et al., 2017].

Clear cell variant of MTC reveals cells with optically clear cytoplasm, which dominate the picture of the tumor or can appear only focally [Chan et al., 2013; DeLellis et al., 2017].

Melanin-producing/pigmented/melanotic variant can be found only in rare cases of MTC, exhibiting cells containing variable amounts of melanin pigment in their cytoplasm, but tumor cells also express strong positive reaction to calcitonin by IHC staining. Melanin pigment could be also found in the extracellular matrix of the tumor [Eng et al., 1989; Chan et al., 2013; Wang et al., 2018].

MTC variant with giant cells shows intermingled pattern of typical small cells with giant, large cells, with bizarre and pleomorphic nuclei, with nuclear pseudo- inclusions, sometimes even multinucleated cells (Figure 6.33). Mitoses can be absent or very few, but stromal amyloid is identified. IHC stainings reveal positive expression for calcitonin and CEA, but negativity for thyroglobulin. Ki67 labeling index is very low (less than 1%) and as such, this subtype has a good prognosis [Kakudo et al., 1978; Chan et al., 2013, DeLellis et al., 2017].

Amphicrine/mucin-producing/mucinous variant is identified only in rare cases. It is characterized by tumor cells that contain mucin and express calcitonin. Tumoral stroma contains extensive mucin secretion and focal areas of Congo Red-positive amyloid [Mardi et al., 2013; DeLellis et al., 2017].

Paraganglioma-like type shows nested architecture delineated by delicate vasculature, mimicking paraganglioma. S100 protein-positive sustentacular-like cells are interspersed [Chan et al., 2013; DeLellis et al., 2017].

Angiosarcoma-like variant of MTC is a tumor with pseudosarcomatous features resembling those of angiosarcomas [DeLellis et al., 2017].

Spindle cell variant of MTC is entirely made up of plump spindle cells arranged in intersecting fascicles, whorls, and packets, mimicking mesenchymal neoplasms, but amyloid-like material is abundant in the background (Figure 6.34). Nuclear pleomorphism is mild and mitotic count is very low [Mondal et al., 2012; Chan et al., 2013].

Carcinoid-like variant of MTC shows histological features resembling intestinal carcinoid, with tumor islands, trabeculae, or glands separated by fibrohyaline stroma [Chan et al., 2013].

Neuroblastoma-like/with rosette formation variant is also a rare subtype of MTC and exhibits a fibrillary matrix and rosettes, resembling neuroblastoma [Tse et al., 2009; Chan et al., 2013].

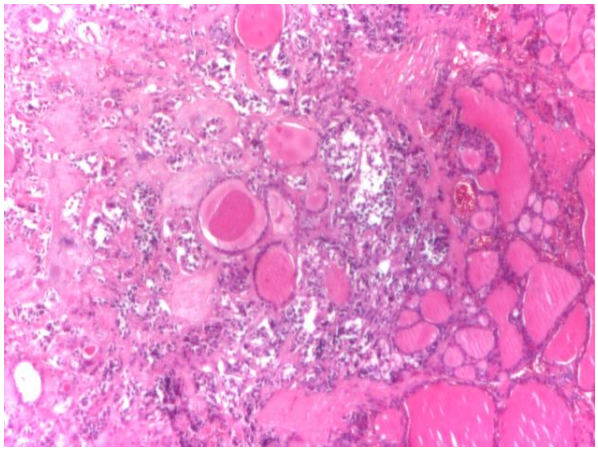


Figure 6.29. Small tumor cells infiltrate between the thyroid follicles from the vicinity (HE, x 40)

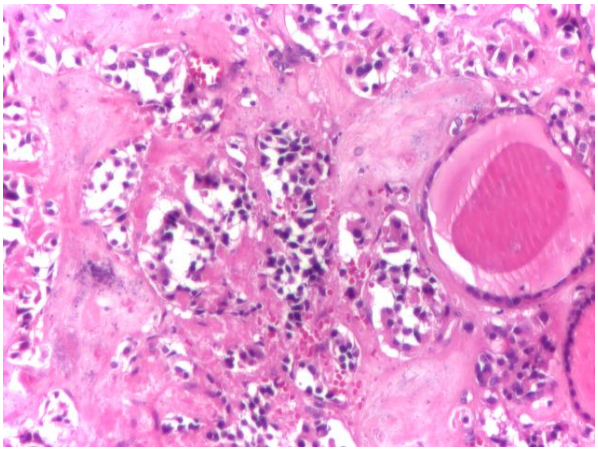


Figure 6.30. Higher magnification reveals small tumor cells and entrapped follicles (HE, x 100)

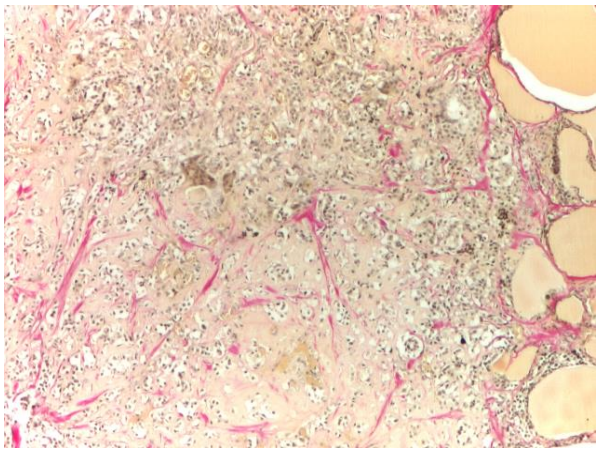


Figure 6.31. Small tumor cells are embedded into amyloid deposits (VG, x 40)

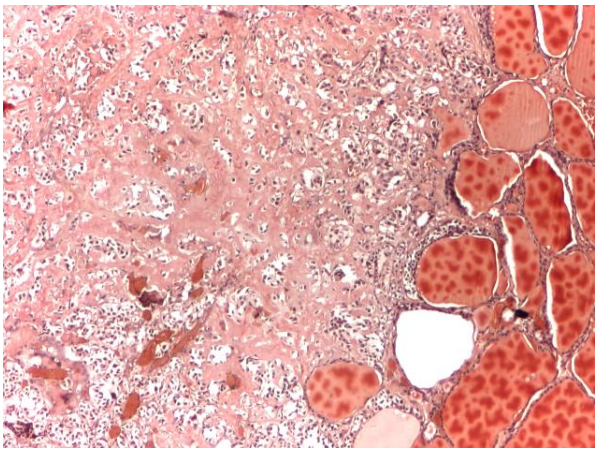


Figure 6.32. Congo Red positive of amorphous amyloid deposits (Congo Red, x 40)

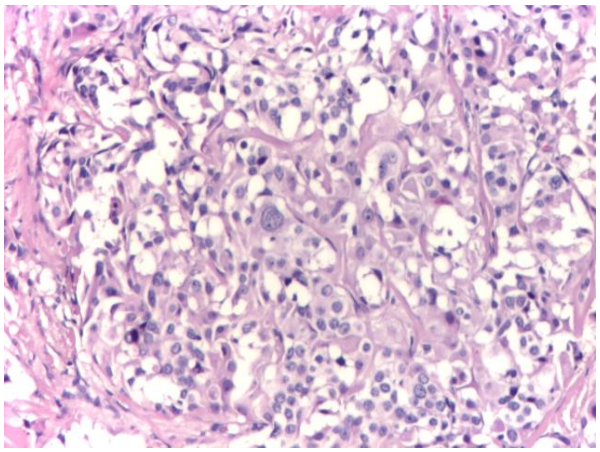


Figure 6.33. MTC giant cells subtype (HE, x 100)

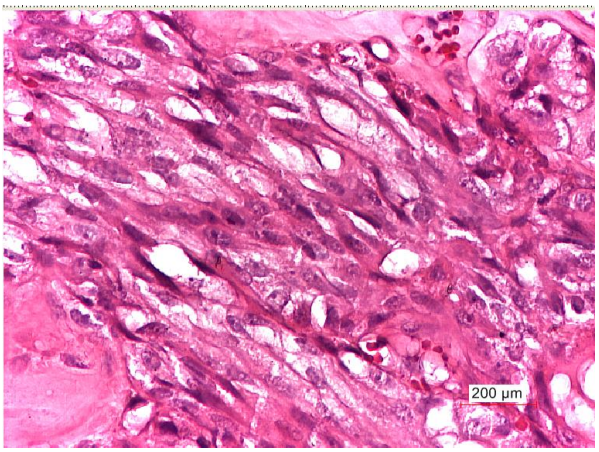


Figure 6.34. MTC spindle cell subtype (HE, x 200)

All the histological variants listed above seem to have no clinical or prognostic significance, except the small cell type, which is more aggressive. The prognostic implication of the giant cell variant is still unsettled [Tse et al., 2009]. In all cases exhibiting one of these histological subtypes of MTC, the correct diagnosis should take into consideration other thyroid pathological entities with which they resemble (Table 6.3).

Table 6.3. Histological subtypes/variants of MTC and their differential diagnosis
[DeLellis et al., 2017, Chan et al., 2013, Rampioni Vinciguerra et al., 2016, Lichiardopol et al., 2016, Eng et al., 1989, Wang et al., 2018, Kakudo et al., 1978, Mardi et al., 2013, Mondal et al., 2012, Tse et al., 2009]

Histological variant of MTC	Thyroid pathology that should be taken into consideration in differential diagnosis
Encapsulated	–
Follicular/glandular	Follicular adenoma or carcinoma; poorly differentiated thyroid carcinoma
Pseudopapillary	Papillary thyroid carcinoma
Oncocytic/oxyphilic	Hürthle cell adenoma and carcinoma
Squamous variant	Primary or metastatic squamous

Histological variant of MTC	Thyroid pathology that should be taken into consideration in differential diagnosis
	cell carcinoma of thyroid gland
Small cells variant	Malignant lymphoma of the thyroid; thyroid metastasis of a small cell lung carcinoma
Clear cell variant	Other clear cell tumors
Melanin-producing/ pigmented/melanotic variant	–
With giant cells	Undifferentiated carcinoma
Amphicrine variant/ mucin-producing/mucinous	–
Paraganglioma-like type	Paraganglioma
Angiosarcoma-like variant	–
Spindle cell	Mesenchymal tumors
Carcinoid-like	Metastatic carcinoid; paraganglioma; follicular neoplasm
Neuroblastoma-like/ with rosette formation	Malignant lymphoma; metastatic neuroblastoma; peripheral primitive neuroectodermal tumor

6.6. HISTOCHEMICAL STAINS AND IMMUNOHISTOCHEMISTRY

Regarding the histochemical stains that could be applied in MTCs, some studies reported that 90% of these cancers show positivity to Grimelius' argyrophil silver staining [Sikri et al., 1985]. Tumor cells exhibit a weak to moderate argyrophilia, but a strong positivity could be occasionally obtained in dispersed cells [Sikri et al., 1985]. Moreover, the tumor cells may also show focal PAS and Alcian Blue reactivity. The PAS positivity does not appear to be related to glycogen even in those tumors with a clear cytoplasm [Desai et al., 2005].

By having so many histological variants, MTC represents the great mimicker of other thyroid cancers. In order to establish a correct diagnosis, immunohistochemistry should be performed in all cases. A wide variety of substances can be demonstrated immunohistochemically in MTC (Table 6.4), but a relatively small number have practical diagnostic or prognostic value. The most useful diagnostic stains include calcitonin, CEA, chromogranin A (CgA), and synaptophisin (Syn) [Nikiforov et al., 2012].

Table 6.4. The "typical" immunophenotypic profile of MTC
[Chan et al., 2013, Nikiforov et al., 2012, Chu et al., 2014]

Usual positive immunostaining	Variable immunopositivity	Usual negative immunostaining
CgA	S100 protein	Thyroglobulin
NSE	Vimentin	CK20
Syn	–	–
CD56	–	–
CK AE1/AE3	–	–
CK7	–	–
TTF1	–	–
Calcitonin	–	–
CEA	–	–

CD: Cluster of differentiation; CEA: Carcinoembryonic antigen; CgA: Chromogranin A; CK: Cytokeratin; MTC: Medullary thyroid carcinoma; NSE: Neuron-specific enolase; Syn: Synaptophysin; TTF1: Thyroid transcription factor 1

MTC cells show positivity for low-molecular-weight cytokeratin (CK); high-molecular-weight CK is rarely expressed in this cancer. MTCs also exhibit positive staining with antibodies directed to neuroendocrine products, but also to CEA, all of them being secreted by tumor cells. As such, positive results are obtained with Neuron Specific Enolase (NSE), CgA (Figure 6.35), and Syn (Figure 6.36) antibodies. More specific is the positive IHC staining with calcitonin antibody (Figure 6.37), which can be identified in approximately 80% of cases [Saad et al., 1984; Rosai et al., 1990], thus confirming the involvement of parafollicular calcitonin-producing C-cells. Moreover, immunostaining of tumor C-cells for calcitonin further helps to identify tumor angioinvasion and extracapsular spreading of tumor cells.

Tumor cells are also characteristically immunopositive for CEA, an antigen that is not usually expressed by thyroid neoplasms derived from follicular cells, and for TTF1 (Figure 6.38), but not for thyroglobulin (Figures 6.39 – 6.40). The presence of thyroglobulin positivity reflects the presence of entrapped non-neoplastic follicles or a neoplastic follicular component in a mixed medullary and follicular cell carcinoma.

MTCs express immunopositivity for a number of other neuropeptides, such as somatostatin, gastrin-releasing peptide, adrenocorticotropic hormone (ACTH), substance P, vasoactive intestinal peptide, and cathecolamine [Chu et al., 2014].

Thus, Duan & Mete (2016) proposed an IHC algorithm that should be used in the diagnosis of a MTC, when the pathologists suspected a neuroendocrine tumor [Duan et al., 2016]. According to them, the following antibodies must be used, in a well-established order: markers for neuroendocrine differentiation [CgA, Syn, CD56, CD57, protein gene product 9.5 (PGP9.5), and neuron-specific enolase (NSE)]; markers for epithelial differentiation: CK AE1/AE3 and CAM5.2; marker for tumor proliferation: Ki-67/MIB-1; markers for site of origin: TTF1, monoclonal CEA, and calcitonin.

It is worth mentioning that medullary thyroid microcarcinomas could be observed as an incidental finding of the pathologist in searching for another histopathological entity in an excised thyroid gland (Figures 6.41 – 6.42).

Furthermore, the diagnosis of a MTC could be very difficult to establish only on HE-stained sections of patients with undetectable levels of serum calcitonin, but in almost half of these cases immunohistochemistry can detect diffuse or focal positivity for calcitonin, CgA and CEA [Gambardella et al., 2019]. However, Zhou *et al.* (2017) and Gambardella *et al.* (2019) found out that this type of MTC has a better oncological outcome than a calcitonin-rich MTC [Zhou et al., 2017; Gambardella et al., 2019].

TTF1 is positive in at least 80% of MTC, and thyroglobulin is negative in MTC cells, but is positive in residual entrapped normal follicles [de Micco et al., 1993].

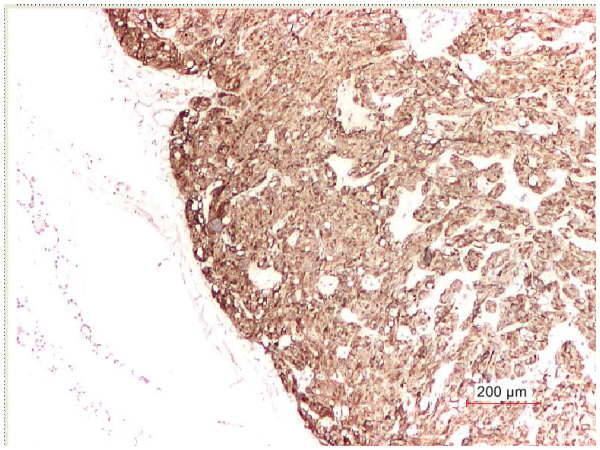


Figure 6. 35. CgA cytoplasmic expression in all tumoral cells (IHC, anti-CgA, x 40)

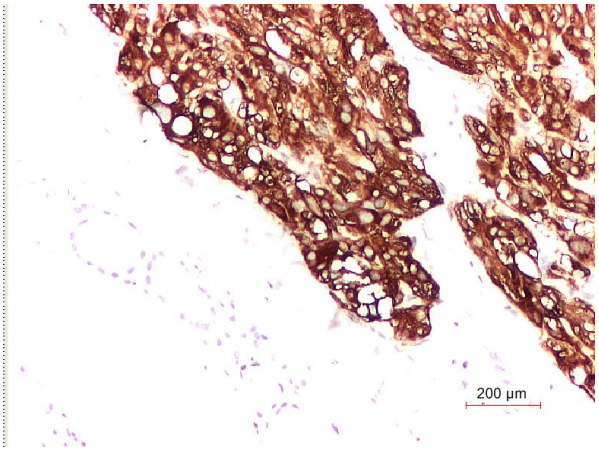


Figure 6.36. Syn strong cytoplasmic expression (IHC, anti-Syn, x 40)

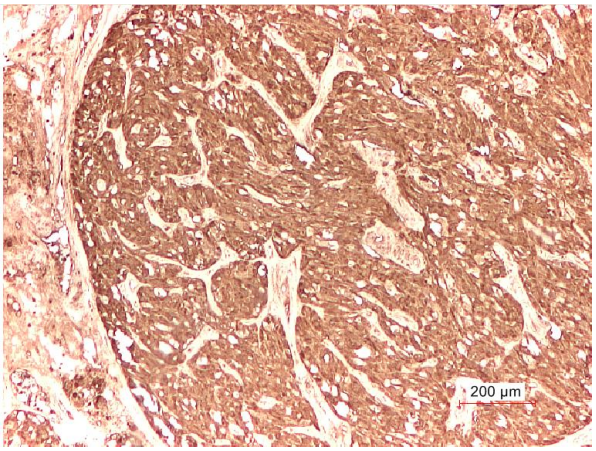


Figure 6.37. Calcitonin strong immunopositivity of all tumoral cells (IHC, anti-calcitonin, x 10)

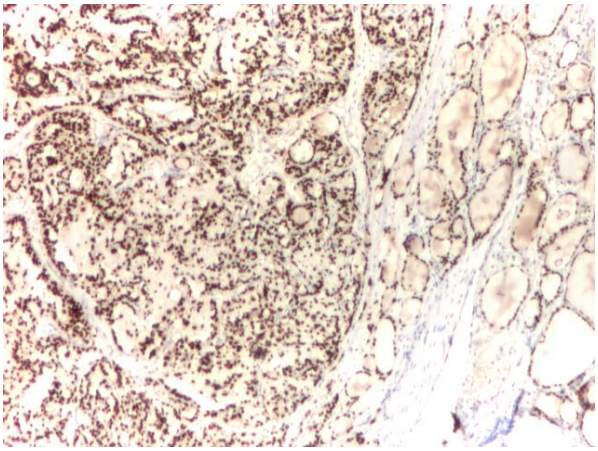


Figure 6.38. TTF1 strong nuclear expression in all tumoral cells (IHC, anti-TTF1, x 40)

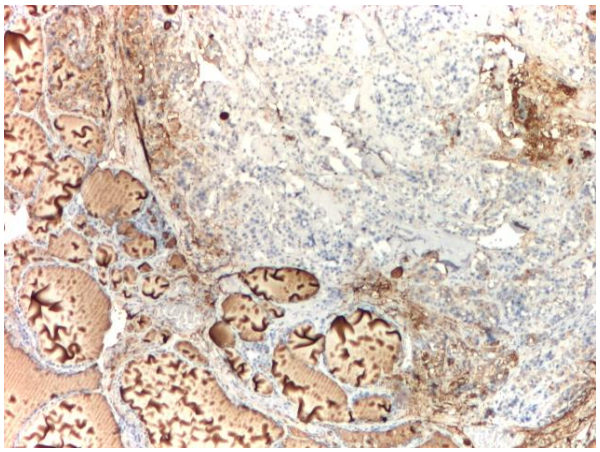


Figure 6.39. Thyroglobulin negative staining in all tumoral cells (IHC, anti-thyroglobulin, x 40)

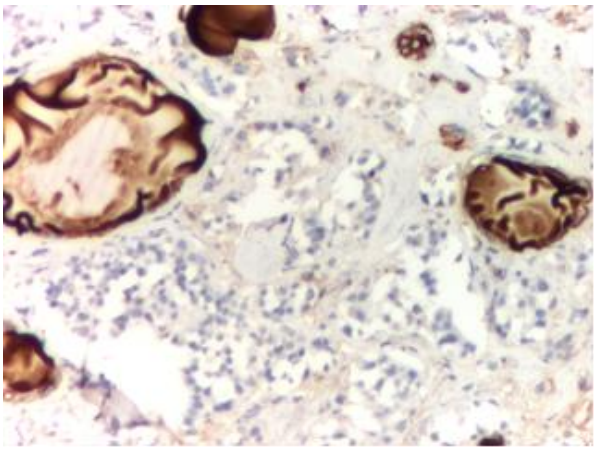


Figure 6.40. Detail thyroglobulin staining (IHC, anti-thyroglobulin, x 100)

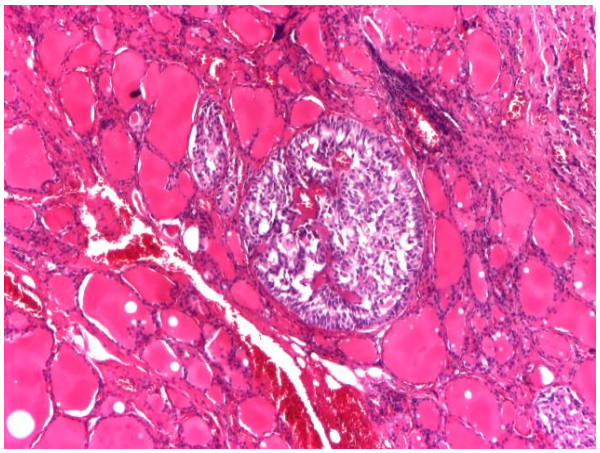


Figure 6.41. MTC microcarcinoma (HE, x 40)

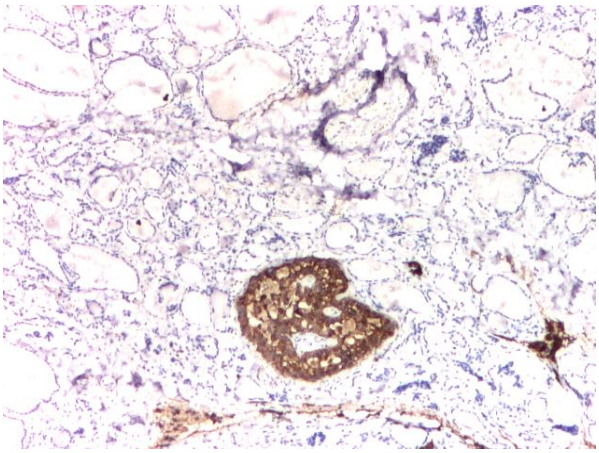


Figure 6.42. MTC microcarcinoma (IHC, anti-Syn, x 40)

Serum calcitonin and CEA levels are usually increased in patients with MTC, compared to patients with papillary, follicular, or anaplastic carcinomas, and correlate with calcitonin and CEA positivity immunostaining (100%) of tumor cells. Aggressive cases of MTCs show persistent elevated CEA levels, but decreased calcitonin serum levels. Usually, MTC expresses low values of Ki67 labeling indexes (Figures 6.43 – 6.44). Higher Ki67 labeling indexes are associated with extrathyroid spread, with lymph nodes and distant metastasis, advanced stage, and low overall survival, the cut-off expression being over 50 cells/mm^2 [Tisell et al., 2003; Mian et al., 2011].

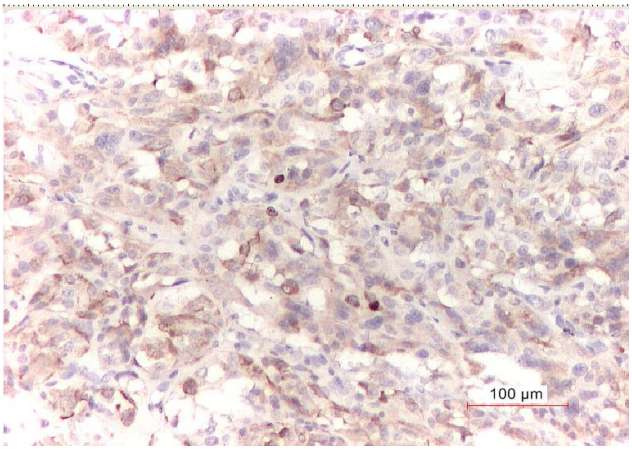


Figure 6.43. Proliferative activity in MTC: (IHC, anti-Ki67, x 100)

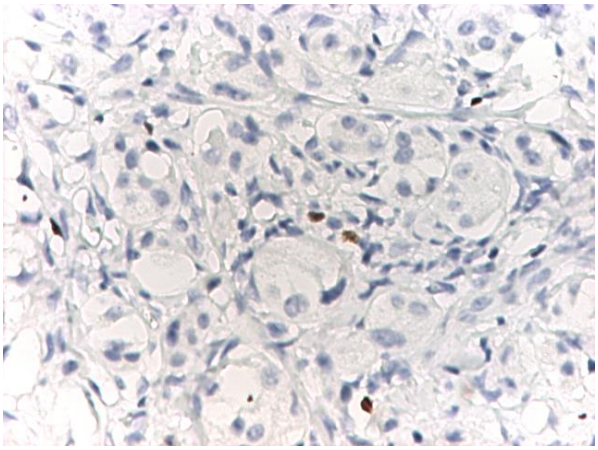


Figure 6.44. Proliferative activity in MTC: (IHC, anti-Ki67, x 200)

6.7. CURRENT UPDATES ON METASTASES

MTC frequently metastasizes and the tumor spreads occur both by hematogenous and lymphatic way. As such, lymphatic and vascular invasion may be seen at the advancing front of the tumor (Figures 6.45 – 6.46).

Regional lymph node involvement occurs early in the evolution of MTCs and affects almost 60% of patients in the moment of their diagnosis [Tanwar et al., 2018]. Especially the neck nodes are affected, *i.e.*, the nodes of the central compartment, tracheal and paratracheal nodes, but also the mediastinal nodes [Desai et al., 2005]. Metastases to cervical lymph nodes are detected in 70% of cases of MTC. In advanced cases, foci of lymphatic invasion may be seen in the contralateral lobe [Rosai et al., 1990]. Zhou *et al.* (2017) found out that larger tumoral masses were associated with higher rate of lymph nodes metastasis, and considered that tumor size represents an independent survival indicator [Zhou et al., 2017].

During the evolution of MTC, distant metastases can be found in almost 40% of patients [Tanwar et al., 2018]. Usually, distant tumor metastases could be detected into the lung, liver, bones and mediastinum [Desai et al., 2005; Sippel et al., 2008; Pacini et al., 2010].

Probably due to longer survival of the patients with MTCs, some unusual metastasizing sites were recently published. Sastry *et al.* (2018) reported a rare case of a MTC metastasizing to the brain 21 years after the thyroidectomy [Sastry et al., 2018]. Tanwar *et al.* (2018) also published an article regarding an even more unusual metastasis of MTC, in the breast - a real challenge for the clinician, radiologist, and pathologist as MTCs can mimic primary invasive lobular carcinoma of the breast, both histopathologically and radiologically [Tanwar et al., 2018]. Gajdzis *et al.* (2018) reported a patient who had undergone thyroidectomy for MTC, but 33 years later he was diagnosed with parotid and intraocular

choroidal metastases of his earlier thyroid cancer [Rosen et al., 2017; Gajdzis et al., 2018; Cameselle-Teijeiro et al., 2018].

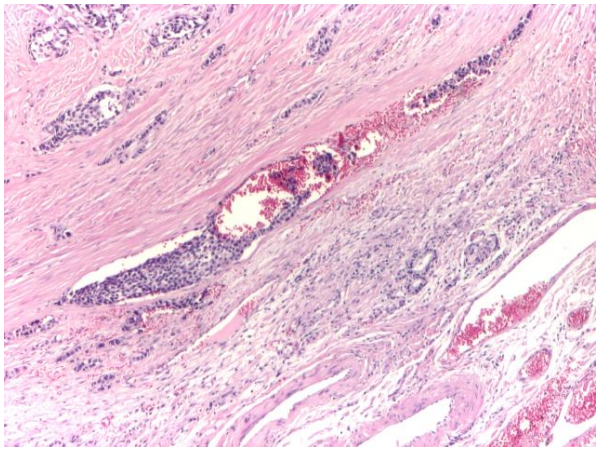


Figure 6.45. Hematogenous dissemination of MTC (HE, x 40)

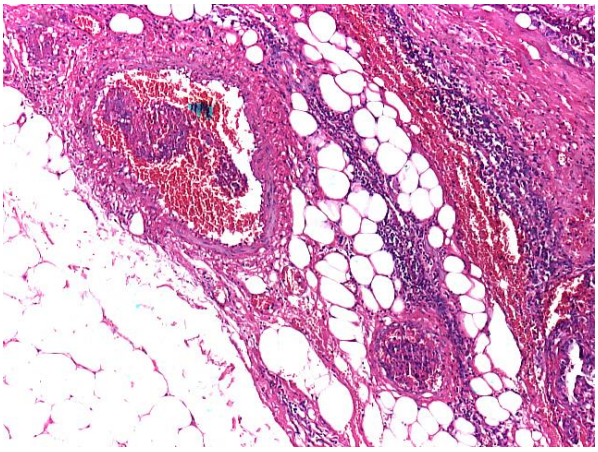


Figure 6.46. Hematogenous dissemination of MTC (HE, x 40)

6.8. CLINICOPATHOLOGICAL PROFILE OF MEDULLARY THYROID CARCINOMA – PERSONAL EXPERIENCE

6.8.1. Introduction

Despite accounting for only 2% to 3% of all thyroid malignancies, MTC is responsible for a disproportionately high number of deaths compared with other thyroid carcinomas due to its relatively aggressive biological behavior [DeLellis et al., 2017]. Among patients presenting with a palpable thyroid nodule, the incidence of clinical cervical lymph node involvement at the time of diagnosis has been reported to be as high as 75% [Thyroid Cancer Survivors' Association (ThyCa), 2014].

The Clinic Emergency County Hospital "Sf. Spiridon" of Iasi is an important center of interdisciplinary diagnosis and treatment of thyroid pathology with an annual addressability of about 450 patients. The area of Moldova is a zone predisposed to thyroid pathology in the context of iodine deficiency and the Chernobyl nuclear disaster. TC reports in our county are infrequent, often focused on PTC as the main form of thyroid tumor [Găleşanu et al., 1989; Mogoş et al., 1995; Ivan et al, 2002; Buzduga et al., 2011; Teodoroiu et al., 2021]. Reported data from our center showed that MTC was identified in 6.74% of TC, more than the European studies [Dal Maso et al., 2017; Teodoroiu et al., 2021].

Although clinical and pathologic variables alone may suffice to predict patient outcome in most cases, the variable and unpredictable behavior of MTC in some cases suggests that other local or ethnic elements may also influence disease progression and survival.

Aim

We analyzed the clinicopathological profile of MTC, aiming to identify the cases with increased oncological risk. Our analysis focused on the possible relationships between a set of clinicopathological characteristics and three parameters expressing tumor extension and aggressiveness (namely thyroid capsule invasion, lympho-vascular invasion, and lymph node metastasis).

6.8.2. Material and methods

The retrospective study comprised 59 consecutive cases of MTC investigated during 2010-2016. The research has been approved by the Ethics Committee of the "St. Spiridon" Clinical Emergency Hospital County Iasi, pursuant to the ethical standards of Helsinki declaration regarding the patients' informed consent for the use of their medical information for scientific purpose.

Patients

All cases were reviewed by three independent pathologists in order to establish the histological variant, tumor stages according to the 2017 WHO Classification, and TNM and AJCC criteria [Greene et al., 2002; De Lellis et al., 2004; Brierley et al., 2017; Lloyd et al., 2017; Amin et al., 2017], and to reassess the main characteristics. Our database included information regarding gender, age (< 55 and respectively ≥ 55 yo), tumor size, histological variant, focality (two or more foci), lympho-vascular invasion, thyroid capsule invasion (defined as microscopic presence of tumor cells into the thyroid capsule), extrathyroidal extension (defined as microscopic presence of tumor cells into perithyroidal soft tissues: adipose tissue, skeletal muscle, sizable vessels and nerves), and lymph node metastasis (including the number of positive lymph nodes). At the histological evaluation we also noted the associated thyroid pathology.

Immunohistochemical exam

The diagnosis of MTC was performed by using immunohistochemical techniques of the following monoclonal antibodies: chromogranin A (5H7 monoclonal mouse anti-chromogranin A antibody, IVD, Novocastra, 1:400 dilution), synaptophisin (27G12 monoclonal mouse anti-synaptophisin antibody , IVD, Novocastra, 1:50 dilution), calcitonin (CL 1948 monoclonal mouse anti-calcitonin antibody, IVD, Novocastra, 1:250 dilution), CD56 (123C3 monoclonal mouse anti-CD56 antibody, IVD, Dako, 1:75 dilution), TTF1 (mAb 8G7G3/1 monoclonal mouse anti-TTF1 antibody , IVD, Dako/Agilent, 1:50) and ki67 (SP6 monoclonal rabbit anti-Ki67antibody, IVD, 1:250) (Table 6.5).

Table 6.5. The antibodies used for MTC IHC staining

Antibody	Manufacturer	Clone	Antigen retrieval	Class	Dilution	Labeling	Cellular localization
Anti-CgA	Novocastra, United Kingdom	5H7	Citrate, pH 6	Monoclonal mouse anti-human chromogranin A	1:400	Neuroendocrine cells	Cytoplasm
Anti-Syn	Novocastra, United Kingdom	27G12	Citrate, pH 6	Monoclonal mouse anti- synaptophisin antibody	1:50	Neuroendocrine cells	Cytoplasm
Anti-calcitonin	Novocastra, United Kingdom	CL 1948	Citrate, pH 6	Monoclonal mouse anti- calcitonin antibody	1:250	C cells	Cytoplasm
Anti-CD56	Dako, Denmark	123C3	Citrate, pH 6	Monoclonal mouse anti-CD56 antibody	1:75	Neuroendocrine cells	Membrane
Anti-TTF1	Dako/Agilent, Denmark	mAb 8G7G3/1	pH 9	Monoclonal mouse anti-TTF1 antibody	1:50	Follicular thyroid cells	Nuclear
Anti-Ki67	ThermoScientific USA	SP6	Citrate, pH 6	Monoclonal rabbit anti-Ki67antibody	1:250	Proliferating cells	Nuclear

Statistical analysis

Data were analyzed using the SPSS V.22-SPSS Inc., IBM Corporation, Chicago, IL, USA). The results of the univariate analysis were reported as mean ± standard deviation for continuous variables. Total count and percent were reported for categorical variables. Chi-squared test (Maximum-Likelihood, Yates, Mantel-Haenszel) was performed for categorical variables and Kruskal-Wallis test for continuous variables. Correlations between predictor and outcome variables were determined using univariate analysis (Spearman Rank test, Gamma) and multiple logistic regressions. The significance level (p-value), which represents the maximum error probability, was considered to be 0.05 (5%); a confidence interval of 95% shows that the decision is correct.

6.8.3. Results

General clinicopathological characteristics

Among the 59 patients in the study, 55 of cases were sporadic MTC aged between 36 and 82 years, and 4 cases of hereditary MTC were aged between 27 and 40 years (with familial tumor aggregation).

The preoperative diagnosis was established based on calcitonin values and clinical evaluation in 21 cases (36%), intraoperatively, by extemporaneous exam, in 9 cases (15%) and postoperatively, by histopathological examination, in 29 cases (49%).

In the study group, 46 (77.9%) were female and 13 (22.1%) male, with a mean age of 59.75 ± 11.12 years, and more than half of the cases (54%) were in the 45-65 age range. 42 (71.19%) were over 55 yo at the time of the diagnosis, whereas 17 were under 55 yo (28.81%).

In 24 cases (40.67%) capsular invasion was detected, and in 9 cases (15.25%) extrathyroidal extension.

The mean tumor diameter was 27.64 mm ± 2.50 mm and range between 2 and 80 mm. T stages were 26 (44.06%) T1, 18 (30.50%) T2, 14 (23.72%) T3, and 1 (1.69%) T4a.

Multifocality was found in 9 cases (15.25%) of MTC.

Vascular invasion was present in 34 (57.62%) cases and perineural invasion in 2 (3.38%) cases.

Lymph node status was N0 23 (38.98%), N1 20 (33.89%) (N1a 4 cases and N1b 16 cases), and Nx 16 (27.12%).

In 9 cases (15.25%) were detected other collision tumors (PTC uni- or multifocal).

Distant metastases were reported in 3 cases: lung (1 case), bone (1 case) and bilateral ovarian and cervical (1 case).

The histopathological exam identified the following categories of MTC: 35 (59.32%) conventional, 12 (20.33%) spindle cell, 5 (8.47%) oncocytic, 2 (3.38%) pseudopapillary, 3 (5.08%) follicular/glandular, 1 (1.69%) with giant cells and 1 (1.69%) with small cells. Accompanying thyroid pathology included nodular goiter - 28 cases (47.45%), colloid goiter - 24 cases (40.67%), and Hashimoto thyroiditis - 7 cases (11.86%).

Complete tumor resection, with negative margins (R0), was histopathologically certified in 54 cases (91.52%). Incomplete tumor resection with positive edges (R1) was identified in 5 cases (8.47%).

For the 21 cases (36%) in which calcitonin was evaluated both preoperatively and postoperatively, serum calcitonin levels show elevated preoperative values, between 38 - 2000 pg/mL and postoperative values normalized (between 2 - 11.5 pg / mL) or persisted at increased levels (between 21 - 2000 pg/mL). Table 6.6 resumes the serum calcitonin level in correlation with the pTN staging.

Table 6.6. Pre- and postoperative calcitonin value corresponding to pTN staging

	Preoperative calcitonin value			Postoperative calcitonin value			
pTN	**order of tens (# 7)**	**order of hundreds (# 10)**	**order of thousands (# 4)**	**normal values (≤ 11,5 pg/ml) (#12)**	**order of tens (# 4)**	**order of hundreds (# 4)**	**order of thousands (# 1)**
T1	3 (14.2%)	5 (23.8%)	1(4.7%)	8 (38.0%)	1 (4.7%)	0 (0%)	0 (0%)
T2	3 (14.2%)	3 (14.2%)	0 (0%)	3 (14.2%)	2 (9.5%)	1 (4.7%)	0 (0%)
T3	1 (4.7%)	2 (9.5%)	3 (14.2%)	1 (4.7%)	1 (4.7%)	3 (14.2%)	1 (4.7%)
N1	0 (0%)	3 (14.2%)	2 (9.5%)	0 (0%)	3 (14.2%)	1 (4.7%)	1 (4.7%)
N0	3 (14.2%)	5 (23.8%)	0 (0%)	9 (42.8%)	0 (0%)	1 (4.7%)	0 (0%)
Nx	4 (19.0%)	2 (9.5%)	2 (9.5%)	3 (14.2%)	1 (4.7%)	2 (9.5%)	0 (0%)

- number of cases;

Relationships between clinicopathological prognostic factors

In evaluating the correlations between clinicopathological factors with prognostic potential, we analyzed the association between age, tumor size, focality, tumor recurrence, histological variant, related thyroid pathology and three parameters considered elements of aggression - namely thyroid capsular invasion, lympho-vascular invasion and lymph node metastasis. The fourth parameter of aggression, namely perineural invasion, was not examined, motivated by the fact that perineural invasion was identified only in two cases.

Thyroid capsular invasion

Our results show the absence of any statistically significant correlation between classical clinicopathological characteristics and thyroid capsular invasion, so that reporting to OR has no statistical relevance (Table 6.7).

Table 6.7. Clinicopathological characteristics of MTC in relation to thyroid capsular invasion

Clinicopathological characteristics	**Thyroid capsule invasion**		**Univariate analysis**	**OR (95% CI)**
	present (# 24)	**absent (# 35)**		
Age at diagnosis				
<55 yo	7 (29.1%)	10 (28.5%)	0.9643	0.971 (0.308-3.054)
>55 yo	17 (70.8%)	25 (71.4%)		
Tumor size (mm)				
< 10 mm	1 (4.1%)	8 (22.8%)	0.0887	5.818 (0.660-51.282)
10 - 40 mm	16 (66.6%)	22 (62.8%)		
> 40 mm	7 (29.1%)	5 (14.2%)		1.925 (0.516-7.177)
Focality of the tumor				
Unifocal	18 (75%)	32 (91.4%)	0.0847	3.555 (0.792-15.957)
Multifocal	6 (25%)	3 (8.5%)		
Tumor recurrence	7 (29.1%)	7 (20%)	0.5260	1.458 (0.452-4.695)
Histopathologic MTC type				
Conventional	12 (50%)	23 (65.7%)	0.2274	1.916 (0.662-5.542)
Other variants	12 (50%)	12 (43.8%)		
Coexisting thyroid pathology				
Colloid goiter	11 (45.8%)	13 (37.1%)	0.7004	0,5455* (0,0967-3,0757)
Nodular goiter	11 (45.8%)	17 (48.5%)		
Hashimoto thyroiditis	2 (8.3%)	5 (14.2%)		

*OR: odd ratio; CI: confidence interval; *p-value <0.05 was considered to be statistically significant * colloid and nodular goiter vs Hashimoto thyroiditis*

Lympho-vascular invasion

Statistical analysis exposed significant correlations between lympho-vascular invasion (present *vs.* absent) and tumor size (p <0.0001, OR = 13.695) (Table 6.8) in tumors larger than 40 mm.

Table 6.8. Clinicopathological characteristics of MTC in relation to lympho-vascular invasion

Clinicopathological characteristics	Lympho-vascular invasion		Univariate analysis	OR (95% CI)
	present (# 34)	absent (# 25)		
Age at diagnosis				
<55 yo	10 (29,4%)	7 (28%)	0,9058	0,933 (0.297-2.927)
>55 yo	24 (70,5%)	18 (72%)		
Tumor size (mm)				
< 10 mm	0 (0%)	9 (36%)	**0.0001**	13.695 (1.662-112.846)
10 - 40 mm	23 (67.6%)	15 (60%)		
> 40 mm	11 (32.4%)	1 (4%)		
Focality of the tumor				
Unifocal	27 (79.4%)	23 (92%)	0.1838	2.981 (0.562-15.790)
Multifocal	7 (20.5%)	2 (8%)		
Tumor recurrence	10 (29.4%)	4 (16%)	0.3427	1.838 (0.516-6.541)
Histopathologic MTC type				
Conventional	19 (55,8%)	16 (64%)	0,3984	0.664 (0.257-1.717)
Other variants	15 (44,1%)	19 (76%)		
Coexisting thyroid pathology				
Colloid goiter	14 (41,1%)	10 (40%)	0,2307	0,25* (0.0442-1.4136)
Nodular goiter	18 (52,9%)	10 (40%)		
Hashimoto thyroiditis	2 (5,8%)	5 (20%)		

*OR: odd ratio; CI: confidence interval; *p-value <0.05 was considered to be statistically significant * colloid and nodular goiter vs Hashimoto thyroiditis*

Lymph node metastasis

There were recorded statistically significant correlations between lymph node metastasis (present *vs.* absent) and tumor size (p <0.0220, OR = 6), and tumor focality (p <0.0230, OR = 9.428) (Table 6.9). Association between these histopathological features indicated a risk rate for lymph node metastasis of 6 for tumors larger than 40 mm, and of 9.42 for multifocal tumors.

Table 6.9. Clinicopathological characteristics of MTC according to lymph node metastasis

Clinicopathological characteristics	Lymph node metastasis		Univariate analysis	OR (95% CI)
	present (# 20)	absent (# 23)		
Age at diagnosis				
<55 yo	8 (40%)	5 (21.7%)	0,1934	0.416 (0.109-1.583)
>55 yo	12 (60%)	18 (78.2%)		
Tumor size (mm)				
< 10 mm	0 (0%)	3 (13%)	**0,0220**	6 (1.081-33.275)
10 - 40 mm	12 (60%)	18 (78.2%)		
> 40 mm	8 (40%)	2 (8.6%)		
Focality of the tumor				
Unifocal	14 (70%)	22 (95.6%)	**0.0230**	9.428 (1.023-86.860)
Multifocal	6 (30%)	1 (4.3%)		
Tumor recurrence	6 (30%)	5 (21.7%)	0.6345	1.38 (0.365-5.215)
Histopathologic MTC type				
Conventional	12 (60%)	12 (52,1%)	0.6060	0.727 (0.216-2.444)
Other variants	8 (40%)	11 (47.8%)		
Coexisting thyroid pathology				
Colloid goiter	8 (40%)	10 (43.4%)	0.9675	1.125 (0.308-4.104)
Nodular goiter	9 (45%)	10 (43.4%)		

Clinicopathological characteristics	Lymph node metastasis		Univariate analysis	OR (95% CI)
	present (# 20)	absent (# 23)		
Hashimoto thyroiditis	3 (15%)	3 (13%)		

*OR: odd ratio; CI: confidence interval; *p-value <0.05 was considered to be statistically significant * colloid and nodular goiter vs Hashimoto thyroiditis*

6.8.4. Discussion

Over the last 20 years, although the incidence of MTC has been steady, mortality has remained unchanged [Lloyd et al., 2017]. A stable incidence of MTC within the increased thyroid malignancy incidence may explain a relative trend toward a decreasing percentage of MTC [Ahmed et al., 2011; Hadoux et al., 2016]. Reports from extensive studies indicate that tumor size, extrathyroidal extension, lymph node and distant metastases may be considered predictors of survival [Kebebew et al., 2000; Lloyd et al., 2017].

We emphasize that, based on the experience in endocrine pathology of the Pathology Department of "Sf. Spiridon" Clinical Emergency County Hospital, the 59 cases of selected MTC are relevant, if we consider that MTC represents only 2-3% of thyroid malignancies [Lloyd et al., 2017]. In parallel, we also analysed the particularities of histological variants of MTC, namely conventional, spindle cell, oncocytic, pseudopapillary, or follicular/glandular, aiming to identify possible differences in the biological potential of aggression.

By correlating the main prognostic factors (gender, age, stage, extrathyroid extension, lympho-vascular invasion, histological subtypes) with the disease evolution (recurrence, nodal or distant metastases), the following aspects have resulted.

Gender

In the analysed group we recorded a higher frequency of females' cases (46 cases - 80%), compared to males (13 cases - 20%), similar data to those presented in the main publications in the field [Lloyd et al., 2017]. The female predominance can be attributed to the hormonal substrate, known as a potential tumor modulator [Wells et al, 2015; Lloyd et al., 2017]. Moreover, in females there was a more advanced stage of the disease (pT2, pT3), older age (> 55 years) and more unfavorable clinical evolution - translated by tumor recurrence (10 cases - 17%) and distance metastasis (2 cases - 3.3%), results consistent with the reports in the field reports [Kebebew E et al, 2000; Wells et al., 2015].

Age

Although the staging of MTC is not influenced by age, the analysis of this parameter in relation to other clinicopathological features has led to additional information that may contribute to deepening knowledge of the biological profile of MTC, with correlation to personalized therapeutic protocols. In our study group with the mean age of approximately 60 yo, we found that patients over the age of 64 had a more severe course, with extensive lymph node metastases (N1b) and high staging. This observation, inconsistent with literature data, opens new perspectives for reconsidering age in assessing the prognosis [Wells et al., 2015].

Tumor size

Tumor size is an important clinicopathological parameter used in tumor staging, bringing information with definite prognostic value on the evolution of the disease, mainly for overall survival, association of lymph node or distant metastasis and tumor recurrence, respectively [Wells et al., 2015; Amin et al., 2016; Lloyd et al., 2017].

In our study group, the size of the tumor nodules was variable, being generally larger in the elderly. Patients over 64 yo had an average size of 40 mm, and patients under 64 yo had dimensions between 10 and 25 mm, closely related to nodal metastasis, an aspect also reported in literature [Kebebew E et al., 2000].

The circumscribed appearance, characteristic of the tumor nodule in MTC in the initial stages of tumor development, was present in most cases in the pT1a, pT1b stage and only in a few cases the pT2 stage. However, this feature is less applicable in the case of tumors classified in the pT3 and pT4 stage, because the delimitation from the periphery is either absent, or only partially present, dominating the infiltrative character.

Although data from the literature indicate the presence of lymph node metastases in the case of tumors smaller than 10 mm (in 10-30% of cases), which benefited from radical/modified radical lymph node dissection, in the analysed group we have no case of node involvement. Of the 17 cases (28.8%) with sizes between 10 and 20 mm, two cases (11.76%) were associated with lymph node metastasis, and of the 21 cases (35.5%) with sizes between 20 and 40 mm, 10 cases (47.6%) had positive lymph nodes; these values are close to those reported in the literature [Lloyd et al., 2017]. In the 12 cases (20.3%) larger than 40 mm, lymph node metastasis was present in 8 cases (66.6%). Consequently, our results indicate a progressive growth percentage in lymph node metastasis in parallel with the increase of tumor size, in line with the results of the main flow of publications [Wells et al., 2015].

Thyroid capsular invasion

In 2004 WHO classification, capsular invasion in association with minimal invasion of perithyroid adipose tissue was introduced as a staging criterion. An important change brought by 2017 WHO classification is the rejection of this parameter in staging [Lloyd et al., 2017]. On the other hand, based on clinical experience, some studies and practice guidelines support the evaluation and reporting of thyroid capsular invasion, considering that it may influence the prognosis in modulating therapy [Doherty et al., 2009; Haugen et al., 2016].

The analysis of classical clinicopathological factors in relation to the invasion of the thyroid capsule did not show statistically significant differences. However, out of the total 24 cases (40.6%) of MTC with capsular invasion, 7 cases (29.1%) were associated with tumor recurrence, and two cases (8.3%) developed distant metastasis. These results recorded in the studied subgroup support the views that capsular invasion is an element of aggression in the evolution of tumor progression, in accordance with literature [Wells et al., 2015].

Lympho-vascular invasion

Of the total 59 cases of MTC, 34 (over 50%) were associated with lympho-vascular invasion. A particular aspect, highlighted with the reevaluation of all cases of MTC, was the frequent occurrence of vascular tumor emboli on the edge of infiltrative area, in the non-tumor thyroid tissue or adipose perithyroid tissue. Thus, for the cases included in the pT1 stage, which have a well-circumscribed delimitation in the periphery, the vascular tumor emboli did not constitute a ubiquitous element. We paid special attention to this aspect, because invasiveness and tumor vascular embolism may themselves be factors that facilitate tumor progression, given that the specific tumor invasion microenvironment provides a setting conductive to angiogenesis [Ralhan et al., 2010; Wells et al., 2015].

Statistical analysis revealed correlations between lympho-vascular invasion and tumor size. This result complements previous comments on tumor size and the association of lymph node metastases by relating directly to the mechanism metastasis. Moreover, as an element of originality, our data support, for the presence of tumor emboli an OR of 13.695, for tumors larger than 40 mm.

Lymph node metastasis

Our results reflect the dynamic relationship within the metastatic sequence, between tumor size, lympho-vascular invasion and tumor lymph nodes metastases. Retrospective analysis of the 59 consecutive cases of MTC, treated by total thyroidectomy and central and/or bilateral laterocervical nodal dissection, confirmed the presence of nodal metastasis in

20 cases (34%), but the frequency was lower compared to other reports - respectively 55%, in a series of 776 cases [Wells et al., 2015].

Considering lymph node metastasis as a parameter of aggression, the statistical analysis showed correlations with tumor size and multifocality.

Focality of the tumor

Multifocality is an important diagnosis element given that the presence of multiple lesions is characteristic of cases of familial MTC. Of the 9 multifocal cases (15.2%) of MTC, 4 cases (44.4%) were classified, based on clinical information, as family MTC. Consequently, our results agree with the results of the main flow of publications [Wells et al., 2015; Lloyd et al., 2017].

Tumor recurrence

At intervals of 12-48 months, we registered locoregional recurrence (stations I, II, III, IV or V) in 20 cases (33%) and retro-pharyngeal in 6 cases (30%). The cases with recurrences presented, at the first surgical intervention, unilateral or bilateral cervical nodal metastasis, aspects that correlate with advanced staging (pT3N1b).

An important aspect analyzed in our study was the disease-free period until the onset of metastasis or recurrence appeared. Patient follow-up showed that the disease-free period was significantly longer in pT1 cases without lymph node metastasis compared to pT2 and pT3 cases with metastasis. The results obtained are similar to the data in the literature [Wells et al., 2015; Lloyd et al., 2017].

Calcitonin

Serum calcitonin is a reliable indicator for preoperative diagnosis and postoperative clinical monitoring of MTC, respectively [Basuyau et al., 2004; Wells et al., 2015]. We recorded an increase in preoperative and postoperative calcitonin values, consistent with staging of pTNM - mainly with the presence of lymph node metastasis. We emphasize that, in cases where surgical treatment provided complete tumor excision and lymph node dissection, calcitonin levels normalized and remained constant during the 3 months of patients' follow-up. Postoperative calcitonin levels remained elevated in association with lymph node metastasis and distant metastasis. These observations are consistent with the data reported in literature that undetectable calcitonin values at two months postoperatively are an excellent predictor of complete remission [Basuyau et al., 2004; Lloyd et al., 2017].

Histopathologic MTC type

The identification of histological MTC subtypes is a constant concern in the routine diagnosis. The statistical analysis did not show significant differences between the histopathological MTC variants and the established aggression parameters. These results are similar to the literature [Lloyd et al., 2017], and argue that histological variants of MTC do not significantly influence prognosis. With strict reference to the histopathological evaluation in MTC, a particular aspect identified was the coexistence of MTC with PTC, with different cell origins. This association, rarely reported in the literature, is diagnosed in our study group in 7 cases (12%), similar to relatively recent reported results, which indicate a percentage of 13.8% [Wells et al., 2015].

6.8.5. Final remarks

In CTM, lympho-vascular invasion and lymph node metastasis are parameters of aggression in relation to the other classical clinicopathological features. The chances of risk for lympho-vascular invasion and lymph node metastasis, expressed by OR, are associated with tumor size over 40 mm. In addition, the chance or risk for lymph node metastasis – calculated as OR - is associated with multifocal primary tumor.

CHAPTER 7.
AUTOIMMUNE THYROIDITIS: FROM THE PATHOGENIC MECHANISM TO DIAGNOSIS

7.1. INTRODUCTION

Autoimmune thyroiditis (AIT) is a term used to describe different pathogenic forms of chronic lymphocytic thyroiditis. The disease is the main cause of acquired hypothyroidism and may be associated with many other autoimmune endocrine and non-endocrine disorders [Jenkins et al., 2002]. It is considered to be a genetic disease caused by the combined effects of human leukocyte antigen (HLA) class II genes and non-HLA genes polymorphisms [Kim et al., 2003].

There is no internationally accepted classification of autoimmune thyroid diseases, as their development is not yet fully understood.

Classically, AIT is considered a histological diagnosis that can be subdivided into chronic lymphocytic thyroiditis, if only lymphocytic infiltration is present, and Hashimoto's thyroiditis, if atrophy and eosinophilic changes in thyroid cells and fibrosis are also seen [Costa et al., 1989].

Taken into consideration the thyroid function and histological findings, the chronic lymphocytic thyroiditis is classified into four categories: (i) oxyphilic chronic thyroiditis, which includes the classical group of Hashimoto's thyroiditis, (ii) mixed chronic thyroiditis, in which the inflammatory infiltrate is more reduced than in the first category, the fibrosis in the interstice is more discrete, and the clinical picture ranges from euthyroidism to hypo- or hyperthyroidism, (iii) hyperplastic chronic thyroiditis, which corresponds to Basedow–Graves disease and progresses with hyperfunction, and (iv) focal chronic thyroiditis, with a discrete focal lymphocytic inflammatory infiltrate, unaccompanied by germinative foci or oxyphilic metaplasia, and in which the thyroid status is euthyroidism [Mizukami et al., 1992].

AIT is subdivided into primary ones (Hashimoto's thyroiditis, chronic lymphocytic thyroiditis, chronic atrophic thyroiditis) and the secondary ones, which are the manifestation of the organ-specific autoimmune diseases of the thyroid [Pal'tsev et al., 1993].

According to other authors, autoimmune thyroid diseases (AITDs) include Graves' disease (GD) and Hashimoto's thyroiditis (HT), both of them being characterized pathologically by infiltration of the thyroid by T- and B-cells, reactive to thyroid antigens, biochemically by the production of thyroid autoantibodies, and clinically by abnormal thyroid functions (hyperthyroidism in GD and hypothyroidism in HT) [Tomer, Huber, 2009]. These researchers admitted that there are additional variants of AITDs that include post-partum thyroiditis, drug-induced thyroiditis, or thyroiditis associated with polyglandular autoimmune syndromes.

A more recent approach classifies chronic AIT into six groups: goitrous (Hashimoto's thyroiditis), characterized by goiter, lymphocytic infiltration, fibrosis, and thyroid-cell hyperplasia; atrophic thyroiditis (primary myxedema), with atrophy and fibrosis; juvenile thyroiditis, characterized by lymphocytic infiltration; postpartum thyroiditis associated with small goiter and some lymphocytic infiltration; silent (painless) thyroiditis, with small goiter and some lymphocytic infiltration; and focal thyroiditis, which is found in 20% of people at autopsy [Weetman et al., 2013].

7.2. ETIOPATHOGENESIS

Etiological insights into autoimmune thyroiditis

The exact etiology of AIT is not fully known [Tomer, Huber, 2009], but it is considered to be multifactorial. The probability of developing an AIT is determined by environmental, genetic, constitutional factors, and the associated disorders [Jenkins et al., 2002; Sava et al., 2013].

Environmental factors play a critical role in the occurrence of AIT in the susceptible population because of the immune system activation. Some of these variables, such as dietary iodine from iodized salt, dairy products, eggs, iodized wholesome added substances, chocolate, and a few multivitamins, act in a particular way [Jenkins et al., 2002; Sava et al., 2013].

Iodine increases the antigenicity of thyroglobulin and thus exacerbates thyroiditis [Jensen et al., 2004]. Iodine has a vital role in thyroid hormone genesis and can trigger thyroid immunity in various ways. In the early phases of immune system formation, high quantities of iodine are quickly oxidized by thyroid peroxidase, which has a specific role in the iodination process and oxidation of iodotyrosine to iodothyronine. This process produces autoreactive products such as lipoic acid and oxygen reactive metabolites. Due to the oxidation of the lipid and protein segments of the cell layer, this oxidative species harm and initiate necrosis of the thyroid cells [Jensen et al., 2004]. Second, the iodination of thyroglobulin intensifies its immunogenicity by making new epitopes or uncovering "mysterious" epitopes that are not expressed in iodine deficiency conditions. These may account for the protective role of iodine deficiency contributing to lessened thyroid autoimmunity and in increased thyroid autoimmunity in areas with extreme iodine intake [Jensen et al., 2004]. Increased iodinated thyroglobulin may encourage antigen take-up and handling by antigen-presenting cells. Also, iodine itself could lead to intercellular adhesion molecule-1 (ICAM-1) expression in the thyrocytes [Jensen et al., 2004]. Thus, iodine overconsumption exacerbates local immune inflammation by immunological and biochemical patterns.

The majority of ecological elements like vitamin D or selenium insufficiencies, medical or accidental exposure to ionizing radiation, medications (beta-blockers, Lithium, Amiodarone, Phenylbutazone, glucocorticoids, Furosemide, Carbamazepine and antiretroviral drugs) can also affect thyroid autoimmunity [Tomer et al., 2009; Sorodoc et al., 2013]. If we refer to selenium as an environmental etiopathogenic factor, the thyroid gland is the largest reservoir of selenium of the whole body [Duntas et al., 2010]. Selenoproteins like glutathione peroxidases (GPxs) and thioredoxin reductases, present at the level of thyrocytes, are able to control the redox state and protect the cells from the oxidative damage; in particular, GPx-3 inhibits the oxidative capacity of hydrogen peroxide. As low selenium levels are associated to immune dysfunction, a reduced selenium assumption is considered a risk factor for AITD development [Effraimidis et al., 2014]. The higher number of AIT cases in women is most likely due to the influence of sex steroids. Estrogen use is associated with a lower risk, and pregnancy with a higher risk for developing hyperthyroidism [Whitacre et al., 2001].

Immunological mechanisms of autoimmune thyroiditis

AIT is a model of both cell and humoral immune disorder. A noteworthy site of autoreactivity is inside the thyroid organ itself. The antigenic structure of immune system reactivity can trigger viral infection that has protein sequence similar to the thyroid organ itself, or a self-protein that is introduced as an antigen [Czarnocka et al., 2011]. The target antigens of the thyroid antibodies are colloid constitutive protein – thyroglobulin (Tg), the enzyme necessary for thyroid hormone synthesis – thyroid peroxidase (TPO), Na^+/I^-

symporter (NIS) and thyrotropin receptor (or TSH-R – thyroid-stimulating hormone receptor) [Czarnocka et al., 2011].

In Hashimoto's thyroiditis, there is a broad invasion of the thyroid by lymphocytes, plasma cells and macrophages. The thyroid follicular cells are destroyed to a variable degree. The rest of the healthy cells will become hyperplastic and will undergo oxyphilic metaplasia and will become Askanazy or Hürthle cells or oncocytic cells, *i.e.*, large cells with abundant eosinophilic granular cytoplasm because of accumulation of altered mitochondria. All types of thyroid autoimmunity are related to a lymphocytic invasion of the thyroid, which will generate both T- and B-cell-interceded autoreactivity. Thyroid autoreactive lymphocytes may also be found in other places like lymph nodes and bone marrow [Prasad et al., 2003]. High amounts of antithyroid antibodies are the principal perceptible sign of immune response in autoimmune thyroiditis.

TPO is the key thyroid enzyme catalyzing both the iodination and coupling reaction for the synthesis of thyroid hormone. It oxidizes iodide ions to iodine atoms that will be incorporated into thyroglobulin for the production of thyroxine (T4) or triiodothyronine (T3), the thyroid hormones. TPO is membrane bound and found in the cytoplasm and in high concentration on the apical microvillar surface of thyrocytes [Iddah et al., 2013]. Anti-TPO autoantibodies, included in IgG class 1 and IgG4 subclasses are found in over 90% of patients with autoimmune hypothyroidism and Graves' disease and are the predominant antibodies in autoimmune hypothyroidism. The serum values of TPO antibodies should be correlated with intracellular interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) levels in thyroid infiltrating lymphocytes and thyrocytes. High levels of these two cytokines, which are involved in humoral and cellular immunity, correlated well with severe AIT [Bossowski et al., 2011].

Thyroglobulin is a 660-kDa glycoprotein made out of two similar subunits of 330 kDa each. It is synthesized in the thyroid follicular cells, released in the follicular lumen, and deposited there as a colloid substance. Each thyroglobulin has around 100 tyrosine residues, and a quarter of them are iodinated. These residues couple with iodine to form T3 and T4 [Iddah et al., 2013]. Thyroglobulin autoantibodies, polyclonal and mainly of IgG class, are found in less than 60% of patients with lymphocytic thyroiditis and 30% of Graves' disease patients [Adil et al., 2003; Iddah et al., 2013].

TSH-R is the prime autoantigen in Graves' disease and atrophic thyroiditis, being located on the basal surface of the thyroid follicular cells [Adil et al., 2003]. In Basedow's disease, thyroid-stimulating antibodies bind to the receptor and stimulate the thyroid cell to produce excessive amounts of thyroid hormones resulting in hyperthyroidism. In patients with atrophic thyroiditis, the major antibody is the TSH-R blocking antibody. After binding to the receptor, this antibody blocks the binding of TSH to its receptor, thus preventing thyroid cell stimulation. Therefore, the thyroid hormone output diminished, the thyroid gland becomes atrophic, and a clinical state of hypothyroidism appears [Szkudlinski et al., 2002; Swain et al., 2005].

NIS, a transmembrane glycoprotein which transports two sodium cations (Na^+) for each iodide anion (I^-) into the cell, mediates the uptake of iodide into follicular cells of the thyroid gland as the first step in the synthesis of thyroid hormone. NIS is another major thyroid autoantigen. Around 33% of Basedow's disease cases and 15% of Hashimoto's cases contain antibodies that restrain *in vitro* NIS iodide take-up [Swain et al., 2005; Czarnocka et al., 2011].

7.3. CLINICAL CHARACTERIZATION

Clinical signs in autoimmune thyroiditis

The thyroid gland appears as firm and enlarged in the goitrous form, while it is not palpable in the atrophic form of AIT. The classic, goitrous form of AIT is less frequent in

men than in women; it develops frequently around fifty years old [Ragusa et al., 2019]. Patients with AIT and goiter may have different local or systemic clinical features. Dysphonia, dysphagia and dyspnea represent the local features, due to the enlargement of the thyroid that narrows the adjacent cervical structures.

About 25-30% of patients have thyroid dysfunctions, ranging from subclinical hypothyroidism, with thyroid hormones at the range levels and high levels of TSH, to overt hypothyroidism [Caturegli et al., 2014; Ragusa et al., 2019]. Systemic clinical pictures occur as a result of primary hypothyroidism. The symptoms and signs of hypothyroidism are many, changeable and not specific, and, due to the wide and deep action of thyroid hormones on organs and tissues [Caturegli et al., 2014; Ragusa et al., 2019]. Different systems, like cardiovascular, pulmonary, hematopoietic, gastrointestinal, urinary, reproductive, neuro-psychiatric, skeletal, skin and appendages, are influenced by hypothyroidism, including AIT hypothyroidism [Caturegli et al., 2014; Ragusa et al., 2019]. However, the symptoms of hypothyroidism are not simple to identify due to an overlap with the aging manifestations.

Ultrasonographic findings in autoimmune thyroiditis

The first basic diagnostic problem arises in patients with only minimally or moderately hypoechogenic pattern. If we do not notice this form in a euthyroid patient, our report will have false positive results in the case of a healthy thyroid. Alternatively, if hypoechogenicity is noticed in a euthyroid patient, we can consider the possibility of an underlying AITD and thus have the chance to recognize hypothyroidism later [Yeh et al., 1996].

The other issue is caused by local hypoechogenicity. This is the most complicated differential diagnosis issue in thyroid ultrasound of a TN. An accurate diagnosis requires the corroboration of clinical, laboratory and cytological data, and, in certain cases, of follow-up results and, in surgically treated patients, possibly with the macroscopic and microscopic pathological findings. There are some essential criteria that allow differentiation. Unlike the TN, in AIT, most of the times, the limits of the lesion are not geometrical, but the hypoechogenic area is connected by echonormal parenchyma not yet affected by thyroiditis. Another finding is the presence of hypoechoic micronodules (1-6 mm) with surrounding echogenic septations, described as pseudonodular or a giraffe pattern [Yeh et al., 1996].

When the main echostructure of the thyroid is not echonormal but rather insignificantly or decently hypoechogenic, then it is a higher possibility to find an AIT. It is also important to analyze the volume of the thyroid as most of the time this gland is enlarged as a multinodular goiter [Yeh et al., 1996]. This means that a normal looking thyroid gland in the presence of numerous hypoechogenic lesions makes the diagnosis of AIT very probable. However, when we have doubts, antibodies and hormone-levels on follow-up investigations will resolve the problem because there is a well-known close correlation between anti-TPO autoantibodies levels and hypoechogenicity [Pedersen et al., 2000; Tam et al., 2015].

7.4. PATHOLOGICAL EXAMINATION

Cytological features of autoimmune thyroiditis

FNAB of the thyroid gland is an accurate diagnostic test used routinely in evaluation of thyroid lesions, either single or in addition to ultrasonographic guidance [Cibas et al., 2008]. The FNAB technique uses a very fine needle (25 or 27gauge) and the aspiration, with or without suction, applied by a syringe. Slides are prepared by expelling and smearing the cells on a slide. Alternatively, or as an adjunct to smears, the needle is rinsed, and the resulting cell suspension used for cytocentrifuge, thin-layer, and/or cell block preparations. The smears are stained with MGG or Papanicolaou stain. The advantages of thin-layer

("liquid-based") preparations over smears include reduced blood; ease in preparation of consistently well-fixed slides, particularly when the FNAB is not performed by a pathologist; and a concentrated specimen that requires less screening time [Cibas et al., 2008]. The Bethesda System for Reporting Thyroid Cytopathology has been widely adopted for reporting the results of thyroid FNABs [Ali et al., 2009]. The FNAB reporting system has six general categories; each of the categories has an implied cancer risk and is linked to an evidence-based clinical management guideline. For a thyroid FNA specimen to be satisfactory for evaluation (and benign), at least six groups of benign, well-visualized follicular cells are required, each group composed of at least 10 cells [Ali et al., 2009].

On smears, the typical image of AIT includes lymphoid cells with follicular epithelial cells with varying degrees of degenerative changes and insignificant colloid in the background (Figures 7.1 – 7.6). The presence of atypical cells is a common feature of autoimmune thyroiditis. Both thyroiditis itself and the dysfunction may cause anisonucleosis and even pleomorphism. The latter may have the appearance of an ATC [Boi et al., 2005].

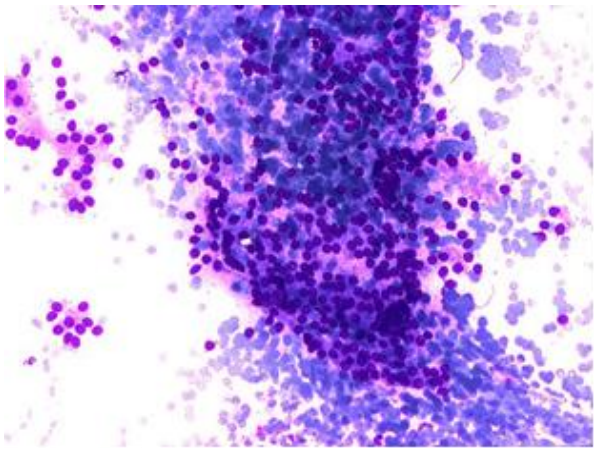


Figure 7.1. Chronic lymphocytic thyroiditis: thyrocytes with monomorphous round nuclei and basophilic cytoplasm admixed with lymphocytes (MGG, x 100)

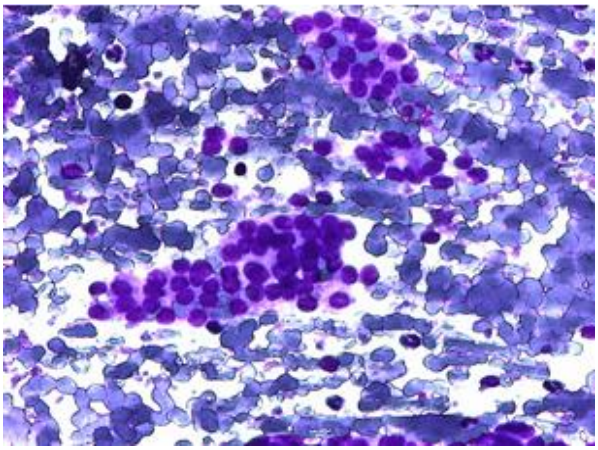


Figure 7.2. Chronic lymphocytic thyroiditis: thyrocytes and lymphoid infiltrate (MGG, x 200)

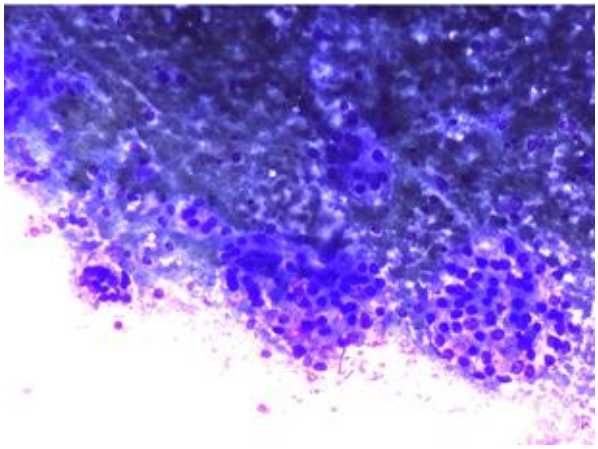


Figure 7.3. Basedow's disease: abundant hematic background and sheets of follicular cells; thyrocytes have discrete nuclear irregularities (MGG, x 100)

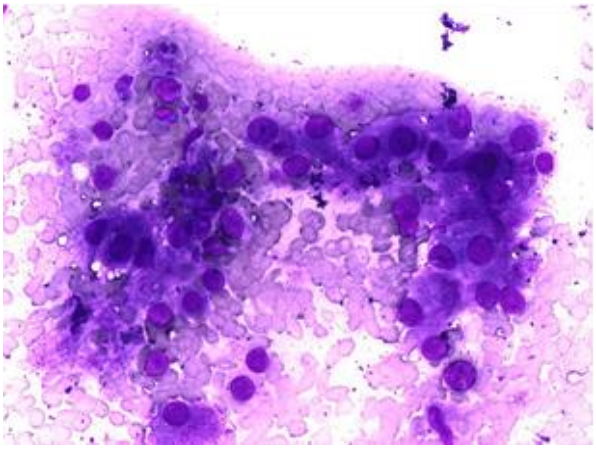


Figure 7.4. Basedow's disease: group of thyrocytes with nuclear irregularities (MGG, x 200)

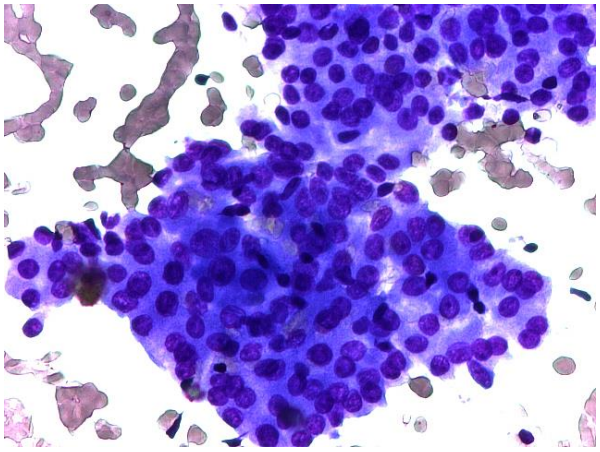


Figure 7.5. Hürthle-cells (oncocytic cells): sheet of oncocytes with nuclear irregularities and eosinophilic cytoplasm (MGG, x 200)

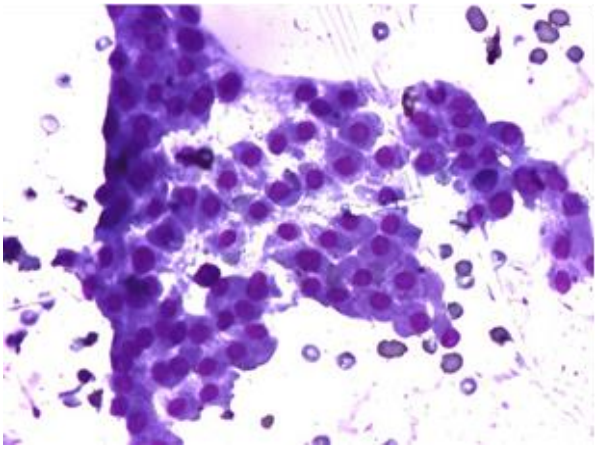


Figure 7.6. Hürthle-cells (oncocytic cells): sheet of oncocytes with large size, distinct cell borders, eosinophilic cytoplasm, and large nucleus with irregularities (MGG, x 200)

A serious differential diagnosis problem is caused by the presence of inclusion and grooves. The positive predictive value of these intranuclear figures is limited in the case of thyroiditis; therefore the diagnosis of a concomitant papillary carcinoma is a great challenge for the cytopathologist in certain cases [Boi et al., 2005].

The presence of naked follicular cells in great number, without structure formation, may be challengeable as to the origin of these cells. They might be misjudged as little lymphocytes (Figures 7.7 – 7.8). The presence or absence of lymphoblast and the absence or abundant presence of colloid is of great help. The other problem is focal AIT close to nodular goiters. In these cases, only scarce lymphocytes are available on the smear. Oxyphilic changes of follicular cells may be seen in various proportions.

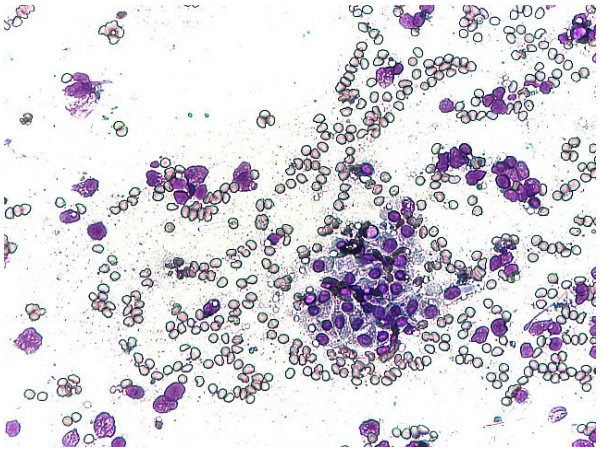


Figure 7.7. Thyreocytes in small sheets associated with frequent lymphocytes (MGG, x 100)

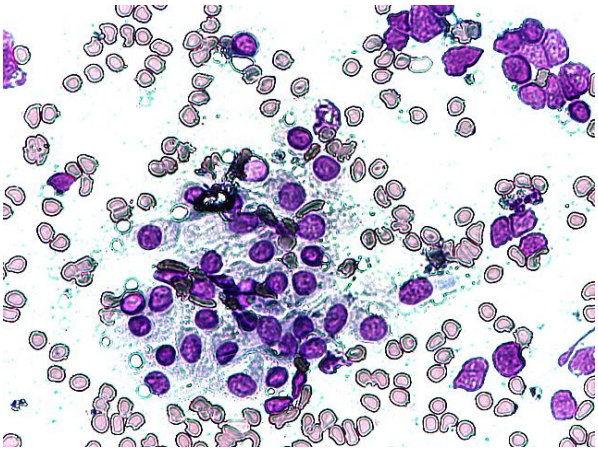


Figure 7.8. Thyreocytes in small sheets associated with frequent lymphocytes (MGG, x 200)

Histopathological principles and dilemmas in AIT

From the morphological point of view, Hashimoto's thyroiditis contains a rich lymphocytic infiltrate predominantly disposed in follicles with germinal center formation. Lymphocytes are predominantly T-cells. Thyrocytes with oxyphilic metaplasia, oncocytes, and large Hürthle cells with abundant eosinophilic cytoplasm may be noticed in their immediate vicinity. Hürthle cells could show moderate nuclear atypia, nuclear hyperchromasia or macronucleoli. As the disease progresses, fibrosis increases and numerous

collagen fiber could be noticed in the interstice. As such, with disease progression, a multinodular appearance developed (Figures 7.9 – 7.11). The lymphoid nodular infiltrate is polymorphic and includes small amounts of T-lymphocytes, laid out on the margins of lymphoid follicles, along with numerous B-lymphocytes and rare macrophages (Figures 7.12 – 7.15). The mitotic rate is high in the germinative centers (Figure 7.16). When the inflammatory infiltrate is abundant, one should rule out a possible progression of the disease towards mucosa-associated lymphoid tissue (MALT) lymphoma, in which case the profile of the lymphoid tumor infiltrate is monoclonal. Focal chronic thyroiditis, which can coexist with other diseases [Sava et al., 2013], reveals discrete chronic inflammatory cell infiltrate, without lymphoid follicles or germinative center. Histological features of oxyphilic metaplasia are discrete. Fibrosis is reduced (Figures 7.17 – 7.18). The inflammatory cell infiltrate is polymorphic, made up of T-cells, B-cells, and randomly scattered macrophages (Figures 7.19 – 7.20).

Unfortunately, thyroid nodules have a similar frequency in both autoimmune and non-AIT patients. The major issue is to diagnose a thyroid nodule in a hypoechogenicity, which may cover a hypoechoic thyroid nodule. This can be done by thorough grayscale investigation and by differentiation between the vascularization of the nodular and the non-nodular part of the thyroid [Loy et al., 2004; Cornianu et al., 2011].

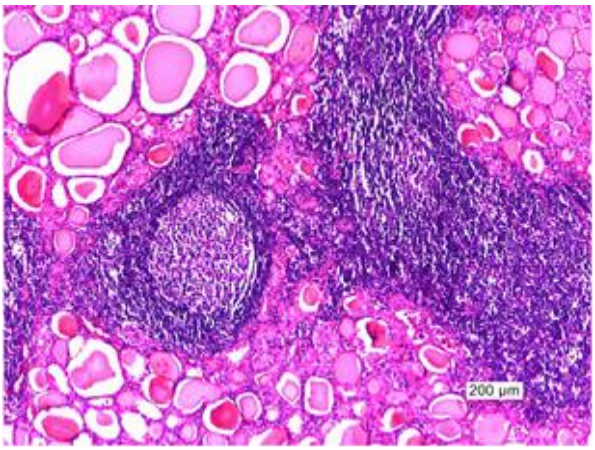


Figure 7.9. Hashimoto's thyroiditis: prominent lymphoid follicles with germinal centers, oxyphilic metaplasia of the epithelium, and abundant colloid (HE, x 40)

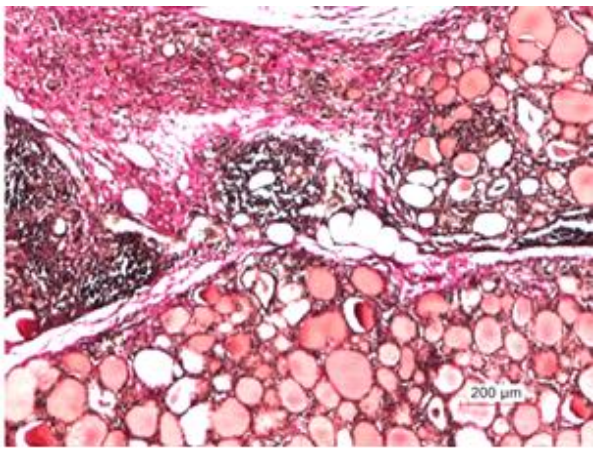


Figure 7.10. Hashimoto's thyroiditis: increased inter-lobular fibrosis produced the atrophy of the follicles nearby (VG, x 40)

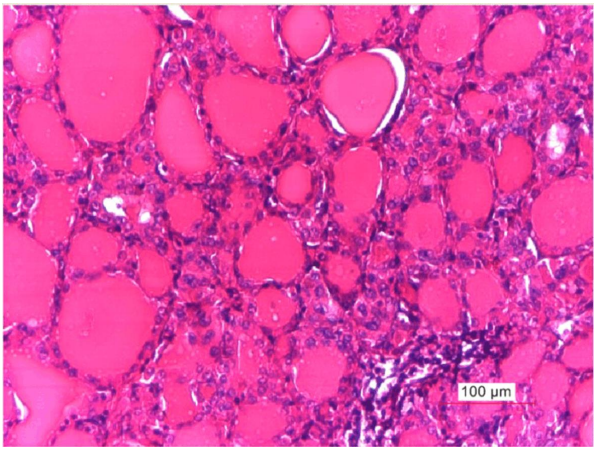


Figure 7.11. Hashimoto's thyroiditis: oxyphilic metaplasia with discrete differences in nuclear size (HE, x 100)

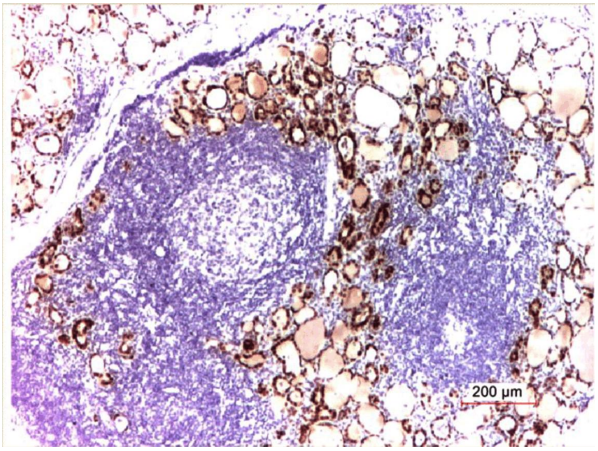


Figure 7.12. Hashimoto's thyroiditis: lymphocytic infil- trate with germinal centre; immunopositivity for TTF1 in oxyphilic areas (IHC, anti-TTF1, x 40)

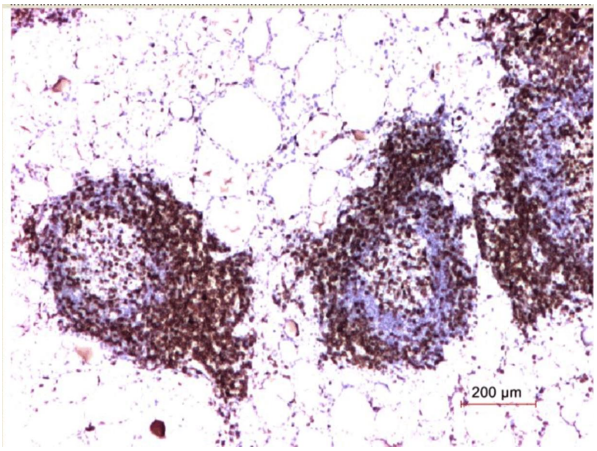


Figure 7.13. Hashimoto's thyroiditis: T-lymphocytes showed immunopositivity for CD3 in the periphery of lymphoid follicles (IHC, anti-CD3, x 40)

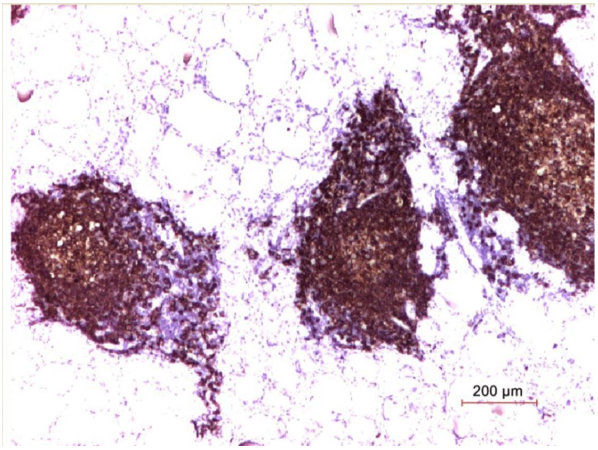


Figure 7.14. Hashimoto's thyroiditis: B-lymphocytes showed immunopositivity for CD20 inside the lymphoid follicles (IHC, anti-CD20, x 40)

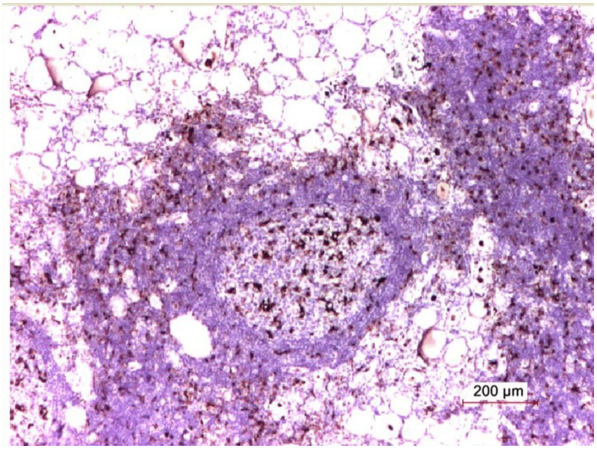


Figure 7.15. Hashimoto's thyroiditis: immunopositivity for CD68 highlights the presence of macrophages (IHC, anti-CD68, x 40)

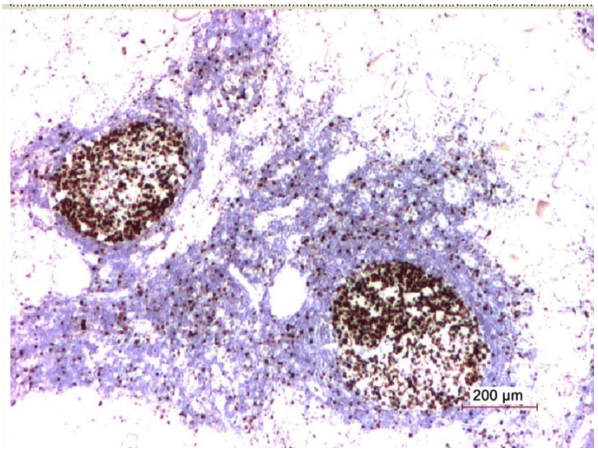


Figure 7.16. Hashimoto's thyroiditis: intense nuclear positivity inside the germinal center of lymphoid follicles, (IHC, anti-Ki67, x 40)

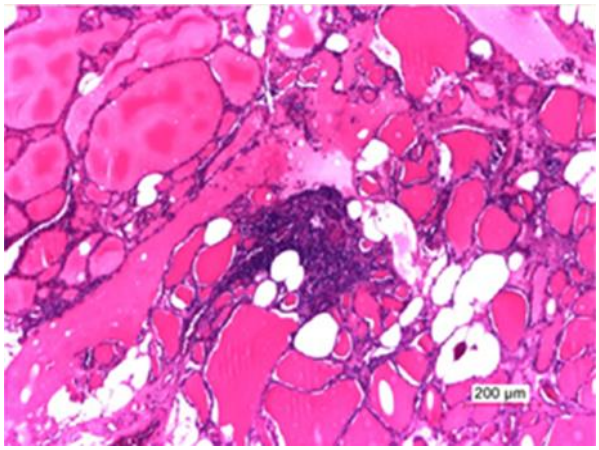


Figure 7.17. Focal chronic thyroiditis: focal aggregates of lymphocytes in, inter- or intra-lobular fibrous tissue (HE, x 40)

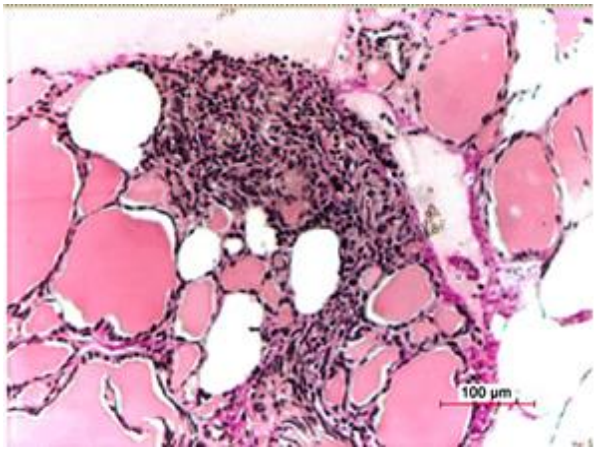


Figure 7.18. Focal chronic thyroiditis: patchy lymphocytic inflammation with small lymphocytes (VG, x 100)

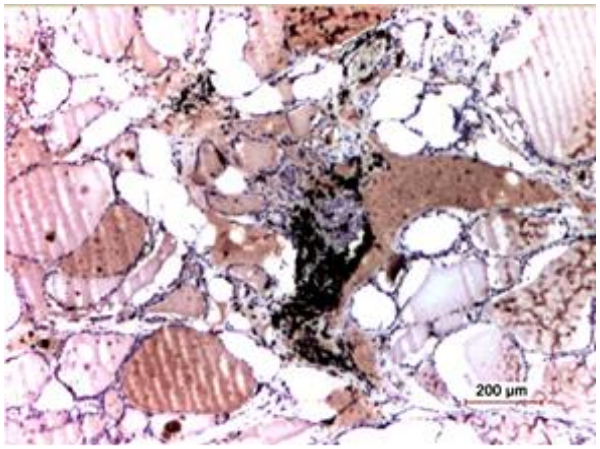


Figure 7.19. Focal chronic thyroiditis: T-lymphocytes showed immunopositivity for CD3 in the chronic inflammatory cell infiltrate (IHC, anti-CD3, x 40)

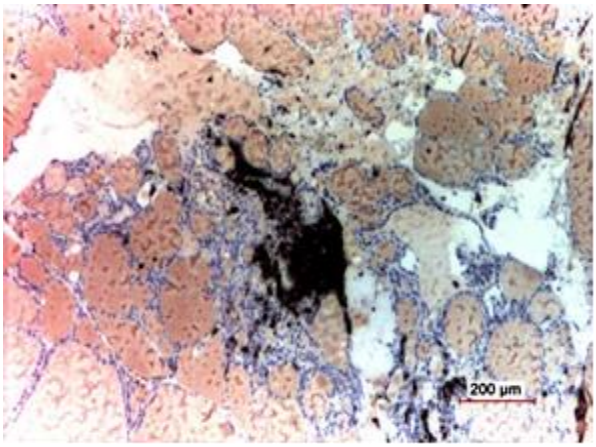


Figure 7.20. Focal chronic thyroiditis: B-lymphocytes showed immunopositivity for CD20 in the chronic inflammatory cell infiltrate (IHC, anti-CD20, x 40)

7.5. DIFFERENTIAL DIAGNOSIS

Hashimoto's thyroiditis versus Basedow's disease

From a clinical point of view, it is easy to differentiate between Hashimoto's thyroiditis and Basedow's disease, even though both of them are considered by some authors to be AIT. The problematic cases are those with only slight hyperthyroidism [Ko et al., 2003]. Some clinicians cannot decide if a patient has or not a thyroiditis, as they cannot decide whether to prescribe the patient a particular thyroid drug or not. It must be kept in mind that these patients typically have a diffuse enlargement of the thyroid [MacDonald et al., 1999].

It is vital to diagnose Hashimoto's thyroiditis due to its progression to hypothyroism and to the fact that it requires long-term thyroxin supplementation. This is especially difficult, since there are two clinical types of a similar autoimmune thyroid disease.

In clinical practice, the progress of a hyperthyroid patient towards Basedow–Graves disorder takes years or even decades. The opposite is also possible, however just occasionally. Additionally, there is a higher risk of extranodal marginal B-cell lymphoma in patients with Hashimoto's thyroiditis probably because the latter is characterized by the presence of lymphoid follicles with germinative centers [MacDonald et al., 1999].

The incidence of carcinoma in patients with Hashimoto's thyroiditis ranges from 0.5% to 23.5% and this fact emphasizes the need for long-term follow-up. FNAB smears of HT reveals oxyphilic cells, invasion of follicles by lymphocytes/plasma cells and moderate amount of colloid in the background. In Basedow's disease, the smears have a hematic background, and there is negligible quantity of colloid, marginal vacuolization, the cells are round, with anisonucleosis and organized in groups, showing a follicular pattern, but, sometimes, there are some clinical cases when the smears are difficult to interpret [Solymosi et al., 2001].

However, Graves' disease – diffuse goiter with hyperthyroidism, ophthalmopathy, or both – is considered to be by some authors a related autoimmune thyroid disease but not AIT [Volpé et al., 1991].

Autoimmune thyroiditis versus de Quervain's thyroiditis

Subacute (de Quervain's) thyroiditis, which is a postviral inflammation of the thyroid characterized by pain and tenderness, is not a form of AIT [Dayan et al., 1996]. There are some problems in distinguishing between AIT and de Quervain's thyroiditis. Most of the

times, the cytological picture and the clinical information about the patient are clear enough. While an AIT may give different clinical pictures, subacute thyroiditis has a deeply specific clinical picture in over 90% of the cases: fever, hard consistency and enlargement of the thyroid, and erythrocytes sedimentation rate exceed 60 mm/h. In some cases, it is exceptionally troublesome or even impossible to make a distinction between the two kinds of thyroiditis. If the clinical picture is clear (patient with either high titers of thyroid autoantibodies or a classical picture of subacute thyroiditis), the absence of typical elements of the cytological picture is not important for the diagnosis [Leung et al., 1988].

MALT lymphomas of the thyroid versus Hashimoto's thyroiditis

Primary thyroid MALT lymphoma is a rare subgroup of thyroid lymphoma, accounting for a quarter of all primary thyroid lymphomas [Pedersen et al., 1996]. MALT lymphomas of the thyroid have the same basis as Hashimoto's thyroiditis and this can lead to cytological differentiation difficulties. MALT lymphoma of the thyroid has a typical clinical appearance. The patient is usually over 60 years of age, the thyroid increases quickly (within two months) in size, acquires a hard consistency, and ultrasonography reveals a diffuse hypoechogenic design. These four components are rarely seen together in Hashimoto's thyroiditis as this disease may appear as a diffuse goiter that grows quickly in younger patients, but in the elderly, it is a gradually progressing illness. In those cases, when it is unrealistic to distinguish between the two conditions, immunocytochemical staining is mandatory [Troch et al., 2008].

Hürthle-cell tumor versus Hashimoto's thyroiditis

In Hürthle-cell tumor, the enlarged nucleoli and the loosely arranged pattern of oncocytes with many dispersed cells are the characteristics of the lesion. The absence of nucleoli or of scattered cells supports a non-tumoral starting point. Despite these cytological signs, in the majority of the cases, we are not ready to make an obvious distinction between these two possibilities, and surgery is hence the most frequent treatment in order to make a decision [Stanciu et al., 2017]. The presence of a well-circumscribed lesion within an absolutely echonormal background rises the suspicion of a tumor, meanwhile a more hypoechogenic lesion is much less likely to be of a cancerous nature [Barbu et al., 2015]. Two other vital parameters have to be analyzed: the changes in the size of the nodule and the changes in the sonographic appearance of the thyroid outside the nodule. Therefore, high titers of thyroid autoantibodies do not exclude the possibility of a concomitant Hürthle-cell tumor. However, the hard consistency of a nodule is a sign which raises the suspicion of a tumor [Stanciu et al., 2017, Montone et al., 2008].

7.6. UNEXPECTED ASSOCIATION IN AUTOIMMUNE THYROIDITIS

7.6.1. Introduction

In AITD there is a putative notion that autoimmune diseases coexist in the same person and in families, although this has been studied only in small groups of subjects with AITD (the most common autoimmune disease) [Boelaert et al., 2010]. Autoimmune diseases associated with increased prevalences of thyroid autoimmunity include type 1 diabetes [Barker, 2006], vitiligo [Laberge et al., 2005], Addison's disease [Kasperlik-Zaluska et al., 1994] and multiple sclerosis [Barcellos et al., 2006]. It is well established that there is significant clustering of AITD within families, with 40% to 50% of patients reporting another family member with a thyroid disorder [Brix et al., 1998]. Additionally, there are marked differences in AITD prevalence between genders with 5-to 10-fold excesses in women for

both Graves' disease and Hashimoto's thyroiditis [Vanderpump et al., 1995]. Although the precise pathogenetic mechanisms are unknown, AITD is believed to reflect a multifactorial mode of inheritance, resulting from an interaction between the products of multiple genes conferring susceptibility (or protection) and various environmental triggers [Boelaert et al., 2010].

Myasthenia gravis (MG) is an autoimmune antibody-mediated disease that affects the neuromuscular junction in different groups of muscles or in all skeletal muscles. Due to immune attacks against various proteins of the postsynaptic membrane the nicotinic acetylcholine receptor (AChR), the muscle-specific tyrosine kinase (MuSK) or the low-density lipoprotein receptor-related protein 4 (LRP4), agrin, tytin, ryanodine receptors, the disease manifests itself as fatigability and weakness of ocular, facial, oropharyngeal, limb and respiratory muscles [Meriggioli et al., 2012].

MG is included among rare diseases, having an estimated prevalence of 7.77 per 100 000 individuals [Orphanet Report Series, 2019]. So far, the origin of autoimmune dysfunction in patients with MG remains unknown; however, thymic abnormalities and the consecutive immunological deficits play important roles in patients with anti-AChR antibodies. Some authors highlighted the fact that there are genetic and hormonal components associated with the production of antibodies [Cataneo et al., 2018].

Over time there have been many classifications of MG, but the clinical severity of the disease has been currently assessed based on the Myasthenia Gravis Foundation of America (MGFA) classification [Jaretzki et al., 2000] into five main classes. Recently, Koneczny & Herbst (2019) classified MG into 10 subtypes according to clinical characteristics, types of detected antibody, and thymus pathology [Koneczny, Herbst, 2019]. This classification reveals a great variability of MG and, as such, it becomes a challenge for all clinicians, as well as for surgeons.

Nonetheless, the most important fact in the management of patients with MG is the risk of developing another autoimmune disease [Nacu et al., 2015]. Such patients can later develop an AIT, i.e., Graves' disease or HT, but there are also rare cases with a second development of autoimmune hemolytic anemia [Tuncer et al., 2003] systemic lupus erythematosus [Nagarajan et al., 2019], and rheumatoid arthritis [Chai et al., 2006]. AIT was the most frequent of 23 associated autoimmune disorders, occurring in 10% of MG patients [Nacu et al., 2015]. The exact pathogenesis, trigger factors and genetic mechanism of MG, as well as MG in relation with other autoimmune disorders are still unknown [Nacu et al., 2015].

Aim

The aim of our study is to investigate the lesional association between two pathologies with autoimmune substrate, MG with anti-AChR antibody positive and HT, and to compare the data with those existing in the literature.

7.6.2. Material and methods

This was a retrospective case series study reviewing demographic, clinical, imaging, laboratory, thymic pathology, and outcome data obtained from medical records of patients with MG with anti-AChR antibodies and concomitant HT, which were recruited from a single surgical unit of a tertiary referral hospital located in the North-Eastern region of Romania. All the patients were admitted and treated in the Third Clinic of Surgery, St. Spiridon Clinical Emergency County Hospital, Iasi, Romania, over a period of 11 years (from January 1, 2000 to December 31, 2010). The research has been approved by the Ethics Committee of the "St. Spiridon" Clinical Emergency County Hospital Iasi, pursuant to the ethical standards of

Helsinki declaration regarding the patients' informed consent for the use of their medical information for scientific purpose.

Patients

All patients were subjects of a thymectomy that was performed for a suspected thymic lesion. For all the patients included in the present study, we analyzed the following: date of thymectomy, patients' gender and age at the time of thymectomy, values of antiAChR antibodies, electrophysiological findings, clinical severity, which was graded according to the MGFA scale at the last admittance.

We also noted: imaging features of the thymus, type of thymic surgery and morphological features of the surgical thymic specimens.

We also considered: the length of time between HT diagnosis and thymectomy, but also the past values of anti-TPO antibodies (normal range: <35 IU/mL); antiTg antibodies (normal range: <35 IU/mL), TSH (normal range: 0.4–5.5 μIU/mL), free thyroxine (fT4) (normal range: 0.9–2.3 ng/dL) at the moment of HT diagnosis. We also noted other autoimmune associated diseases that were detected in these patients throughout their life.

Immunohistochemical exam

Three pathologists reviewed all the histological sections and new immunohistochemical (IHC) stainings were decided to be carried out on the representative paraffin blocks. As such, histological sections with a thickness of 3 μm were dried for one hour at 65°C before the pretreatment procedure of deparaffinization and rehydration. The epitope was retrieved in citrate buffer, pH 6.5, or in alkaline buffer (depending on the antibody we used) in water bath at 95°C for 30 minutes. Before immunostaining the sections, endogenous peroxidase activity was blocked. We used the following antibodies: anti-CKAE1/AE3 (Dako, Denmark), anti-p63 (ImmunoLogic, Netherlands), anti-CD5 (Novocastra, UK), anti-CD20 (Dako, Denmark), anti-CD23 (Novocastra, UK), anti-CD68 (Novocastra, UK), anti-Ki67 (ThermoScientific, USA) and anti-p63 (ImmunoLogic, Netherlands) (Table 7.1). After incubation, the reaction was visualized with UltraVision™ Quanto Detection System Horseradish Peroxidase (HRP), using 3,3'-Diaminobenzidine (DAB) chromogen as a substrate. Sections were counterstained with Mayer's Hematoxylin for nuclear counterstaining. The reaction was considered positive only when a brown cytoplasmic, membranous, or nuclear immunostaining was detected.

Table 7.1. The antibodies we used for IHC staining of the analyzed thymic pathologies

Antibody	Manufacturer	Clone	Antigen retrieval	Class	Dilution	Labeling	Cellular localization
Anti-CK AE1/AE3	Dako, Denmark	AE1/AE3	Citrate pH 6	Monoclonal mouse anti-human CK AE1/AE3	1:50	Epithelial cells	Cytoplasm
Anti-CD5	Novocastra, United Kingdom	4C7	Citrate pH 6	Monoclonal mouse anti-CD5 antibody	1:100	T cells	Membrane
Anti-CD20	Dako, Denmark	L26	Citrate pH 6	Monoclonal mouse anti-human CD20cy	1:150	B cells	Membrane
Anti-CD23	Novocastra, United Kingdom	1B12	Citrate pH 6	Monoclonal mouse anti-CD23 antibody	1:100	Follicular dendritic cells	Membrane
Anti-CD68	Novocastra, United Kingdom	514H12	pH 9	Monoclonal mouse anti-CD68 antibody	1:100	Macrophages	Cytoplasm and membrane
Anti-Ki67	ThermoScientific USA	SP6	Citrate pH 6	Monoclonal rabbit anti-Ki67antibody	1:250	Proliferating cells	Nuclear

Antibody	Manufacturer	Clone	Antigen retrieval	Class	Dilution	Labeling	Cellular localization
Anti-p63	ImmunoLogic, Neederland	4A4	Citrate, pH 6	Monoclonal mouse anti-human p63	1:200	Epithelial cells	Nuclear

CD: Cluster of differentiation; CK: Cytokeratin.

7.6.3. Results

Following the case analysis, a number of four patients were identified, three females and one male (F:M ratio, 3:1) (Table 7.2). The mean age of the patients at the time of their thymectomy was 40.25 years, but the female patients were older (47.33 years), while the male patient was in his youth (19 years). 75% of all patients presented moderate or severe MG, and 100% of them showed anti-AChR antibodies and a decrement greater than 25% on electromyographic investigations (Table 7.2).

All patients have been diagnosed in their past medical history with HT by a full thyroid panel (high TSH values, low fT4 values, and anti-TPO antibodies) (Table 7.3). AIT responded well to the treatment with Euthyrox.

The two lesions with autoimmune mechanism were diagnosed at different or concomitant stages of the disease, as follows: for Case No. 1, HT preceded MG with one year, in Case No. 2 and Case No. 4, the two autoimmune diseases appeared simultaneously, and in Case No. 3, MG was diagnosed four years before HT. In Case No. 4, alongside of MG and HT, our patient was also diagnosed with a hemolytic anemia [very low hemoglobin (Hb) count (4.5 g/dL), reticulocytosis, high values for lactate dehydrogenase (LDH) and indirect bilirubin, and positive Coombs test] at the time of his thymectomy (Tables 7.2 and 7.3).

Table 7.2. Clinical characteristics and diagnostic work-up for MG of our patients at the moment of myasthenia diagnosis

Case No.	Patient's age at thymectomy [years]	Gender	MG – history and work-up study			
			Length of time between MG diagnosis and thymectomy	MG type	Anti-AChR antibody [nmol/dL]*	Electromyographic decrement#
1.	54	F	4 years	IVA	12	25%
2.	28	F	6 month	IIA	6	62%
3.	60	F	8 years	IIIA	16	32%
4.	19	M	1 year	I	4	20%

AChR: Acetylcholine receptor; F: Female; M: Male; MG: Myasthenia gravis [MG Foundation of America (MGFA) class]. *Normal range: ≤0.25 nmol/dL; #Normal muscle produces a decrement up to 8%.

Table 7.3. History and diagnostic work-up for HT of our patients at the moment of their chronic thyroiditis diagnosis

Case No.	Patient's age at thymectomy [years]	Gender	Chronic autoimmune HT – history and work-up study at the moment of diagnosis					Other AID
			Length of time between HT diagnosis and thymectomy	TPO ab [IU/mL]	Tg ab [IU/mL]	TSH [μIU/mL]	fT4 [ng/dL]	
1.	54	F	5 years	556	53	2	1.2	-
2.	28	F	6 months	176.7	47	6.53	1.1	-
3.	60	F	4 years	184	62	8.41	0.93	-

Case No.	Patient's age at thymectomy [years]	Gender	Chronic autoimmune HT – history and work-up study at the moment of diagnosis					Other AID
			Length of time between HT diagnosis and thymectomy	TPO ab [IU/mL]	Tg ab [IU/mL]	TSH [μIU/mL]	fT4 [ng/dL]	
4.	19	M	1 year	63	46	2.95	0.97	Hemolytic anemia: Hb ↓ (4.5 g/dL); Reticulocytosis; LDH ↑; Indirect bilirubin ↑; Positive Coombs test

F: Female; fT4: Free thyroxine (normal range: 0.9–2.3 ng/dL); Hb: Hemoglobin; HT: Hashimoto's thyroiditis; LDH: Lactate dehydrogenase; M: Male; Tg ab: Anti-thyroglobulin antibodies (normal range: <35 IU/mL); TPO ab: Serum anti-thyroid peroxidase antibodies (normal range: <35 IU/mL); TSH: Thyroid-stimulating hormone (normal range: 0.4–5.5 μIU/mL.

In our series, we found four MG subtypes: early-onset myasthenia gravis (EOMG), late-onset myasthenia gravis (LOMG), thymoma-associated myasthenia gravis (TAMG), and ocular-associated myasthenia gravis (OAMG), all of them being associated with anti-AChR antibodies, and different thymus pathology, i.e., atrophic thymus with calcification or with cystic dilatations of Hassall's corpuscles, thymic follicular hyperplasia, or B2 invasive thymoma (Table 7.4).

Table 7.4. Imaging, surgical, pathological and outcome characteristics of our patients at the time of their thymectomy

Case No.	Age [years]	Gender	Thoracic CT scan	Surgery	Pathological report	Type of MG	Outcome
1.	54	F	Enlarged antero-superior mediastinum with a heterogeneous thymic tissue	T	Atrophic thymus with calcification of Hassall's corpuscles (Figures 21-22)	LOMG	Death – MSOF at 60 days
2.	28	F	Nodular thymus (Figures 23 -24)	T	Thymic follicular hyperplasia (Figures 25-31)	EOMG	Complete remission of MG
3.	60	F	Mediastinal mass invasive into the left mediastinal pleura – suggestive for thymoma (Figure 32)	T	B2 invasive thymoma (Figures 33-41)	TAMG	Complete remission of MG
4.	19	M	Mediastinal mass suggestive for thymoma (Figures 42-43)	T	Atrophic thymus with cystic dilatations of Hassall's corpuscles (Figures 44-47)	OAMG	Complete remission of MG

CT: Computed tomography; EOMG: Early-onset myasthenia gravis; F: Female; LOMG: Late-onset myasthenia gravis; M: Male; MG: Myasthenia gravis; MSOF: Multisystem organ failure; OAMG: Ocular-associated myasthenia gravis; T: Thymectomy; TAMG: Thymoma-associated myasthenia gravis.

Thoracic CT revealed a heterogeneous mediastinal mass and established the correct diagnosis only in 25% of cases, i.e., in the case of invasive thymoma (Table 7.4; Figures 7.23 - 7.24, 7.32, 7.42 – 7.43). The gross features of thymic surgical specimens also revealed heterogeneous morphological appearances: atrophic thymus in two cases (Figure 7.21), a tumor mass in one case (Figure 7.33) and a nodular thymus in another case (Figure 7.25).

The pathological exam showed a heterogeneous pattern of the thymic lesions, ranging from atrophy to thymic follicular hyperplasia and invasive thymoma (Table 7.4; Figure 7.22, Figures 7.26 – 7.31, Figures 7.34 – 7.41). Atrophic thymus also expressed different morphological changes of Hassall's corpuscles: calcification (Figure 7.22) or cystic dilatations (Figures 7.44 – 7.47). Thymic lympho-follicular hyperplasia (TLFH) expressed an increased density of lymphoid follicles with activated germinal centers and different dimensions, which expanded thymic medulla and disrupted the normally epithelial network as could be seen with CK AE1/AE3 immunostaining. In the follicular dendritic cell network, there were cells showing CD23 immunopositivity (Figures 7.26 – 7.31).

In B2 thymoma, tumoral epithelial cells, setting in a background of abundant lymphocytes, expressed CK19 immunoreactivity, very high values (80%) for Ki67 labeling index, but most of the nuclear staining represented T lymphocytes, and strong and diffuse immunopositivity for p63. Also, IHC staining revealed the characteristics of the intratumoral population of lymphocytes: CD20 immunopositivity of B-lymphocytes infiltrate and strong CD5 immunopositivity of T-lymphocytes infiltrate (Figures 7.34 – 7.41).

Regarding the outcome of our patients, we found out a complete remission of MG in 75% of our patients undergoing a total thymectomy, but one patient (25% of all cases) died at 60 days after surgical intervention due to a multisystem organ failure (MSOF).

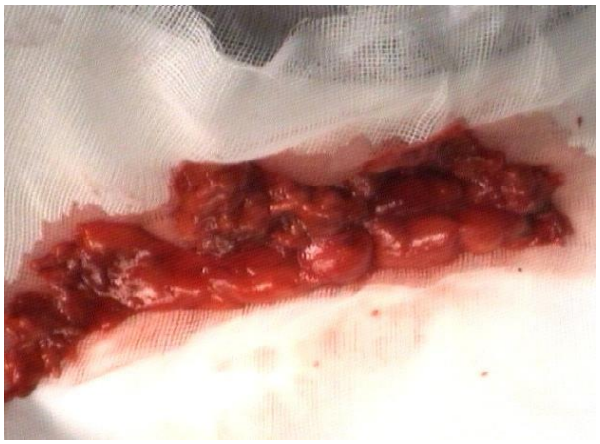


Figure 7.21. Case 1 surgical specimen: nodular aspect of the thymus gland

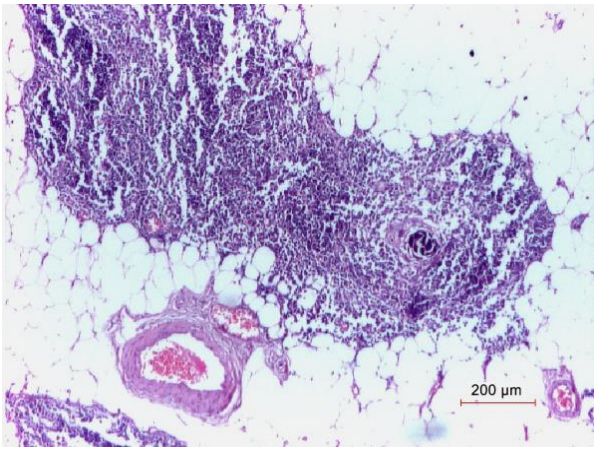


Figure 7.22. Case 1 morphological features: atrophy of the thymus with calcification of a Hassall corpuscle (HE, x 40)

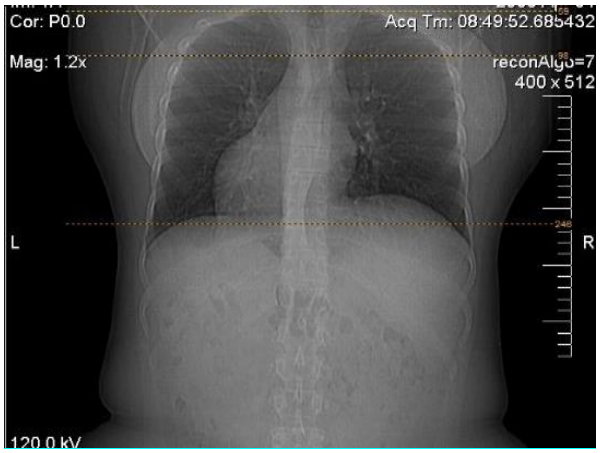


Figure 7.23. Case 2 thorax CT: enlarged heterogenous thymic gland (coronal plane)

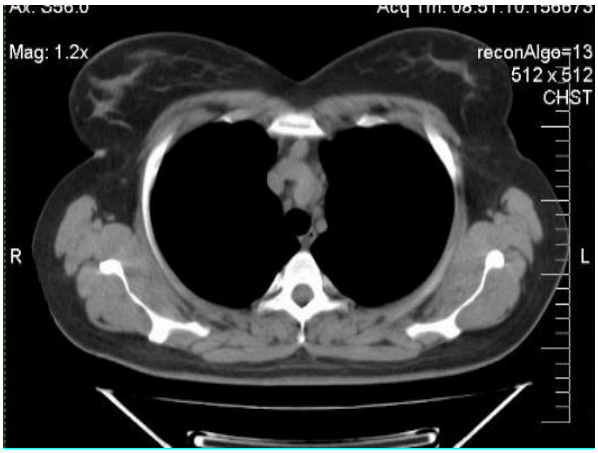


Figure 7.24. Case 2 thorax CT: some nodules may be distinguished from adipose tissue (axial plane)

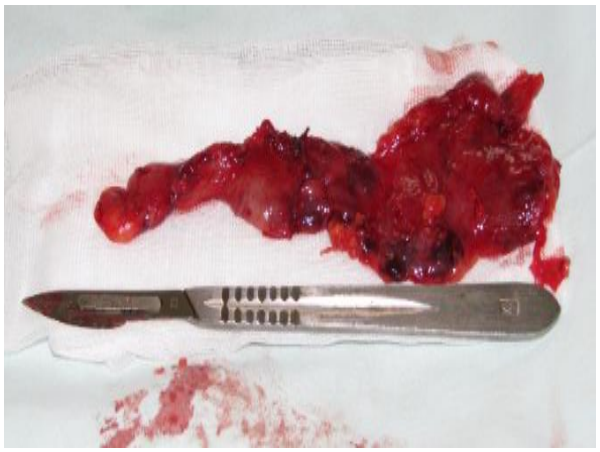


Figure 7.25. Case 2 surgical specimen: thymic tissue with 9.5 x 5.5 x 2.5 cm in size, and weighed 65 g

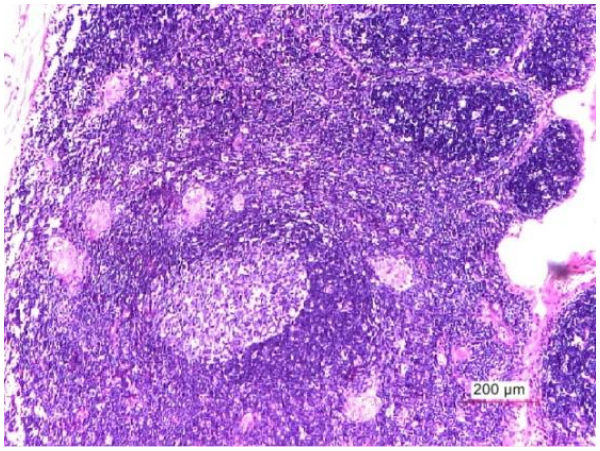


Figure 7.26. Case 2 TLFH: thymic tissue with increased density of lymphoid follicles with hyperplastic germinal centers and occasional Hassall corpuscles (HE, x 40)

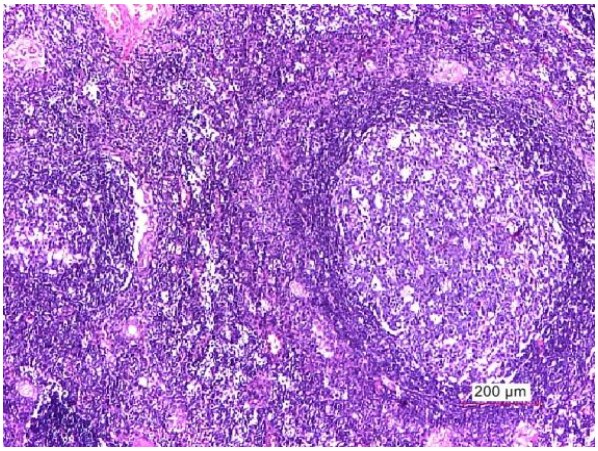


Figure 7.27. Case 2 TLFH: higher magnification revealed active germinal center (HE, x 100)

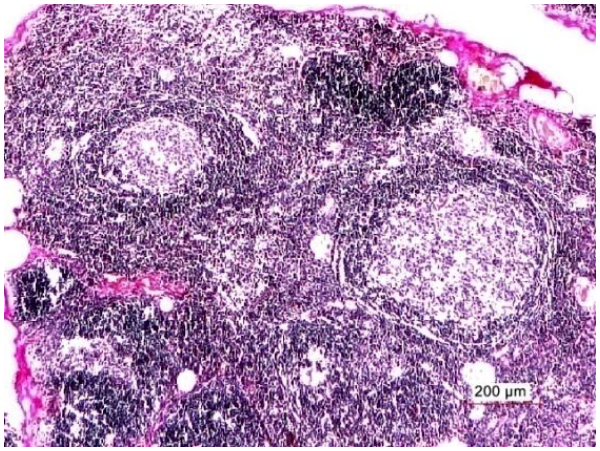


Fig 7.28. Case 2 TLFH: many lymphoid follicle, small and big, with prominent germinal centers and expanded thymic medulla (VG, x 40)

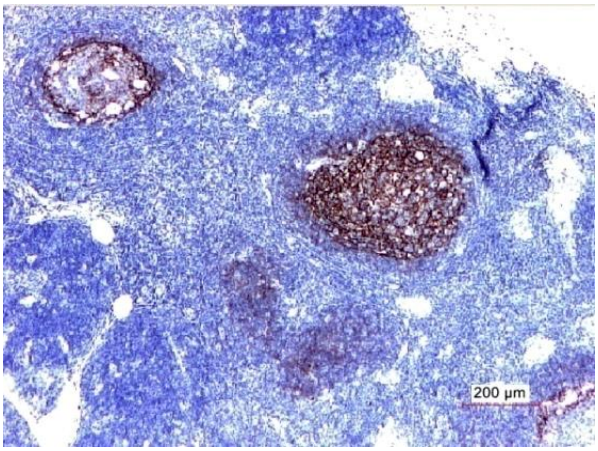


Figure 7.29. Case 2 TLFH: lymphoid follicles from thymic medullary area showed CD23 immunopositivity in the follicular dendritic cell network (IHC, anti-CD23, x 40)

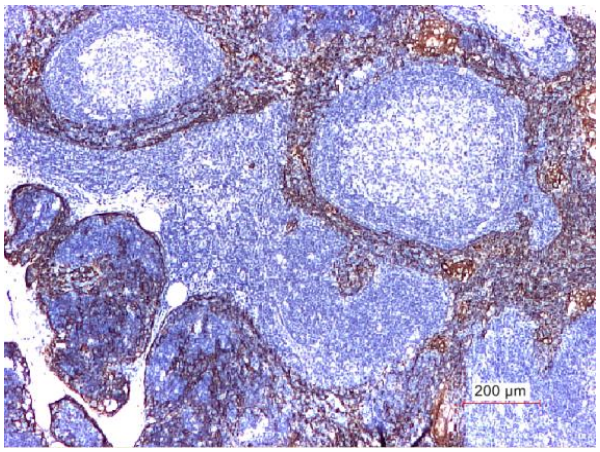


Figure 7.30. Case 2 TLFH: Immunopositivity of the thymic epithelial cells revealed disrupted the normally epithelial network and epithelial hyperplasia around the reactive follicle (IHC, anti-CKAE1/3, x 40)

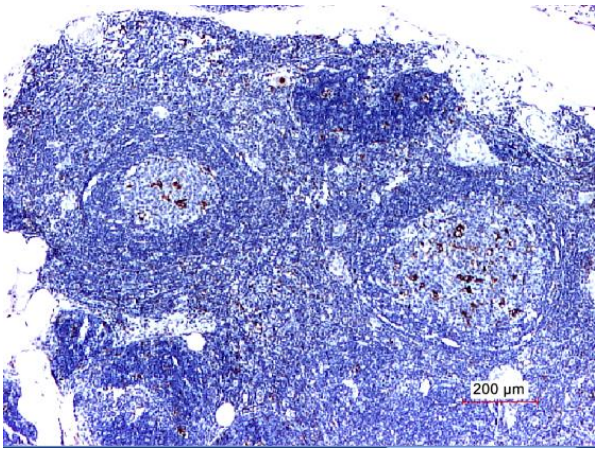


Figure 7.31. Case 2 TLFH: CD68 immunopositivity identified few macrophages in cortical and medullary regions (IHC, anti-CD68, x 40)

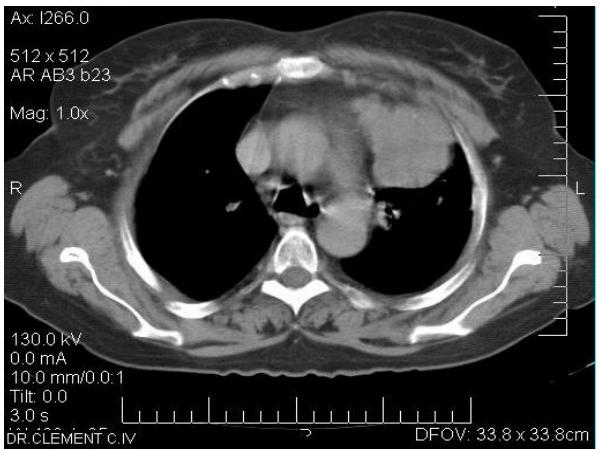


Figure 7.32. Case 3 B2 thymoma: thorax CT showing a lobular anterior mediastinal mass, infiltrating the adjacent pleura (axial plane).

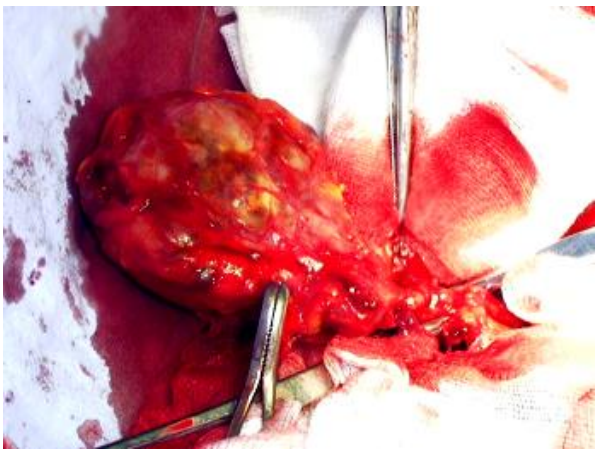


Figure 7.33. Case 3 B2 thymoma: surgical specimen revealed a pink-tan, solid tumor showing multiple nodules, with 6.1cm x 3.5cm in maximal dimensions.

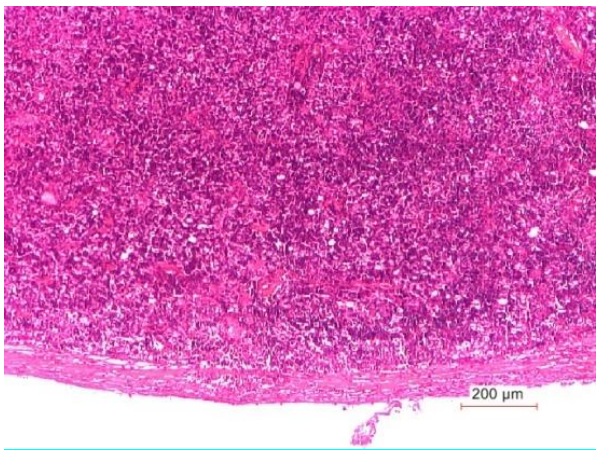


Figure 7.34. Case 3 B2 thymoma: two distinct cellular populations consisting of clusters of large polygonal neoplastic epithelial cells and numerous lymphocytes (HE, x 40)

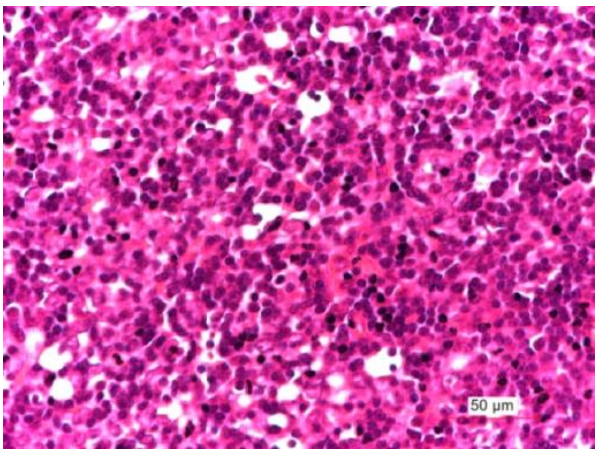


Figure 7.35. Case 3 B2 thymoma: epithelial cells with hypochromatic nuclei and small nucleoli, and uniform lymphocytes with scant cytoplasm, round nuclei, and inconspicuous nucleoli (HE, x 100)

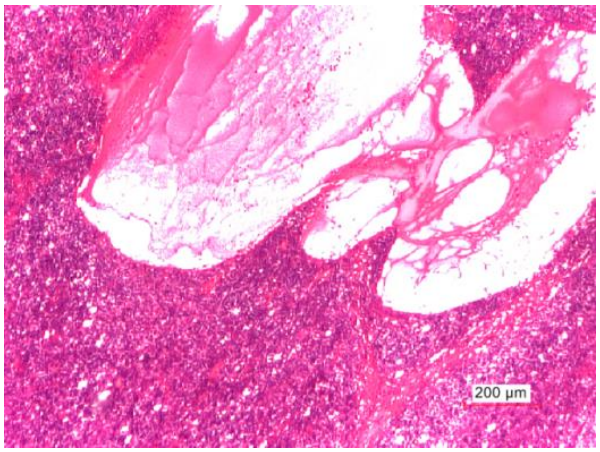


Figure 7.36. Case 3 B2 thymoma: perivascular spaces centered by a venule, surrounded by a clear space containing proteinaceous fluid (HE, x 40)

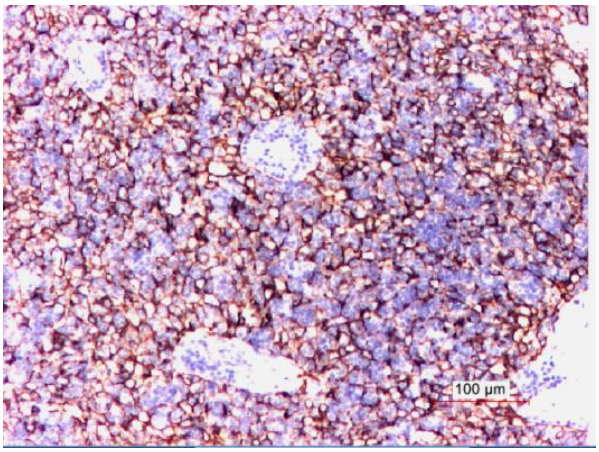


Figure 7.37. Case 3 B2 thymoma: cytokeratin 19 positivity in neoplastic epithelial cells (IHC, anti-CK19, x 100)

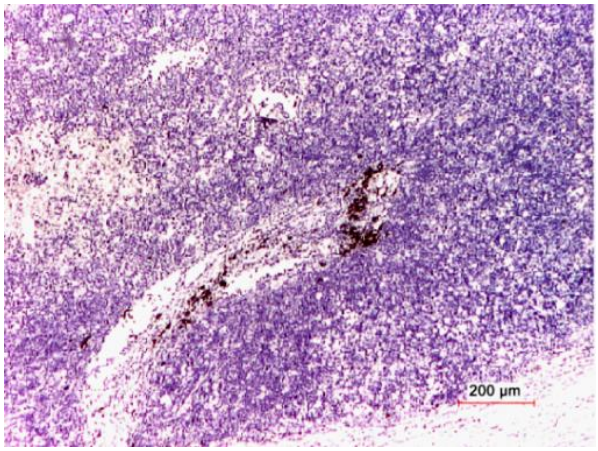


Figure 7.38. Case 3 B2 thymoma: CD20 immunostaining positive in B lymphocytes infiltrate (IHC, anti-CD20, x 40)

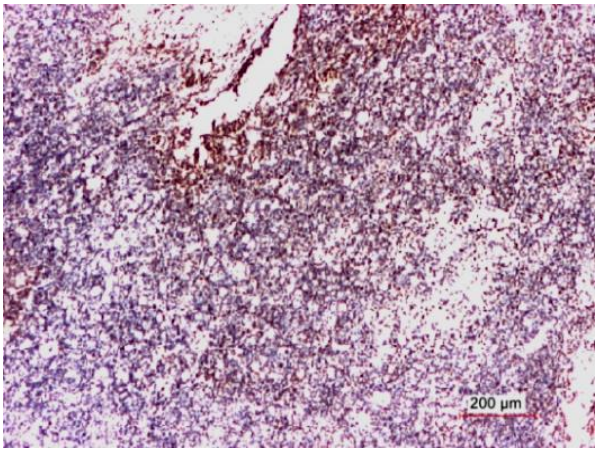


Figure 7.39. Strong CD5 immunopositivity of T lymphocytes infiltrate, but negative in the epithelial neoplastic component (IHC, anti-CD5, x 40).

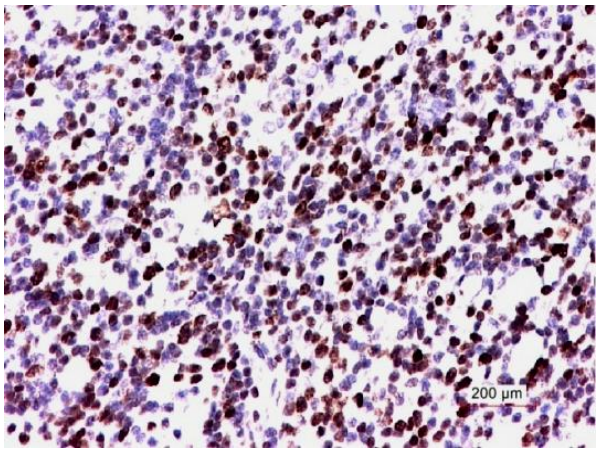


Figure 7.40. Case 3 B2 thymoma: Ki67 labelling showed high nuclear staining in T lymphocytes and some larger epithelial cells (IHC, anti-ki67, x 200)

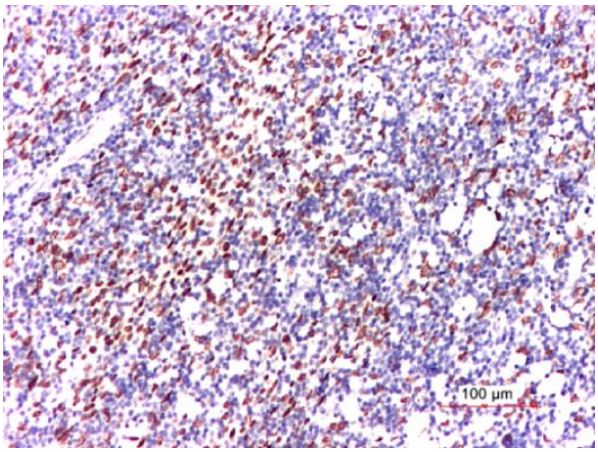


Figure 7.41. Case 3 B2 thymoma: strong and diffuse immunopositivity for p63 in tumoral epithelial cells (IHC, anti-p63, x100).

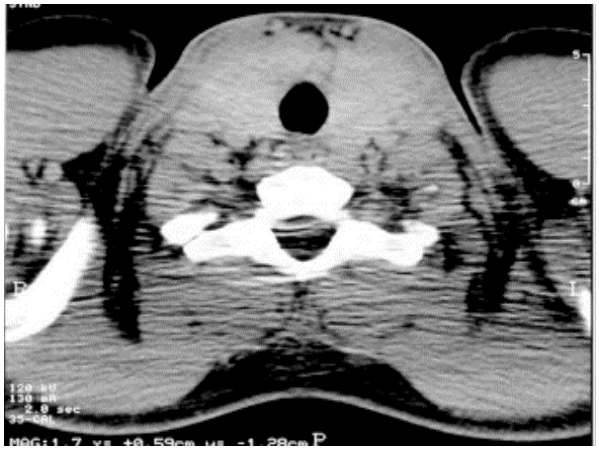


Figure 7.42. Case 4 thymic athrophy: CT scan images of the neck demonstrated a heterogeneously enlarged thyroid gland (axial view)

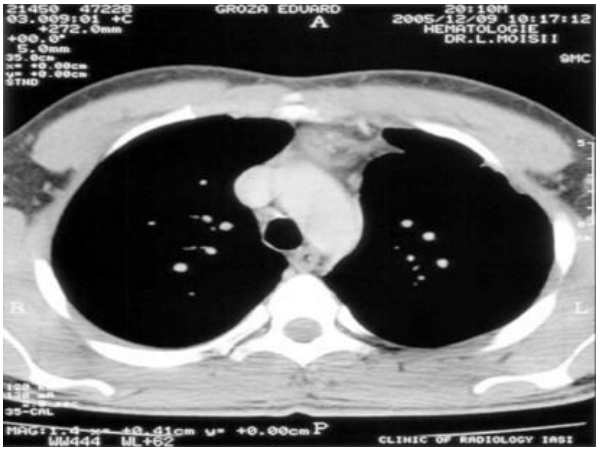


Figure 7.43. Case 4 thymic athrophy: CT scan images of the thorax revealed a nodular mediastinal mass suggestive for thymoma (axial view).

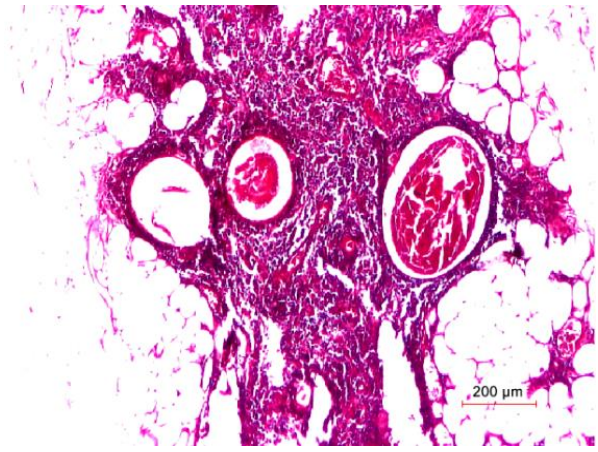


Figure 7.44. Case 4 thymic athrophy: extensive reduction in the thymic cortex with many cystic structures, filled with homogeneous eosinophilic material (HE, x 40)

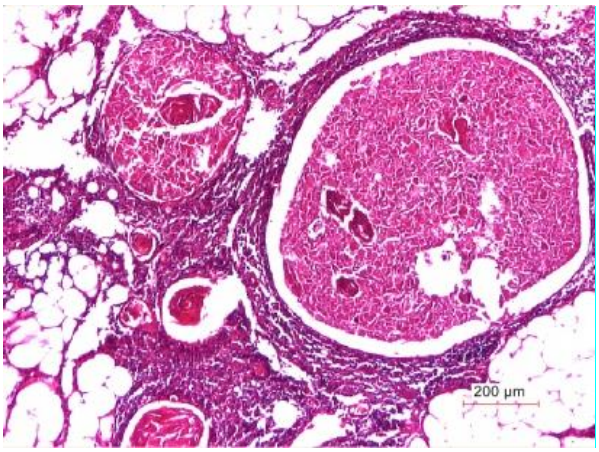


Figure 7.45. Case 4 thymic athrophy: huge cystic dilatation filled up with cellular detritus (HE, x 10)

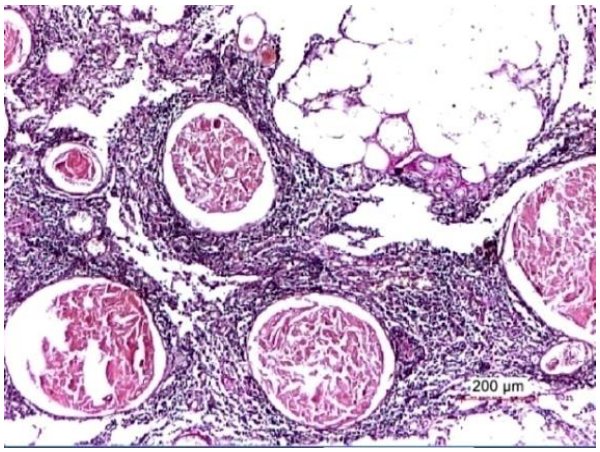


Figure 7.46. Case 4 thymic athrophy: thymic medulla with extremely large cystic dilatation filled with heterogeneous amorphous material (VG, x 40)

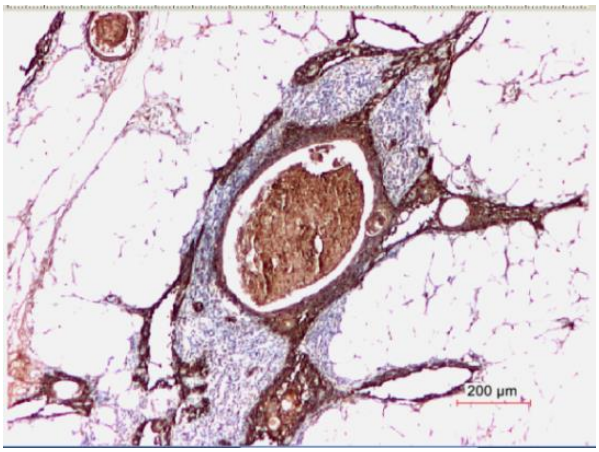


Figure 7.47. Case 4 thymic athrophy: strong immunopositivity for CKAE1/3 in thymic cystic epithelial cells confirmed the cystic transformation of Hassall's corpuscles (IHC, anti-CKAE1/3, x 40)

7.6.4. Discussion

MG can be associated in 15% of cases with another autoimmune disease, i.e., thyroid disease, rheumatoid arthritis, and systemic lupus erythematosus and this association could signify a possible common basis for all these diseases, as well as their impact on the intensity and treatment of MG [Tanovska et al., 2018].

Patients diagnosed with MG can associate all kinds of morphological and functional thyroid disorders, including AITs [Lin et al., 2017], but Graves' disease and HT are more prevalent in patients with MG than in the general population [Kanazawa et al., 2007].

The association between MG and HT has been reported since the 1960s, but it is still rare, as the literature mentions a percentage ranging between 1.1% and 9% [Chen et al., 2013, Kubiszewska et al., 2016].

The first studies which identified the MG and HT association have been published especially during the second half of the 20th century [Daly et al., 1964; Singer et al, 1966; Mizutani et al., 1991]. In 1972, Cheah & Tan presented a case of 64-year-old Chinese woman with MG and HT, and hypothesized that MG could be an autoimmune disorder [Cheah et al., 1972]. At the beginning of the 21st century, especially in the last five years, due to the emergence of new methods of investigation, some series of new cases have been published [Kubiszewska et al., 2016].

AITs and MG present some common elements, as both pathologies appear because of a deficient immune response against self-structures. From a morphological point of view, HT contains a rich lymphocytic infiltrate predominantly disposed in follicles with germinal center formation and prominent T-cells associated [Kubiszewska et al., 2016]. Chronic autoimmune thyroiditis should be treated, otherwise a hypothyroidism could develop, and severe complications could be added to those expressed by the associated MG, such as extensive intracerebral calcification leading to death [Sava et al., 2013].

However, AITs often accompany MG and may influence its evolution. These two diseases can occur at the same time or one of them can precede the other [Mohamed et al., 2017]. Moreover, some authors claim that AIT, especially HT, has a high risk of being subsequent to MG [Yeh et al., 2015]. In our series, in two cases, these two autoimmune diseases appeared simultaneously, in another case MG preceded HT by four years, and in the fourth case HT preceded MG by one year.

Patients with MG could also develop other autoimmune diseases than HT. In our series, the youngest patient was also diagnosed with hemolytic anemia at the time of his thymectomy. Arellano et al. (2017) reported another, even rarer association, presenting the case of a 69-year-old patient with idiopathic pulmonary fibrosis associated to his HT and MG [Arellano et al., 2017].

A study from 2016 reports investigating 343 consecutive patients with MG and it found that 9% of this cohort presented HT and MG, most female (67.7%), their mean age at onset of MG being 40.4 years. Two subtypes of MG (EOMG and LOMG) were identified [Kubiszewska et al., 2016]. We got similar results in terms of age and gender of patients, yet we identified all four forms of MG expressing anti-AChR antibodies: EOMG, LOMG, TAMG, and OAMG, as reported by Koneczny & Herbst (2019) [Koneczny et al., 2019].

Prior to the first surgery for thymus removal in patients with MG, which was performed in 1941 [Blalock et al., 1941], the idea that these patients had structural abnormalities of the thymus appeared based on the histopathological aspects identified on the autopsy specimens. Since then, microscopic analysis identified the presence of benign tumors, hyperplasia, or the persistence of an atrophic thymus in patients who have died in hospitals due to MG. As far as we know, there are two articles reporting the morphological aspects of the thymic surgical specimens in correlation with the type of MG [Utsugisawa et

al., 2011; Koneczny et al., 2019], but our study is the only one presenting histopathological and IHC images of thymic pathology in patients with MG and concomitant HT. An interesting fact is that we found different thymus pathologies for each subtype of MG.

Romanian studies report thymoma in MG patients, based on histopathological and IHC findings. Cornea et al. (2009) reported a case of microscopic thymoma, which is a nodular hyperplasia of the thymic epithelium in a patient with eye-related symptoms of MG, which aggravated in two years of evolution [Cornea et al., 2009]. Another case of B1 thymoma was reported in a MG patient and the study concluded that of all IHC markers, only p53 can predict a more aggressive evolution [Cornea et al., 2012]. Our case with HT, MG and invasive B2 thymoma expressed high Ki67 labeling index and strong p63 immunopositivity. These two markers could be the proof for a poor prognosis expressed as tumor recurrence, but if we followed the case her outcome was good.

In our small cohort, we noticed that LOMG and OAMG were associated with an atrophic thymus, each case displaying specific features (calcification of Hassall's corpuscles in LOMG, and cystic dilatations of Hassall's corpuscles in OAMG). EOMG exhibited TLFH, with thymic epithelial hyperplasia, and TAMG expressed an invasive thymoma.

Some studies show that thymus plays an important role in MG pathogenesis with antibodies against the AChR of skeletal muscles. These antibodies are produced in B-cells, production which depends on T-cells. It is likely for a non-tolerogenic thymopoiesis to generate in the thymus specific T-cells for AChR, due to an aberrant function of thymic epithelial cells. However, the generation of these T-cells specific for AChR is not the cause of MG, as these cells are also found in healthy people. It seems that MG is triggered by the activation of these potentially specific AChR-specific Tcells. Intra-thymic activation of AChR-specific T-cells is probably limited to certain types of MG patients: those with EOMG in whom the thymus presents TLFH and some patients in whom MG is associated with a thymoma.

Most thymomas and atrophic thymuses of the patients with LOMG do not present this T-cell activation process [Utsugisawa et al., 2011]. Since we have identified particular morphological changes in the atrophic thymus of MG patients, we suggest that the constituent cells of these histological structures could also play a role in the pathogenesis of MG, at least in MG with anti-AChR antibodies and concomitant HT.

Some papers signal thymic abnormalities in approximately 95% of patients with MG; thymic hyperplasia in up to 65%, thymomas in up to 21%, and normal or regressive thymus in 9%, i.e., atrophic and replaced with fat tissue, and persistent thymus in 5% of cases [Tanovska et al., 2018]. On the contrary, we found 50% of our patients with atrophic thymus, 25% of them with thymoma, and 25% with TLFH, yet our series includes only a few cases.

Nikolic et al. (2013) revealed that most patients identified with anti-AChR antibodies present TLFH or thymoma, and patients identified with anti-MuSK antibodies most often reveal an atrophic thymus [Nikolic et al., 2013]. Nevertheless, in our patients with MG and anti-AChR antibodies and concomitant HT, atrophic thymus was identified in 50%, with a particular morphological expression of the Hassall's corpuscles. Some researchers indicate that Hassall's corpuscles differentiate from medullary thymic epithelial cells after they lose autoimmune regulator expression [Wang et al., 2012]. It could then be extrapolated that not only thymocytes, which interact with thymic epithelial cells [Speck-Hernandez et al., 2018] can have a role in MG pathogenesis, but also the cells of Hassall's corpuscles could play a role in this process, especially since their function is to train thymocyte subsets to transform into CD4+ CD25+ regulatory thymic T-cells, which modulate the immune response and have implications in some autoimmune diseases [Watanabe et al., 2005].

Recently, Mikušová et al. (2017) examined 95 human thymic tissue samples to identify the structure and role of Hassall's corpuscles. The authors reported that most of

Hassall's corpuscles are heterocellular and consist of thymic epithelial cells, macrophages, interdigitating dendritic cells, myoid cells, and, occasionally, mast cells and lymphocytes. Regarding the potential functions of Hassall's corpuscles, the authors found out that these structures contained high concentrations of B-lymphocytes and B-cell lymphoma 2 (BCL2)-positive lymphocytes, suggesting a role in the regulation of lymphopoiesis [Mikušová et al., 2017].

Brinkane et al. (2003) recommend chest X-ray and thoracic CT scan in patients with AIT to search for a thymic mass. On the other hand, these authors suggest imaging and laboratory investigations for AIT in patients with a thymic mass identified on CT or magnetic resonance imaging (MRI) scans [Brinkane et al., 2003]. However, in our series, HT was identified based on clinical manifestation (hypothyroidism) and also on a full thyroid panel, but the diagnosis was finalized concomitantly, before or after the diagnostic of a MG.

Although both MG and HT are autoimmune diseases, their treatment is different. In the case of HT, the treatment aims to regulate the level of thyroid hormones and therefore the patients receive a substitution treatment (Euthyrox given orally throughout their life), to which our patients have responded well. Also, thyroid dysfunction may increase the risk of hypertension. As such, hypertension should be treated, with special awareness that a treatment resistance could occur. In this case, the clinician should search for other causes (atherosclerotic renovascular changes, or systemic amyloidosis) [Costache et al., 2017; Costache et al., 2018].

In cases with MG, drug therapy consists in medications that increase neuromuscular transmission (anticholinesterase agents) and immunomodulating treatments, i.e., glucocorticoids, plasmapheresis, immunoglobulins, and monoclonal antibodies [Cataneo et al., 2018].

In the unfavorable evolution of the two diseases, the surgical treatment is of choice. In MG, surgical treatment is done by simple or extended thymectomy [Zielinski et al., 2010] and its effect is an improvement of clinical outcome. In HT, thyroid ablation is recommended when a defined thyroid nodule is present. However, it is interesting to note that in both cases the excised organ revealed germinal centers with B-lymphocytes participating in the pathogenic response [Lopomo et al., 2017].

After the first thymectomy in 1941, it was subsequently found that surgery has greater benefits in patients with non-thymomatous MG, with remission rate higher than those with non-surgical treatment [Cataneo et al., 2018]. Except for one case who died 60 days after surgery due to a multiple organ insufficiency, all other patients from our series showed complete remission of MG after total thymectomy, emphasizing once again the importance of surgery in patients with MG.

7.6.5. Final remarks

Despite the limited cohort of patients, this study is the only one reporting thymic histopathological and IHC investigations in patients with anti-AChR antibodies-positive MG and HT. These two diseases occurred together, which raises new questions regarding autoimmune developing factors. Considering the wide range of thymic morphological changes revealed by the study, we can hypothesize that thymus is involved in the pathogenic mechanism of anti-AChR antibodies-positive MG and concomitant HT.

7.7. SELENIUM AND AUTOIMMUNE THYROIDITIS – EXPERIMENTAL EVIDENCE

7.7.1. Introduction

Selenium (Se_{79}^{34}) was first isolated in 1817 by Jacob Berzelius but its importance in human health and ecosystem was recognized barely in 1957. The name is derived from Selene, the Greek goddess of the moon and is a metalloid of the same family as sulfur and oxygen [Mehdi et al., 2013]. Se is present in the environment (water, soil and air) in very low concentrations (<1µg/g). Se is an essential element for human health. Food as source of Se in human nutrition can contain inorganic (selenite) or organic (Se-amino acids, Se-methylated and Se-proteins) forms of Se [Mehdi et al., 2013].

Se is an essential micronutrient important in many aspects of human health [Verma et al., 2011] which plays a major part in optimal endocrine response, immunomodulation and inflammatory process [Dharmasena, 2014]. Se is incorporated into cysteine forming the 21st amino acid used during protein synthesis. Selenoproteins have vital functions in the body: essential antioxidant enzymes that fight cancer, regulators of thyroid function, structural proteins in sperm required for fertility, and reduce virulence associated with certain viral infections [Weeks et al., 2012].

The effect of Se in humans is concentration-dependent, ranging from an antioxidant activity in the nanomolar-micromolar range to a potentially prooxidant activity at concentrations higher than required for selenoprotein synthesis [Negro et al., 2008].

The thyroid is the endocrine gland with the highest Se content because it expresses specific selenoproteins. Se status appears to have an important impact on thyroid metabolism (selenoproteins have a major role in the synthesis and action of thyroid hormones) and thereby seems to be involved in thyroid pathology [Bhuyan et al., 2012; Balazs et al., 2013]. Most of known selenoproteins are expressed in the thyroid gland: glutathione peroxidases (GPXs), thyoredoxin reductases (TRs) and iodothyronine deiodinase (type D1, D2 and D3). The thyroid contains more Se per gram of tissue than any other organ [Effraimidis et al., 2014].

As most human autoimmune disorders, AIT (chronic lymphocytic thyroiditis/Hashimoto's thyroiditis) results from a combination of genetic predisposition and environmental triggers [Rose et al., 2002]. Low birth weight, iodine and selenium excess or deficiency, reproductive span, parity, stress, seasonal variation, radiation, smoking, allergy, viral and bacterial infections have an important role in the development of AITD [Prummel et al., 2004].

Clinical and epidemiologic evidence point to excessive iodine intake as the environmental agent responsible for the thyroid autoimmunity induction [Foley et al., 1992; Markou et al., 2001]. The role of iodine in the homeostatic regulation of thyroid function was first demonstrated over 50 years ago. However, the precise mechanism of regulation remains unclear [Wolff et al., 1948]. High doses of iodide suppress the functional activity of the thyrocytes (Wolff-Chaikoff effect), inhibiting the iodination of the thyroid protein fraction and decreasing the concentration of thyroid hormones in serum [Paul et al., 1988]. It has been demonstrated that a single injection of a high dose of iodide inhibits the biosynthesis of thyroid hormones at several levels [Denef et al., 1996; Vitale et al., 2000]. The sensitivity to the stimulating action of thyroid-stimulating hormones decreases and the expression and activity of TPO (the enzyme catalyzing iodination of thyroglobulin in the presence of iodide and hydrogen peroxide) are suppressed [Cardoso et al., 2001]. Finally, the NADPH-oxidase reaction producing hydrogen peroxide (the limiting step in the iodide metabolism) is also suppressed [Mahmoud et al., 1986; Vitale et al., 2000]. The necrotic effect is increased in

case of Se deficiency [Contempre et al., 1993; Contempre et al., 1996]. However, thyroid cells have their own antioxidant system. Thus, in the case of iodine excess, the expression of antioxidative enzymes increases [Chiu-Ugalde et al., 2012].

In a recent cross-sectional, prospective European study [Krassas et al., 2014] a linear correlation of Se levels and Se protein P was found in patients with thyroid disorders, indicating a less than optimal Se status. Patients with Graves' disease and HT had significantly lower Se levels compared with patients with non-autoimmune disease. Adequate Se intake assures thyroid hormone synthesis and metabolism and also protects the gland from damage from excessive iodine exposure [Krassas et al., 2014].

Long time before genetic characterization could be envisioned, medical research on thyroid disease had evolved according to the existing techniques since the 1890s [Moncayo et al, 2020]. Early researchers investigating goiter concentrated on the clinical description of cases including their evolution and surgical therapy. Since the 1950s, experimental researchers have attempted to develop animal models that could resemble human disease, e.g., thyroiditis [Moncayo et al., 2020]. In 1956, Danziger and Elmergreen presented a model of a homeostatic mechanism of the thyroid-pituitary relation based on a mathematical negative-feedback model, thus paving the way for the use of laboratory methods [Danziger et al., 1956]. In 1957 Witebsky et al. have clearly shown that the normal thyroid is susceptible to invasive attack by lymphoid elements following auto-immunization and that the resulting lesions have many features in common with those present in the human gland. In these studies, rabbits were injected intra-dermally into the footpads with homologous thyroid extracts incorporated into the aqueous phase of a complete Freund adjuvant; circulating thyroid antibodies and thyroiditis of variable severity were observed within 2-5months in a large proportion of the animals [Terplan et al., 1960]. The resulting lesions have been described in detail by Terplan & Witebsky in 1960 and in many respects resemble those seen in the Hashimoto gland [Terplan et al., 1960].

The first iodine‑induced thyroiditis has been transient in most experimental animals, except for genetically modifed animals prone to develop AIT, such as non-obese diabetic (NOD) mice. They present important areas of destroyed thyroid tissue which are replaced by inflammatory tissue [Kolypetri et al., 2010]. Experimental autoimmune thyroiditis (EAIT) has been used to simulate human autoimmune thyroid disease for decades [Arata et al., 2006]. EAIT can be easily induced in genetically susceptible strains of mice by excess iodine ingestion [Rasooly et al., 1996] or by immunization with mouse thyroglobulin [Čiháková et al., 2004]. However, iodine excess alone has also been used to induce EAIT in insusceptible murine models, including Wistar rats [Pitsiavas et al., 1997; Gao et al., 2013], as well as in other animals [Bagchi et al., 1985].

Aim

The aim of the present study was to assess the effects of inorganic Se supplementation on thyroid morphology in EAIT induced by the administration of potassium iodide (KI) in Wistar rats.

7.7.2. Material and methods

Animals

A total of 48 Wistar adult rats (24 females weighing 160±20 g and 24 males weighing 180±20 g) were used for the present study. The animals were obtained from the 'Victor Babes' National Institute of Research Development in the Pathology Domain and Biomedical Sciences (Bucharest, Romania). Wistar rats were housed under standard conditions at the Biobase for research animals of „Grigore T. Popa" University of Medicine and Pharmacy

(Iasi, Romania) and were fed with standard food. The rats were housed in clean and ventilated polyurethane cages; 2 rats were placed in each cage. All rats were maintained under standard conditions of temperature (20±4℃), relative humidity of 55±10%, and light/dark cycles of 12/12 h consecutively. Access to food and water was ad libidum. The acclimatization of the rats lasted 7 days prior to the study initiation.

The study was approved by the Ethics Committee of „Grigore T. Popa" University of Medicine and Pharmacy with respect the EEA Agreement subject to EU Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes.

EAIT and Se administration

As AIT is more common in females (3:1) [Roubaty et al., 1990; Cui et al., 2014; Hassanin et al., 2013], it was investigated whether the same susceptibility of the female sex also occurs in the animal model of Wistar rats. The animals were randomized into groups according to four treatment regimens: C0, two control groups for each sex; C1, two (male and female) groups that received KI for 56 days (0.2 mg per animal in drinking water); C2, two (male and female) groups that received concomitant KI and sodium selenite (0.5 mg/kg body weight of sodium selenite administered in drinking water) for 56 days; and C3, two (male and female) groups that received KI for 56 days and afterwards sodium selenite for another 56 days (Table 7.5).

Even though no dose-finding study was performed, a previous report [Risher et al., 2011] concerning sodium selenite administration in Wistar rats has shown consistent toxic effects of sodium selenite at a dose of >1 mg/kg body weight (using the same administration method: ad libitum in drinking water) [Risher et al., 2011; Solcan et al., 2013].

Table 7.5. Study group allocation and treatment regimens

Factors	C0	C1	C2	C3
Gender (males/females)	6/6	6/6	6/6	6/6
KI administration	No	56 days of KI	56 days of KI	56 days of KI
Na-Se administration	No	No	56 days of Na-Se, concomitant with KI administration	56 days of Na-Se, after the KI administration
Total days of treatment/observation	7 days	56 days	56 days	112 days

KI: potassium iodine; Na-Se: sodium selenite.

Tissue collection and analysis

General anesthesia was performed with a combination of ketamine (60 mg/kg body weight) and xylazine (8 mg/kg body weight) administered intraperitoneally. Thyroid tissues were collected for pathology analysis, in accordance with the Council Directive 63/2010/EU on the protection of animals used for scientifc purposes, after 7 days in control groups, 56 days in C1 and C2 groups, and 112 days in C3 groups. Tissue samples were harvested from the neck area containing the anterior muscular plan, the thyroid and parathyroid tissue and the tracheal tissue with the cartilage ring. In order to keep the thyroid intact, tissue samples were fixed in 10% formaldehyde at 24℃ for 24 h. The tissue specimens were processed and interpreted at the Department of Pathology, „Sf. Spiridon" Clinical Emergency County Hospital (Iasi, Romania). The tissue samples were embedded in paraffin, cut into 4µm thick sections, and stained with HE and VG. The sections were examined using Nikon Eclipse E600 (Nikon Corporation) equipped with the Nikon Digital Net Camera DN100 image capture system (Nikon Corporation) and the Lucia Net program with morphometric analysis software (morphometric software LUCIA Net v.16.2®; Laboratory Imaging R.R.O., with NIS Elements 3.0). The morphometric analysis of the thyroid tissues was made from

successive images captured from each lobe (digital pictures of 10-15 non-adjacent fields) with x 4, x 10 and x 20 lenses, and x 10 eyepiece magnification. The interpretation of histopathological sections and the acquisition of images for all studied animals were performed by a single examiner.

Morphologic parameter assessment

The mean size of thyroid follicles was assessed by measuring the maximum diameter of over 20 thyroid follicles per case in various areas of the thyroid gland, using x 10 objective. The mean size of the thyroid follicular epithelium was assessed by measuring the size of the follicular epithelium in fixed positions (at 12, 5 and 7 o'clock). For each case the inflammation, follicular morphology and fibrosis were evaluated also using a scoring system from 0 to 3 for each of the parameters: presence of inflammation, vascular congestion, resorption vacuoles, interfollicular space and interstitial collagen deposits (Table 7.6). A final score was calculated as the sum of the inflammation score, vascular congestion, fibrosis and resorption vacuoles scores: normal thyroid morphology (final score 0‑3), mild thyroiditis (final score 4‑6), moderate thyroiditis (final score 7‑9) or severe thyroiditis (final score 10-12) (Table 7.7).

Statistical analysis

All statistical analyses were performed using SPSS v24.0 software (IBM Corp.). Skewness and kurtosis (-2<P<2) tests, the tests of normality in frequentist statistics, were used to examine the distribution of continuous variables. For multiple comparisons of normally distributed data, two-way ANOVA was performed with Tukey's HSD post hoc test. If the normality assumption was not satisfed, Kruskal-Wallis test and Dunn-Bonferroni post hoc test were carried out. Associations between categorical variables were assessed by Chi-square test.

Table 7.6. Scoring system used for the pathological evaluation of the EAIT

	Scoring system				
Parameter	**0**	**1**	**2**	**3**	**Adapted references**
Inflammation (INF)	NM	Mild DTF, few Ly in 2-3 TF	Moderate DTF, INF 10-40% TA	Severe DTF INF > 40% TA	Roubaty et al., 1990 Cui et al., 2014 Ruwhof et al., 2001
Vascular congestion (VC)	NM	Mild VD in capsular vessels	Moderate VC in capsular and intraglandular vessels in 10-40% TA	Severe VC in capsular and intraglandular vessels in > 40% TA	Hassanin et al., 2013 Zhu et al., 1995
Resorption vacuoles (RV)	NM	RV < 10% FC	RV ≈ 10-40% TA	RV > 40% FC	Hassanin et al., 2013 Zhu et al., 1995
Interfollicular space (IS)	NM	IS < 10% TA	IS ≈ 10-40% TA	IS > 40% TA	Hassanin et al., 2013 Zhu et al., 1995
Interstitial collagen deposits (ICD)	NM	Discret TCC	Thick TCC, with fine PCC	Thick TCC, PCC, PFC	Hassanin et al., 2013 Zhu et al., 1995 Amara et al., 2010 Jacobson et al., 1997 Xiang et al., 2018

EAIT: experimental autoimmune thyroiditis; NM: Normal morphology; DTF: destruction of thyroid follicles; INF: inflammation; Ly: lymphocytes; TF: thyroid follicles; TA: total area; VD: vascular distension; VC: vascular congestion; RV: resorption vacuoles; FC: follicular cavities; IS: interfollicular spaces; ICD: interstitial collagen deposits; TCC: thyroid capsule collagenization; PCC: pericapsular collagenization; PFC: perifollicular collagenization

Table 7.7. Total score in EAIT
(adapted Zhu et al., 1995; Jacobson et al., 1997; Amara et al., 2010; Hassanin et al., 2013; Xiang et al., 2018)

Thyroid morphology	Score
NM	0-3
Mild AIT	4-6
Moderate AIT	7-9
Severe AIT	10-12

EAIT: autoimmune thyroiditis; NM: Normal morphology

7.7.3. Results

Mean size of thyroid follicles

Regarding the size of thyroid follicles, the morphometric analysis revealed the following aspects.

The analysis of the entire study group showed that the mean value (56.48±17.05 µm x100) was far different from the median value (52.40 µm x100); Skewness (skw = 2.105) and Kurtosis (krt = 6.605) test results >2 suggested that the assumption of normality was not satisfied for the entire range of values; however, in C0, C1, C3, for both male and female subgroups, continuous values were confirmed.

Figures 7.48 – 7.73 illustrated relevant aspects for each group and allowed the comparison between groups.

The male rat thyroid morphology (Figures 7.48, and 7.50 - 7.52) showed that C1 group had higher mean value of thyroid follicles than C0 group (73.82 vs. 50.13 µm x100) and C2 group (73.82 vs. 53.74 µm x100). In C3 group, the mean value was higher than that recorded in the control group C0 (65.86 vs. 50.13 µm x100). The lowest mean value of the thyroid follicles was registered in the control group C0 (Figure 7.48).

In female rats, the highest mean value of thyroid follicles was recorded in C1 group (57.56 µm x100) and the lowest in C2 group (47.32 µm x100; Figures 7.48, 7.54, 7.56, 7.64, 7.70, and 7.72).

The results of two-way ANOVA showed no statistically significant differences in the mean size of thyroid follicles analyzed by sex and intervention group (Figure 7.48).

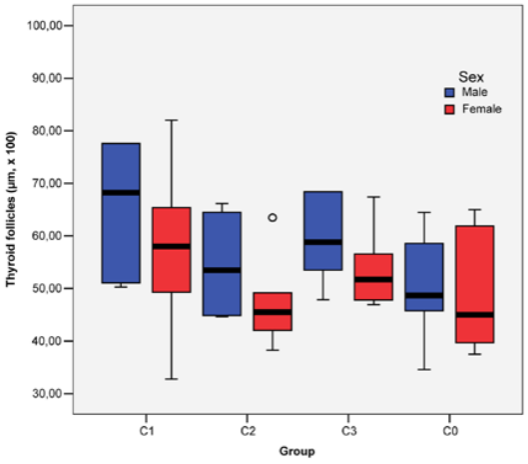
Mean size of the thyroid follicular epithelium

The values for the size of follicular epithelium in the entire study group were homogeneous, thus significance tests could be applied for these continuous variables: The mean value (3.55±0.80 µm x400) was close to the median value (3.29 µm x400); Skewness (skw = 0.472) and Kurtosis (krt = -0.649) test results were comprised in the interval [-2, +2].

In male rats, the highest mean value of the thyroid follicular epithelium was recorded in C1 group (only KI administration; 4.56 µm x100) and the lowest mean value in C2 group (3.13 µm x100) (Figures 7.53 and 7.59).

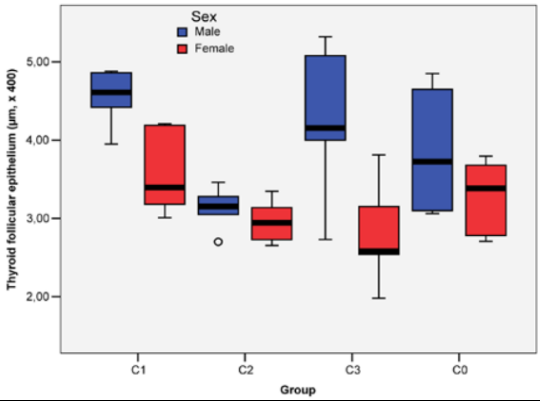
In female rats, the highest mean value of the thyroid follicular epithelium was recorded in C1 group (3.56 µm x100) and the lowest mean value was recorded in C3 group (2.77 µm x100) (Figures 7.49, 7.55, 7.57, 7.65, and 7.73).

Two-way ANOVA results showed statistically significant differences in the mean size of thyroid follicular epithelium analyzed by sex and intervention group only between C1 (only KI administration) and C2 (concomitant KI and sodium selenite administration) groups (Figure 7.49).



C1	p-values		C2	p-values		C3	p-values		C0	p-values	
	C2	0.149		C1	0.149		C1	0.877		C1	0.099
	C3	0.877		C3	0.491		C2	0.491		C2	0.997
	C0	0.099		C0	0.997		C0	0.377		C0	0.377

Figure 7.48. Comparison of the mean value of thyroid follicles (µm, x100) evaluated by sex between the study groups (median values are indicated by a bold line for each group).



C1	p-values		C2	p-values		C3	p-values		C0	p-values	
	C2	**0.001**		C1	**0.001**		C1	0.104		C1	0.177
	C3	0.104		C3	0.220		C2	0.220		C2	0.133
	C0	0.177		C0	0.133		C0	0.993		C0	0.993

Figure 7.49. Comparison of the mean value of thyroid follicular epithelium (µm, x400) evaluated by sex between the study groups (median values are indicated by a bold line for each group).
Values in bold correspond to $p<0.05$.

Inflammation assessment

The results on thyroid inflammation revealed that there were no significant differences between sex or treatment regimens in the study groups (C1, p=0.333; C3, p=0.296; Table 7.8).

Vascular congestion

In male rats, significant differences were found between groups: in C1 group, severe vascular congestion was observed in 66.7% of male rats; in C2 group, 50% of rats had moderate vascular congestion; whereas in C3 group, 50% of male rats had normal vascular morphology (p=0.001; Table 7.8).

In female rats, significant differences were also identified between groups: in C1 group, the same percentage of cases (33.3%) presented severe, moderate and mild vascular modifications; in C2 group, 50% of female rats had moderate vascular congestion; whereas in C3 group, 83.3% of the cases had normal vascular morphology (p=0.049). However, within each of the study groups, no statistically significant differences in terms of sex were confirmed (C1, p=0.513; C3, p=0.212; Tables 7.8 and 7.9).

Table 7.8. Comparison of thyroid morphology results between male and female rats in each treatment group

		C0		**C1**		**C2**		**C3**	
Parameter	**Score**	**Males (No/%)**	**Females (No/%)**	**Males (No/%)**	**Females (No/%)**	**Males (No/%)**	**Females (No/%)**	**Males (No/%)**	**Females (No/%)**
INF	**0 NM**	6/100	6/100	6/100	4/66.7	6/100	6/100	5/83.3	6/100
	1 Mild INF	-	-	-	1/16.7	-	-	1/16.7	-
	2 INF ≈ 10-40%	-	-	-	1/16.7	-	-	-	-
	3 INF > 40%	-	-	-	-	-	-	-	-
p-value		-			0.333		-		0.296
VC	**0 NM**	6/100	6/100	-	-	-	-	3/50	5/83.3
	1 Mild VD	-	-	1/16.7	2/33.3	3/50	3/50	-	1/16.7
	2 Moderate VC	-	-	1/16.7	2/33.3	3/50	3/50	1/16.7	-
	3 Severe VC	-	-	4/66.7	2/33.3	-	-	2/33.3	-
p-value		-			0.513		-		0.212
RV	**0 NM**	6/100	4/66.7	-	-	6/100	6/100	1/16.7	6/100
	1 < 10% FC	-	2/33.3	2/33.3	3/50	-	-	3/50	-
	2 ≈ 10-40% FC	-	-	2/33.3	2/33.3	-	-	1/16.7	-
	3 > 40% FC	-	-	2/33.3	1/16.7	-	-	1/16.7	-
p-value		0.439			0.766		-		**0.036**
IS	**0 NM**	6/100	6/100	-	-	6/100	6/100	4/66.7	6/100
	1 < 10% TA	-	-	3/50	4/66.7	-	-	-	-
	2 ≈ 10-40% TA	-	-	1/16.7	2/33.3	-	-	2/33.3	-
	3 > 40% TA	-	-	2/33.3	-	-	-	-	-
p-value		-			0.290		-		0.439
ICD	**0 NM**	6/100	5/83.3	-	1/16.7	-	-	1/16.7	5/83.3
	1 Discrete TCC	-	1/16.7	2/33.3	5/83.3	3/50	-	2/33.3	1/16.7
	2 Thick TCC, with fine PCC	-	-	4/66.7	-	2/33.3	3/50	3/50	-
	3 Thick TCC, PCC, PFC	-	-	-	-	1/16.7	3/50	-	-
p-value		0.296			**0.043**		0.122		**0.049**
EAIT Score	**0-3 NM**	6/100	6/100	-	-	4/66.7	1/16.7	2/33.3	6/100
	4-6 Mild AIT	-	-	1/16.7	3/50	2/33.3	5/83.3	3/50	-
	6-9 Moderate AIT	-	-	3/50	3/50	-	-	1/16.7	-
	10-12 Severe AIT	-	-	2/33.3	-	-	-	-	-
p-value		-			0.223		0.079		**0.049**

NM: Normal morphology; DTF: destruction of thyroid follicles; INF: inflammation; TA: total area; VD: vascular distension; VC: vascular congestion; RV: resorption vacuoles; FC: follicular cavities; IS: interfollicular spaces; ICD: interstitial collagen deposits; TCC: thyroid capsule collagenization; PCC: pericapsular collagenization; PFC: perifollicular collagenization: EAIT: experimental autoimmune thyroiditis; **Bold font indicates p<0.05**

Table 7.9. Association matrixes of morpho-pathological parameter results assessed within each study group

Males	C1	C2	C3	C0	Females	C1	C2	C3	C0	Males vs. females
VC scoring										
C1	-				C1	-				0.513
C2	**0.049**	-			C2	0.301	-			-
C3	**0.020**	**0.029**	-		C3	**0.025**	**0.011**	-		0.212
C0	**0.001**	**0.002**	0.050	-	C0	**0.007**	**0.002**	0.500	-	-
C1+2+3				**0.001**	C1+2+3				**0.049**	0.407
RV scoring										
C1	-				C1	-				0.766
C2	**0.007**	-			C2	**0.001**	-			-
C3	0.661	**0.036**	-		C3	**0.001**	-	-		**0.036**
C0	**0.007**	-	**0.036**	-	C0	0.050	0.439	0.439	-	0.439
C1+2+3				**0.001**	C1+2+3				0.050	**0.036**
IS scoring										
C1	-				C1	-				0.290
C2	**0.007**	-			C2	**0.002**	-			-
C3	**0.025**	0.439	-		C3	**0.002**	-	-		0.439
C0	**0.007**	-	0.439	-	C0	**0.002**	-	-	-	-
C1+2+3					C1+2+3					
ICD scoring										
C1	-				C1	-				**0.043**
C2	0.393	-			C2	**0.004**	-			**0.043**
C3	0.497	0.439	-		C3	**0.003**	**0.040**	-		0.122
C0	**0.002**	**0.007**	**0.014**	-	C0	**0.003**	**0.040**	-	-	0.296
C1+2+3				**0.001**	C1+2+3				0.050	0.050
EAIT scoring										
C1	-				C1	-				0.223
C2	**0.025**	-			C2	0.105	-			0.079
C3	**0.049**	0.393	-		C3	**0.002**	**0.019**	-		**0.049**
C0	**0.007**	0.439	**0.049**	-	C0	**0.002**	**0.019**	-	-	-
C1+2+3				**0.046**	C1+2+3				**0.033**	0.474

VC: vascular congestion; RV: resorption vacuoles; IS: interfollicular spaces; ICD: interstitial collagen deposits; EAIT: experimental autoimmune thyroiditis; **Bold font indicates p<0.05**

Resorption vacuoles

In male rats, significant differences were confirmed between the groups: in C1 group, resorption vacuoles assessment revealed equal percentages of cases (33.3%) with score 1 (<10%), 2 (10-40%) and 3 (>40%); in C2 group, all cases had normal morphology; and in C3 group, 50% of the male rats had resorption vacuoles <10% (p=0.001; Table 7.8).

In female rats, significant differences were also found between groups: in C1 group, 50% of female rats had resorption vacuoles <10%; and in C2 and C3 groups, all cases had normal morphology (p=0.05; Tables 7.9 and Figures 7.66-7.67). Significant sex differences were observed only in C3 group (p=0.036; Table 7.8).

Interfollicular space

All rats (regardless of sex) in C1 group (only KI administration) presented interfollicular spaces.

The interfollicular space score for C1 male rats (treated only with KI) was significantly different than that in C2 group (concomitant KI and Se administration,

p=0.007), C3 group (subsequent KI and Se administration, p=0.025) and C0 group (control, p=0.007) (Table 7.9). Scores of 2 (10-40% of glandular surface) and 3 (>40% of glandular surface) were particularly recorded in 50% of male rats (one rat with score 2 and two rats with score 3), whereas all the female rats presented only scores of 1 (<10% of the glandular surface) and 2 (Table 7.8).

Interstitial collagen deposits

In male rats, 66.7% of the cases in the C1 group had moderate fibrosis; in C2 group, 50% of male rats had mild collagen deposits and only 33.3% moderate fibrosis; whereas in C3 group, only 33.3% of cases had mild collagen deposits and 50% moderate fibrosis (p=0.001; Table 7.8).

In female rats, significant differences between groups were also confirmed: in C1 group, 83.3% of the rats had discrete collagen deposits; in C2 group, 50% had important collagen deposits; and in C3 group, 83.3% had normal morphology (p=0.05; Table 7.9). Concerning the morphology of interstitial collagen deposits, significant sex differences were observed only within C1 (p=0.043) and C3 (p=0.049) groups (Table 7.8).

Thyroiditis final score

In males, significant differences between treatment regimens were confirmed: in C1 group (only KI administration), 50% of the rats developed moderate thyroiditis and 33.3% severe thyroiditis; in C2 group (concomitant KI and Se administration), 33.3% of the male rats developed mild thyroiditis; and in C3 group (subsequent KI and Se administration), 50% of cases had mild thyroiditis and 16.7% moderate thyroiditis (p=0.046; Table 7.9 and Figure 7.58).

Female rats demonstrated significant differences in overall thyroid morphology: in C1 group, 50% of cases developed moderate thyroiditis; in C2 group, 83.3% of female rats had mild thyroiditis; whereas all cases in C3 group had normal morphology (p=0.033; Table 7.9 and Figures 7.60, and 7.62 – 7.63).

Regarding final thyroiditis score, significant sex differences were recorded only in C3 group where all females had normal thyroid morphology, similar to the female control group (p=0.049; Table 7.8 and Figures 7.68 – 7.69).

Figure 7.50. C1 male rat: thyroid tissue (HE, x 40)

Figure 7.51. C1 male rat: thyroid tissue (VG, x 40)

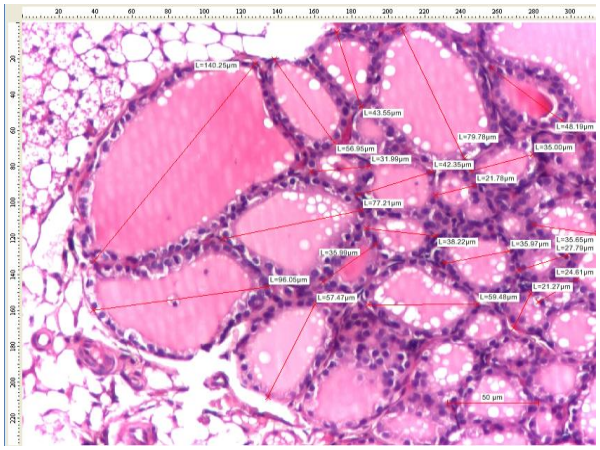


Figure 7.52. C1 male rat: measurements of the maximum diameter of 20 thyroid follicles (HE, x 100)

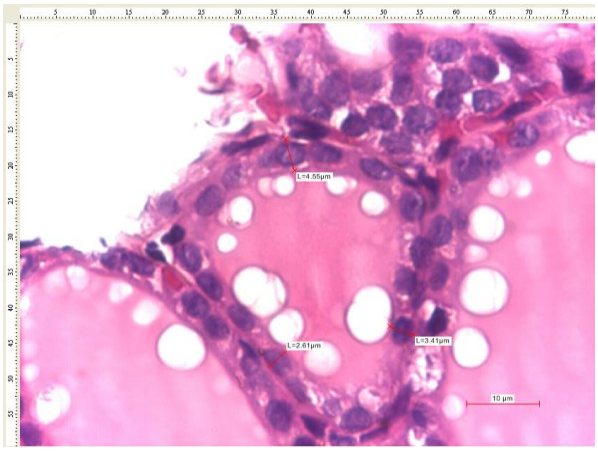


Figure 7.53. C1 male rat: measurements of heights follicular epithelium (HE, x 400)

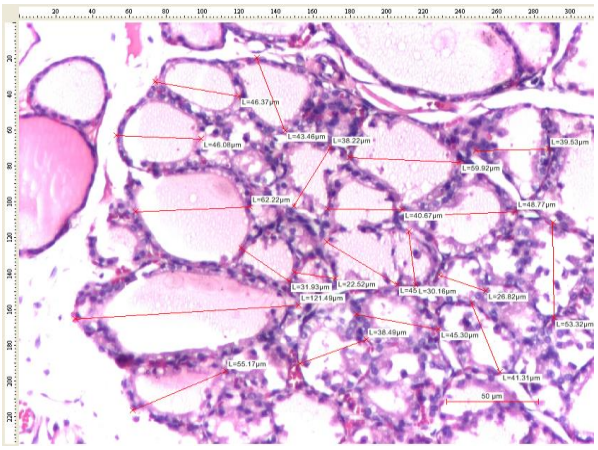


Figure 7.54. C2 female rat: the maximum diameter of thyroid follicles (HE, x 100)

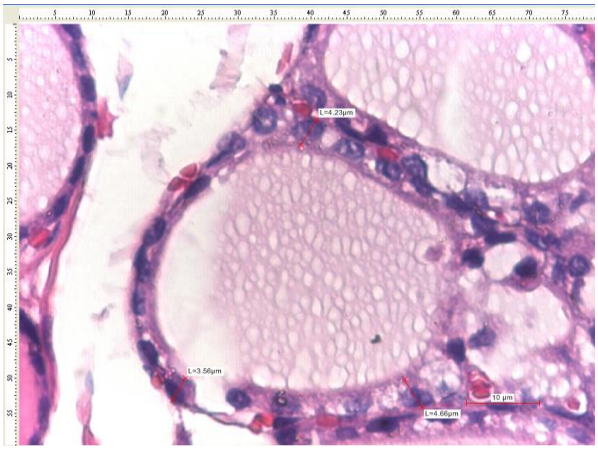


Figure 7.55. C2 female rat: height of follicular epithelium (HE, x 40)

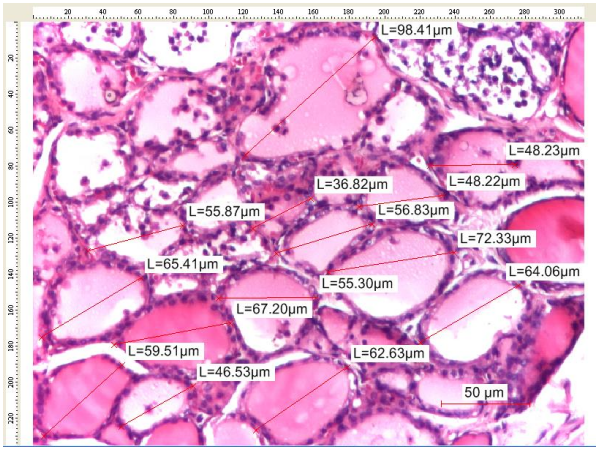


Figure 7.56. C0 female rat: maximum diameter of thyroid follicles in control group (HE, x 100)

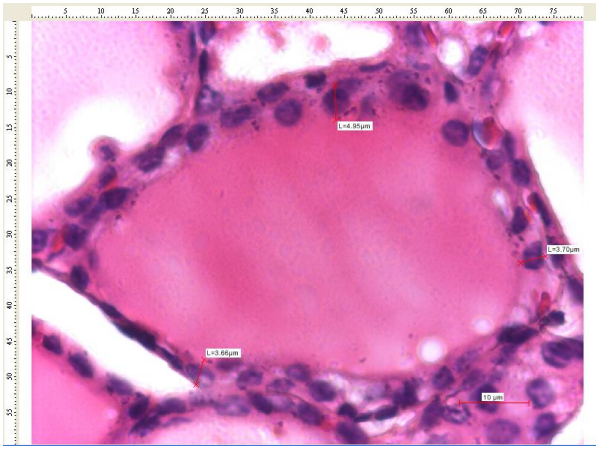


Figure 7.57. C0 female rat: height of follicular epithelium in control group (HE, x 400)

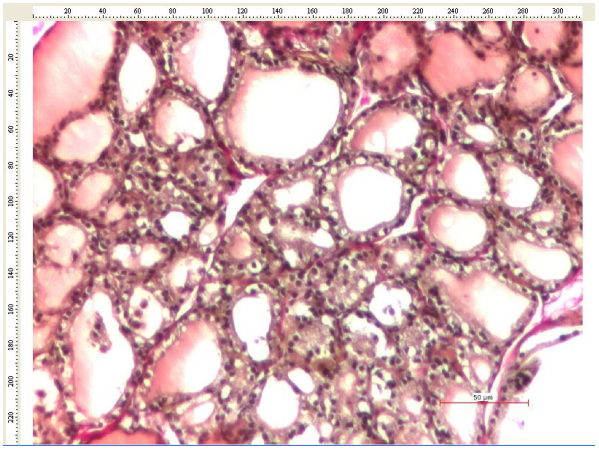


Figure 7.58. C1 male rat: fibrosis, inflammation, vascular congestion and resorption vacuoles (VG, x 200)

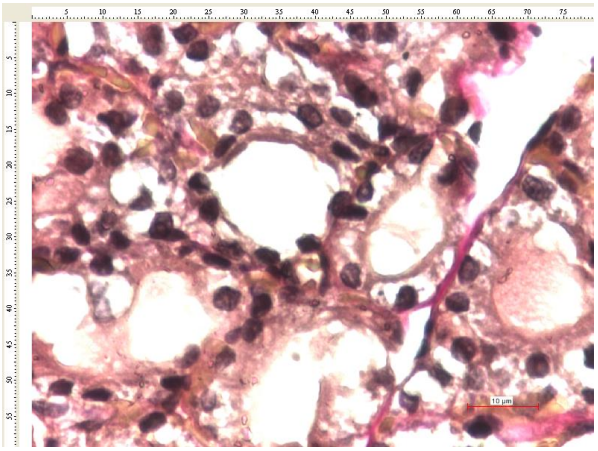


Figure 7.59. C1 male rat: follicular epithelium details (VG, x 400)

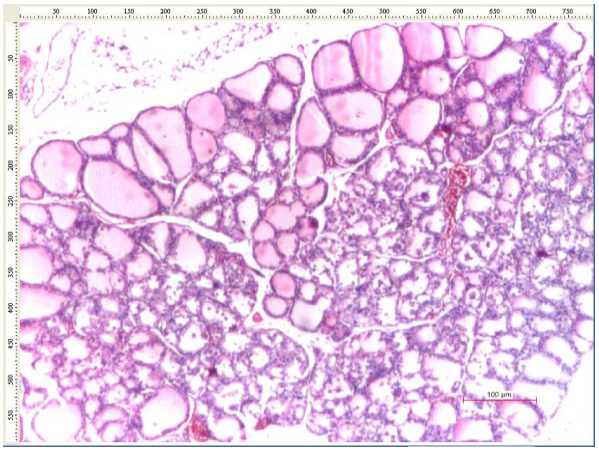


Figure 7.60. C2 female rat: thyroid tissue (HE, x 40)

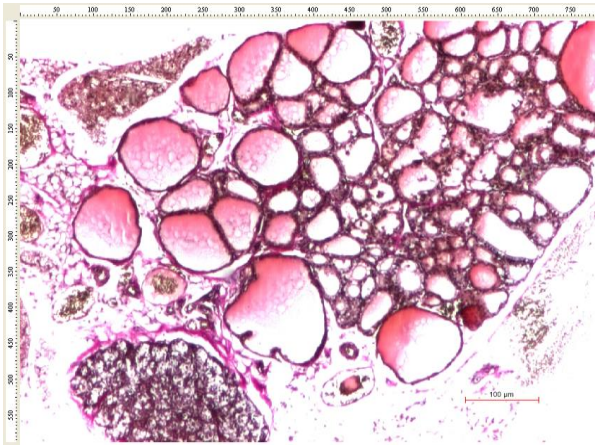


Figure 7.61. C2 female rat: thyroid tissue (VG, x 40)

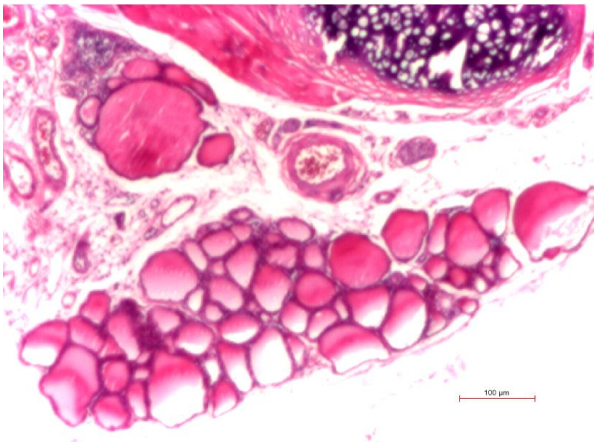


Figure 7.62. C3 female rat: thyroid tissue (HE, x 40)

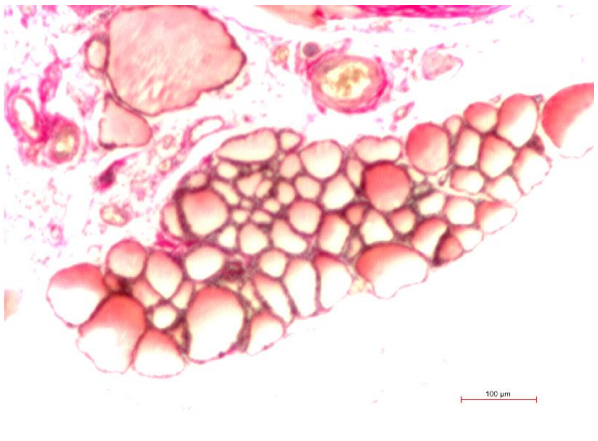


Figure 7.63. C3 female rat: thyroid tissue (VG, x 40)

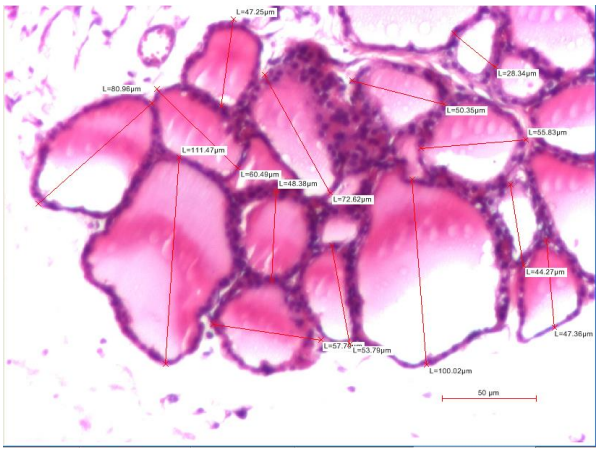


Figure 7.64. C3 female rat: measurements of the maximum diameter of thyroid follicles (HE, x 100)

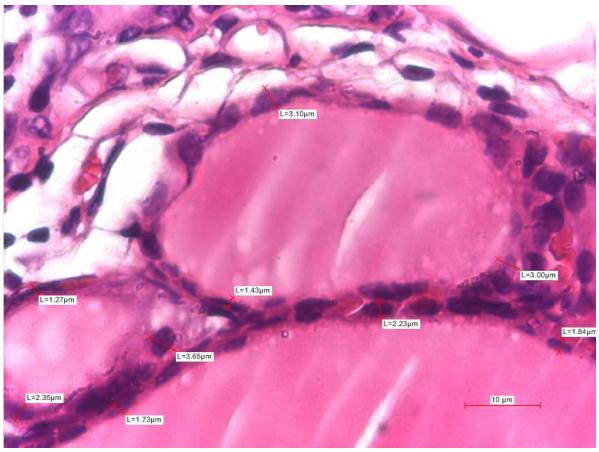


Figure 7.65. C3 female rat: height of follicular epithelium (HE, x 400)

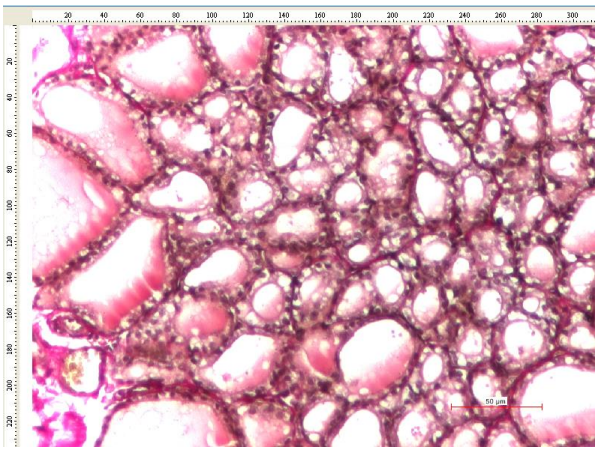


Figure 7.66. C3 female rat: evaluation of resorption vacuoles, vascular cogestion and fibrosis (VG, x 200)

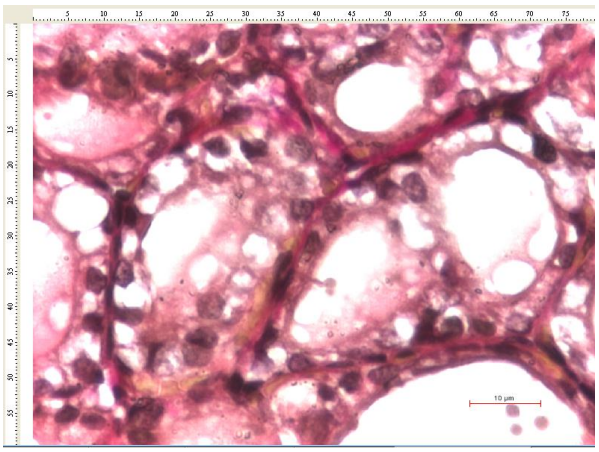


Figure 7.67. C3 female rat: follicular epithelium details (VG, x 400)

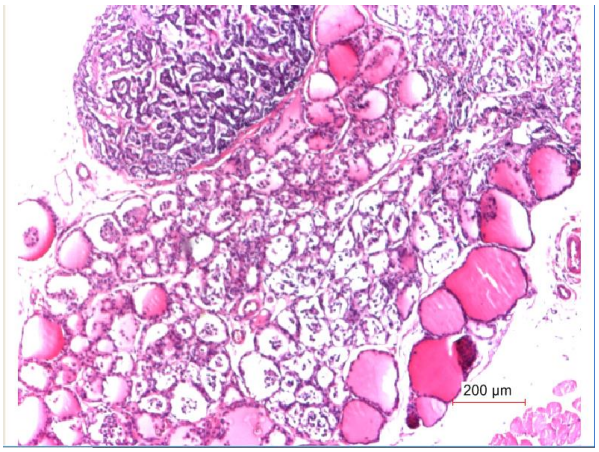


Figure 7.68. C0 female rat: thyroid and parathyroid tissue (HE, x 40)

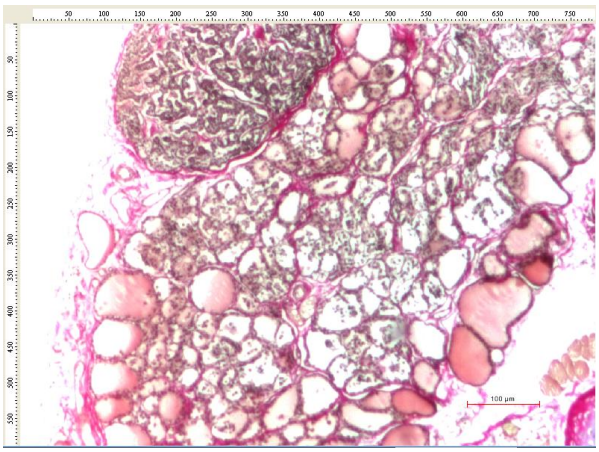


Figure 7.69. C0 female rat: thyroid and parathyroid tissue (VG, x 40)

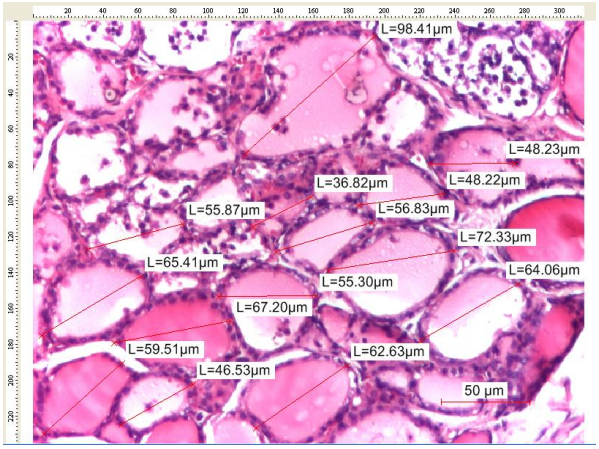


Figure 7.70. C0 female rat: measurements of the maximum diameter of thyroid follicles (HE, x 100)

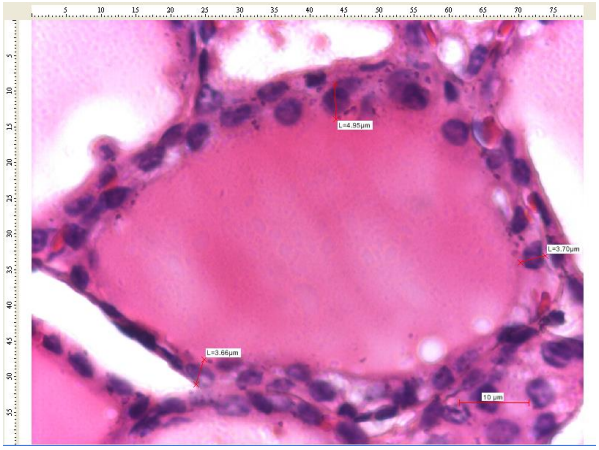


Figure 7.71. C0 female rat: height of follicular epithelium (HE, x 400)

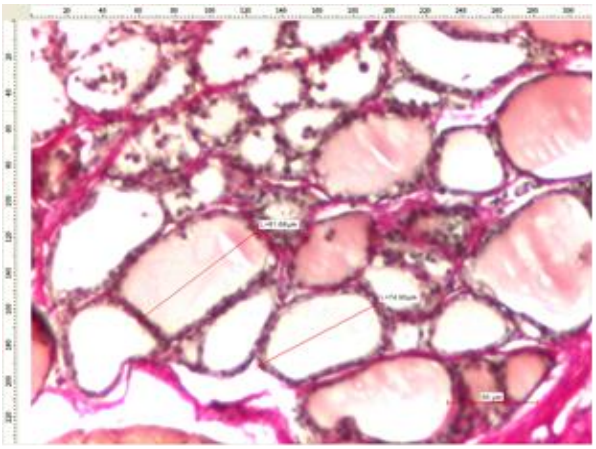


Figure 7.72. C0 female rat: evaluation of resorption vacuoles, vascular cogestion and fibrosis (VG, x 200)

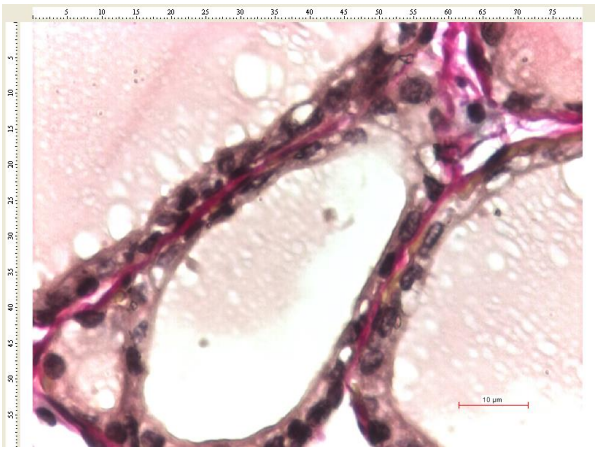


Figure 7.73. C0 female rat: follicular epithelium details (VG, x 400)

7.7.4. Discussion

This study confirms the protective role of Se on thyroid morphology in iodine-induced EAIT in Wistar rats. Se effect was more evident in female rats, where Se administration after iodine exposure determined negligible thyroid morphology changes, the thyroid gland keeping a normal aspect. Iodine has a great impact in thyroid autoimmunity induction and modulation. Several studies assessed the incidence of thyroid antibodies and autoimmune hypothyroidism in patients located in iodine-rich or poor areas [Vanderpump et al., 1995; Laurberg et al., 1998; Effraimidis et al., 2014]. After iodine prophylaxis in iodine-deficient areas, a 4-times increase in anti-thyroid antibodies was reported [Fountoulakis et al., 2007]. According to a Danish survey, following the administration of an iodine dose of 500µg/day for 6 months, AIT occurred in 20% of the healthy population under study [Nielsen et al., 2014].

The use of NOD.H2h4 mice for genetically determined EAIT led to the same outcome. Higher iodine doses in mice conduced to higher disease incidence and severity [Rose et al., 2002]. Several possible mechanisms by which iodine could trigger AIT have been described: higher iodine exposure leads to higher thyroglobulin iodination, higher antigenicity (immunogenicity) by creating new iodine-containing epitopes or by discovering

cryptic epitopes, facilitating antigen exposure (antigen processing or antigen presentation) and increasing T-cell receptor binding and activation of T cells [Rose et al., 2002].

Secondly, higher iodine exposure is conducive to higher reactive oxygen species (ROS) in thyrocytes. ROS may increase the expression of intracellular adhesion molecule 1 in the thyroid follicular cell, which subsequently attracts immunocompetent cells to the thyroid gland. The binding of ROS to the phospholipidic membrane may damage the thyroid and trigger auto-antigens release [Fountoulakis et al., 2007]. In addition, iodine excess promotes apoptosis of thyroid follicular cells by inducing the expression of TRAIL (TNF-related apoptosis-inducing ligand) necrosis factor and its receptor, death receptor-5, in the thyroid. Iodine impacts the immune system cells by increasing dendritic cell maturation, T cells count, and the production of immunoglobulins, according to the in-vitro evidence [Fountoulakis et al., 2007].

In the present study, the EAIT in adult male and female Wistar rats was induced by KI administration in the drinking water. Most studies in the literature, which sought to induce EAIT, used genetically modified animal models, NOD.H2h4 [Rose et al., 2002; Kolypetri et al., 2010] and BB/W (Bio Breeding/Worcester) rats [Yanagisawa et al., 1986; Li et al., 1993]. NOD mice and BB/W rats are animal models generally used for the study of type 1 diabetes. NOD.H2h4 is a genetically animal model predisposed to develop EAIT over time. Iodine administration increases the prevalence of EAIT, earlier occurrence, and a more severe form of disease [Barin et al., 2005]. A similar study was performed on BB/W rats by looking at the effect of excess iodine on thyroid function and on immunological phenomena that trigger AIT. The results of the study showed an increase in the number of dendritic cells and lymphocyte infiltrate in animals receiving additional iodine in drinking water [Li et al., 1993]. Both the increase in the number of dendritic cells and the lymphocyte infiltrate are possible mechanisms involved in triggering EAIT.

In a study on NOD.H2h4 mice of different ages, it was observed that the prevalence and severity of EAIT increased with age in genetically predisposed animals [Barin et al., 2005]. In the present study, only young adult Wistar rats were used, with no genetic predisposition to influence the onset or the form of the disease. This was also preferred in order to have a homogenous group and to avoid possible age-induced changes in the experiment.

Regarding the presence of thyroid inflammation in both male and female Wistar rats, no significant changes between the treatment regimens were described, the results being similar to those in the control group. The absence of inflammation could be explained by the short period of iodine administration or by insufficient iodine quantities. Similar studies that obtained inflammatory changes had administered iodine up to 12 weeks [Zhu et al., 1995; Barin et al., 2005; Lupachik et al., 2007].

The evolution of follicular epithelium size shows potential benefits of Se treatment as favorable, statistically significant differences were observed between the measured parameter in the group treated with concomitant sodium selenite and KI administration, in comparison with the group administered with iodide only.

Significant changes were observed in the groups treated with KI and Se compared with the KI treated groups, with forms of thyroiditis less aggressive in both males and females treated with Se. In rats with sequentially KI and sodium selenite administration, the same favorable outcomes were not obtained as in the case of the concomitantly treated groups; this effect was only observed in males. The females initially treated with KI and subsequently with Se had a surprising evolution, the results being almost identical to those of the control group, as they no longer had EAIT. Experimental studies showed that the antigen that initiates EAIT in animal models is thyroglobulin, regardless of species (studies in mice, rats and birds) [Ruwhof et al., 2001]. The thyroglobulin allografts affect the susceptibility to thyroiditis. Moreover, modulating genes related to the X-chromosome have been highlighted,

which could explain the different responses in EAIT not only in animals, but also in humans [Amara et al., 2010]. The effectiveness of Se supplementation proved to be different depending on the time of treatment initiation and gender.

AITD is highly prevalent, with the highest female-to-male ratio among all autoimmune diseases [Jacobson et al., 1997]. There is a large body of evidence that moderate amounts of estrogen may enhance immunologic reactivity to self-antigens [Kincade et al., 1994; Xiang et al., 2018]. However, as AIT is frequently diagnosed after menopause, the X-chromosome seems to be the source of enhanced susceptibility rather than sexual hormone levels. For example, X-chromosome inactivation has been associated with AITD [Yin et al., 2007]. However, there have been reports in men that confirm a connection between estradiol levels (or estradiol to testosterone ratio) and thyroid autoimmunity [Chailurkit et al., 2013; Chen et al., 2017].

In males, Se supplementation has been shown to be more effective with concomitant administration of KI. Males have been presented with less aggressive forms of the disease than those who had received successive administration (initially with KI and subsequently with Se). Se administration, in both concomitant and then successively treated groups, contributed to milder forms of AIT, compared with the group not supplemented with Se.

Moreover, an extremely important aspect was observed in the groups of female rats in which, unlike male rats, Se supplementation proved to be very effective. In the group of females treated successively, the thyroid morphological aspect was identical to the morphological appearance of the control group, which did not show AIT, thus advocating the remission of the KI-induced disease. Concomitant administration resulted in a significant improvement in thyroid morphology.

Overall, the results of the present study revealed the effectiveness of Se supplementation in both co-administration with KI and sequential administration in female and male sex alike. Significant sex differences were recorded in the groups initially treated with KI and subsequently with Se ($p=0.049$): While in females histological appearance of the thyroid was normal in the whole group, in males only 33% had normal thyroid, the rest having mild (50%) or medium (17%) thyroiditis. In the rest of the study groups, the differences were not statistically significant (Table 7.8).

This was especially observed in females due to the hormonal features involved in the AIT pathogenesis. It is known that estrogen increases (while androgen decreases) the response of the hypothalamic-pituitary-adrenocortical axis to stress, and activation of this axis is more pronounced in women than in men, which explains the higher incidence of AITD in women [Falgarone et al., 2012].

There are a number of limitations in the present study. A low number of Wistar rats were used in each study group, although valid for statistical analysis following previous scientific research protocols. In addition, a Se dose-finding study was not performed; however, no clinical signs of Se toxicity were observed during the study at the administered dosages.

7.7.5. Final remarks

EAIT induction by KI administration to Wistar rats had a greater impact on males than on females. Although the latter are more prone to the disease, it is the males that develop more severe forms, estrogens playing a modulating role.

The effectiveness of Se supplementation resulted in improved forms of EAIT. The timing of Se administration has also been proven to be important. Concurrent administration of KI and sodium selenite stimulated the normalization of thyroid morphology in most cases.

SECTION III.
FUTURE PROJECTS IN THE PROFESSIONAL, ACADEMIC AND SCIENTIFIC FIELD

Medicine, the art of healing, arouses a keen interest in the general population, being a dynamic, stressful profession, with suspense, uncertainty, wear and tear, emotions, suffering, but also joy. Medicine is a bold profession, but an imperfect science, with permanent changes in the level of knowledge, with errors and risks, which largely depends on experience, skills, intuition or flair. In order to provide patients with optimal care, practice and knowledge is needed, based on the accumulation of information that defines the evolution of the medical field. In correspondence with the information avalanche, at full professional maturity we accumulate, through upgrading and self-improvement, several times more theoretical and practical knowledge than at the initial moment, of the debut in medical training.

This process of formation requires responsibility and involvement, passion and compassion, perseverance and understanding, curiosity and vision - medicine being a road with a horizon always open. In the university career, it is necessary to establish some principles and objectives that are outlined according to the final goal, personal priorities, individual potential *vs.* the achievements expected by those close to you, the specifics of the profession and the socio-professional environmental factors.

III.1. DEVELOPMENT FOCUSED ON PROFESSIONAL ACTIVITY

In today's society, many managers expect personnel to be able to monitor their own performance and know how to adapt to higher workloads, stressful situations, or changes in the entity.

That's why it's important to know the things that influence your performance, as well as how your behavior affects those around you.

The fact that you know how to learn new things and what you should improve is a very valuable thing at work, and is a goal to be achieved professionally.

In this new stage of professional progress, I will consider the following principles and objectives:

- assuming a personal career strategy in line with the „Grigore T. Popa” University of Medicine and Pharmacy, Iasi, superimposing the individual goals with the institutional objectives;
- constant improvement of one's own skills and competences at the quality standards in higher education, aiming at increasing the prestige of the „Grigore T. Popa” University of Medicine and Pharmacy, Iasi;
- advancement of personal research in parallel to complementary research, directing the efforts both towards the fundamental field of pathology and the clinical area, facilitating the translation of the results from the fundamental research to the medical clinic line;
- active participation in projects and actions initiated by faculty and university for research and structural funds.

III.2. DEVELOPMENT FOCUSED ON ACADEMIC ACTIVITY

Teaching Pathology in a medical university requires a permanent training and the continuous acquisition of new knowledge. The constant development is essential for the teacher in order to provide an image of the contemporary scientific world. This update requires a variation to the level of knowledge of each category of subject, from the early years of studentship to residents and specialists, appropriately adjusting the volume of detailed information and the complexity of the data, connections with other medical fields and the practical applicability of theoretical notions. Even if the main field of teaching has descriptive notions, I will pursue the integration of Pathology in the broad context of related sciences: cell biology, histology, biochemistry, genetics, physiology, immunology, microbiology, virology, parasitology and oncology.

The development of the Pathology Discipline requires the consolidation of the logistics for teaching and research, as well as the selection of young teachers - a goal that must be aimed at doctors and residents in the field of Pathology, whose open and comprehensive vision can be beneficial to optimize the educational process and research.

Main objectives for the development of academic activity:

- to increase teaching infrastructure quality through educational project fundraising and to improve teaching aid archive through quality macroscopic and microscopic specimens in all study fields;
- to create an imaging database posted on the E-learning platform of Grigore T. Popa" University of Medicine and Pharmacy, beneficial for students, graduates and postgraduates, using the portfolio existing within the Pathology Discipline of UMPh and "St. Spiridon" Clinical Emergency County Hospital Iasi and by collaborations with other groups from Romania and abroad, which I intend to develop;
- promoting educational strategies adapted to the specifics of the discipline, recognizing the importance of the student-centered teaching process, their responsibility and involvement;
- optimization of modern teaching methods, respecting the main didactic principles: the principle of integrating theory with practice, the principle of systematic and continuous learning, the principle of intuition, the principle of conscious and active participation of the learner, the principle of thorough acquisition of knowledge, skills and inverse connection;
- supporting students in accordance with their potential, needs and aspirations, stimulating students who show an attitude of stagnation or capping in learning;
- conducting courses, credited in continuing medical education (CME) by the Medical Council for graduates of medical schools in various specialties;
- improving the training curricula in the pathology residency and other related specialties, by collaborating with the teaching staff from the medical universities in the country.

III.3. DEVELOPMENT FOCUSED ON RESEARCH

Research is one of the basic components of our professional and academic development. At present, internationally, there is an open competitive academic culture, with official university hierarchies and differentiated funding. The main criterion according to which faculties and universities are evaluated and ranked, both in the national education system and in international ranking system, is the scientific research. In addition to increasing international visibility, performance in scientific research is funded additionally and differently, which implicitly leads to institutional development. These are the reasons why research is considered as the first criterion in the promotion and evaluation of teachers, which can sometimes create frustration, given that the essence of our profession is that of a teacher.

Main objectives for the development of research activity:

- collaboration in research activity in the field of endocrine pathology, dermatopathology, digestive pathology, gynecology and forensic medicine with other centers of excellence in the country and abroad;
- use of data obtained within research studies, by writing more articles whose novelty and impact ensure acceptance in high-impact factor journals;
- involvement in competitions for international scientific projects and in accessing European funds for research, by using personal expertise in the field of pathology;
- participation in prestigious national and international scientific events, in order to constantly update information and research in the field of pathology and increase visibility by publicly supporting personal or collective research;
- supporting students and residents with research skills, in the development of a "core of scientific research" with real activity within the Pathology Discipline, with the role of reservoir for future doctoral students and post-doctoral researchers of the faculty, according to the existing model in prestigious universities abroad.

For the future, I intend to continue and expand some of the research themes that I have developed after I finished the PhD thesis or some research project in which I was a part of an international multicenter teams but, also, to start new, promising ones, as follows:

III.3.1. Non-differentiated thyroid carcinomas – the adversaries that can be revealed

Histological classification of thyroid cancers originating from follicular cells is proposed to keep separate the well-differentiated thyroid cancer group of papillary and follicular thyroid carcinomas from the less frequent but clinically aggressive histologic types, such as poorly differentiated and anaplastic thyroid carcinomas [JAES/JSTS, 2018; Abe et al., 2021]. Anaplastic thyroid carcinoma (ATC) is an uncommon carcinoma representing 1 to 4% of all thyroid cancers [Smallridge et al., 2012]. Causative factors for ATC remain unknown, although they might at least partly overlap with those of well-differentiated thyroid carcinomas (DTC), as suggested by the frequent co-occurrence of well-differentiated components in ATC cases [Akaishi et al., 2019; Xu et al., 2020]. ATC is considered the end point of follicular cell-derived cancer progression and despite its heterogeneous morphology is defined by the presence of high-grade features and lack of follicular cell differentiation [Lloyd et al., 2017; Abe et al., 2021]. ATC is a rare orphan disease that is refractory to therapeutic efforts. More than 80% of ATC patients already have disease progression to the surrounding organs and/or distant metastasis at their initial presentation [Akaishi et al., 2019; Abe et al., 2021], and such extraordinarily rapid disease progression sometimes precludes the initiation of therapeutic attempts. In cases in which effective management cannot be provided, it is not unusual for an individual with ATC to die within several days of receiving the diagnosis [Lam et al., 2000].

Poorly differentiated thyroid carcinoma (PDTC) is even scarcer than ATC and its incidence varies worldwide, as a possible consequence of environmental factors and classification criteria [Tallini et al., 2011; Akaishi et al., 2019]. In a large series from a single institution, the incidence of PDTC was 3% of primary thyroid carcinoma and slightly more half the incidence of ATC [Lam et al., 2000]. The criteria for PDTC are still somewhat controversial [Tallini et al., 2011; Volante et al., 2016; Xu et al., 2020]. Its features, as outlined in the Turin consensus [Volante et al., 2007], define the prototype of a thyroid carcinoma that is both high grade and poorly differentiated [Tallini et al., 2011] and have been embraced by the WHO classification [Volante et al., 2007; Lloyd et al., 2017].

It is also important to note that even minor components of either PDTC or ATC in an otherwise well-differentiated carcinoma are impacting negatively on patients' prognosis, and these should be mentioned in the pathological report. In fact, the prognosis of patients with thyroid carcinomas having a component of either PDTC or ATC at the threshold of 10% were shown to bear a prognosis similar to predominant poorly differentiated /anaplastic ones [Dettmer et al., 2011; Wong et al., 2020].

The 7th edition of the TNM classification stratified ATC patients simply by the existence of extrathyroidal extension of the primary tumor and distant metastasis. All ATC patients were thus classified as having a T4 tumor and stage IV disease [Sobin et al., 2009]. In the revised 8th edition, ATC tumors are classified as the same T category as DTCs [Brierley et al, 2017]. All ATC patients are still classified into stage IV, as in the 7th edition. No further changes in the stage stratification according to the tumor are made by the 8th edition; instead, the presence of nodal metastasis is regarded as a factor that moves the patient into stage IV B. At the same time, there are no changes in the PDTC classification between the 7th and 8th editions of AJCC [Brierley et al., 2017].

The differences between biological behavior of DTC and PDTC or ATC, are grounded through sequences of the carcinogenic mechanism, with consequences on cellular cycle with increase of proliferation (overexpression of cyclin D1, Ki67, p53 and lack of expression of p27) [De Lellis et al., 2004; Pešutić-Pisac et al., 2008; Seybt et al., 2012; Liu et al., 2015], adhesive morphological status of cells (altered the expression of E-cadherin, claudin-1 and beta3-tubulin) [Rocha et al., 2003; Muller et al., 2009; Abd et al., 2012; Roque et al., 2013; Ceyran et al., 2015] and influence on the tumoral microenvironment (transformation of the expression of periostin or fibronectin in tumoral cell and stroma) [Liu et al., 2015].

In this context, I propose to study and compare:

- the prognoses of ATC and PDTC patients by applying the 7th and 8th editions of the TNM classification with subclassification into two categories, that of patients with de novo ATC and PDTC developed tumors *vs.* DTC with tumor progression;
- the morphological profile of coexisting DTC preceding the development of PDTC and ATC, compared to conventional DTC, in order to define an aggressive molecular tumor profile of well-differentiated tumors on which oncological therapy can intervene as a priority.

III.3.2. Role of retinoblastoma (RB1) tumor suppressor expression in poor outcomes of medullary thyroid carcinoma

Medullary thyroid carcinoma (MTC) is originated from the parafollicular clear cells (C-cells) of the thyroid gland [Cakir et al., 2009]. Although MTC accounts only 4% of all thyroid carcinomas, it is accountable for about 13% of deaths resulting from thyroid cancer [Kebebew et al., 2000]. Besides a sporadic form that occurs in the majority of cases, in the remaining 25–30% of patients MTC develops as part of the hereditary Multiple Endocrine Neoplasia type 2 syndrome (MEN2) [Cakir et al., 2009]. While localized disease may be treated surgically, with a 10-year survival rate of 95% [Links et al., 2015], MTC may also act as an aggressive malignancy. Around 50% of patients have lymph node metastases and 10% have distant metastases at the time of diagnosis [Links et al., 2015]. Overall survival (OS) for MTC patients is 86% at 5 years and 65% at 10 years [Institute NC, 2016].

In recent years, in clinical practice for the treatment of patients with advanced progressive MTC, were introduced the multikinase inhibitors (vandetanib and cabozantinib) which inhibit the kinase activities of key pathogenic tyrosine kinases [Wells et al., 2012; Schoffski et al., 2012]. High rates of partial response and/or disease control have been

reported with both agents in Phase III studies [Elisei et al., 2013; Alonso-Gordoa et al., 2015]. While representing an important improvement in the field, it is notable that complete responses in these late-stage patients are not observed, resistance develops over time, toxicities reduce tolerability, and OS has not been shown to be improved [Wells et al., 2012; Elisei et al., 2013; Links et al., 2015]. These features point to a critical need for new therapies for patients with progressive MTC and for better selection of patients for treatment.

Because approximately 60% of sporadic MTCs do not harbor RET mutations, additional oncogenic drivers have been investigated [Musholt et al., 2005]. One candidate alternative pathway for MTC development is the CDK/RB cell-cycle regulatory pathway. The retinoblastoma (RB1) tumor suppressor has been reported to be mutated in many human cancers such as sarcomas, glioblastomas, and small-cell and non-small-cell carcinomas of the lung [Shew et al., 1989; Cance et al., 1990; Xu et al., 1991; Ichimura et al., 1996; Cagle et al., 1997]. Numerous studies in mice involving genetic inactivation of the RB pathway have shown that neuroendocrine cells exhibit specific sensitivity to loss of cell-cycle regulation. It has been confirmed, for example, that germline heterozygous loss of rb and other members of the pathway, including cyclin dependent kinase inhibitors (CDKIs) p18 and p27, results in a high rate of MTC in mice [Cote et al., 2015]. Concomitantly to the loss of p18 and p27 the incidence of MTC occurrence in RET transgenic mice increases [Flicker et al., 2012]. In addition, homozygous deletion of Rb1 in the mouse thyroid in a p53 null background produces an aggressive MTC phenotype [Song H et al., 2017]. Furthermore, overactivation of CDK5 in mice resulted in a MTC phenotype through an RB-mediated mechanism [Pozo et al., 2013]. In sporadic and hereditary MTC, loss of a section of the p arm of chromosome 1 that includes the genes encoding p18 and E2F2 has been commonly found [van Veelen et al., 2009]. These reports together suggest that the CDK/RB pathway may be important in the development of human MTC. Post-translational inactivation of RB through phosphorylation by cyclin kinases leads to release of E2F transcription factors from their binding to RB, and to progression of the cell cycle. To date, only a single frameshift mutation in RB has been reported in 54 human MTCs in the COSMIC database and/or reported in the literature [Uzilov et al., 2016]. Somatic inactivating p18 mutations have been found in a subset of human MTC samples [van Veelen et al., 2008], as has p18 loss of heterozygosity (LOH) [Grubbs et al., 2016]. A number of small immunohistochemistry (IHC) studies have been performed to determine RB protein expression in thyroid neoplasms, including MTC tissue samples, with RB presence varying from 29% to 100% [Figge et al., 1991; Holm et al., 1994; Anwar et al., 2000; Tavangar, 2008; Valenciaga et al., 2017]. Some of these studies correlated RB positivity to benign neoplasms [Tavangar, 2009], while others found no association with the clinical course in MTC samples [Anwar et al., 2000].

In this context, I propose to study:

- the clinicopathological profile of MTC with unfavorable evolution;
- the RB expression in MTC with poor outcomes.

III.3.3. Homeobox B9 (HOXB9) and LRRN4 expression in colon adenocarcinoma. The role in predict differentiation and clinical outcome.

Homeodomain-containing (HOX) proteins belong to the homeobox superfamily comprising a highly conserved 61-amino acid homeobox domain. In total, 39 HOX gene members have been identified and ordered into four clusters (A, B, C, and D), which are located on four different chromosomes (7p15, 17p21, 12q13, and 2q3) [Abate-Shen, 2002]. In addition to their critical roles in development, increasing numbers of studies demonstrated that HOX family genes are associated with tumorigenicity [Chen et al., 2003; Grier et al,

2005; Shah et al., 2010] and progression including tumor growth and angiogenesis [Chen et al., 2003, Rhoads et al, 2005, Winnik et al, 2009, Sun et al, 2013].

HOXB9 was known to induce tumor angiogenesis, invasion and lung metastasis in breast carcinoma [Hayashida et al, 2010], as well as being an important prognostic factor for clinical outcomes of breast cancer patients [Chiba et al, 2012; Morgan et al, 2012; Seki et al, 2012; Shrestha et al, 2012]. The activator E2F3 A and repressor BMI1/PRC1 mediate HOXB9 expression in Hodgkin lymphoma cells [Rosenwald et al., 2007; Zhussupova et al., 2014]. The Wnt/TCF4 pathway directly targets HOXB9 in lung cancer to mediate lung adenocarcinoma metastasis [Nguyen et al., 2009]. The ubiquitin-like protein FAT10 regulates HOXB9 expression via the b-catenin/TCF4 pathway in hepatocellular carcinoma [Yuan et al., 2014]. HOXB9 was also found to induce epithelial-to-mesenchymal transition (EMT), a process that is associated with tumor invasion and resistance to chemotherapeutic drugs and radiations by accelerating DNA damage responses [Nguyen et al, 2009; Hayashida et al, 2010]. HOXB9 induction of EMT is mediated by activation of the Wnt signaling pathway [Hayashida et al, 2010]. Recently, a report showing that decreased expression of HOXB9 is related to a poor overall survival in patients with gastric carcinoma, identifying an opposite role of HOXB9 in cancer [Sha et al, 2013], suggested that HOXB9 may play a diverse role during cancer progression under various circumstances.

LRRN4 (leucine rich repeat neuronal 4), a recently identified member of leucine rich repeat neuronal protein family (NLRR), has been reported to be expressed in various tissues. At present, LRRN4 has been investigated mainly in the central or peripheral nervous system [Bando et al., 2005; Bando et al., 2012]. A recent study shown that the aberrantly low expression of LRRN4 was closely associated with the dilated cardiomyopathy [Li et al., 2017], which reminded us that aberrant LRRN4 expression might also play a role in other diseases. LRRN4 has not been studied in cancers, but several other members of NLRR have been reported in some cancers. The expression of LRRN1 was upregulated in gastric cancer tissues, related to a poor prognosis [Liu et al., 2019] and negative regulator of ALK signaling in neuroblastoma [Satoh et al., 2016]. NLRR1, NLRR3 and NLRR5 were found to have different biological functions among the neuroblastoma subsets [Hamano et al., 2004].

Colon carcinoma (CC) is one of the leading malignancies worldwide and is the fourth cause of death in cancer patients [Zhan et al., 2014]. Although early diagnoses and therapeutic strategies have been well established for colon cancer patients, invasion, metastasis and recurrence of the disease are still challenging. As for molecules associated with prognosis in colon cancer, the silent mating-type information regulation 2 homologue 1 (SIRT1), has been found associated with good prognosis in colorectal cancer [Jung et al, 2013]. CALU and CDH11 are candidate stromal biomarkers of prognostic significance in colon cancer as well [Torres et al, 2013].

However, the detailed molecular mechanisms accounting for the functions of HOXB9 and LRRN4 in different cancers are unknown and there are only a few studies regarding the clinical relevance of their expression with colon adenocarcinoma progression in human [Zhan et al., 2014; Zhang et al, 2022].

In this context, I propose to study:

- the prognostic role of HOXB9 and LRRN4 in colon adenocarcinoma progression and their clinicopathological significance.

III.4. FINAL REMARKS

"Grigore T. Popa" University of Medicine and Pharmacy Iasi, one of the oldest higher education institutions in the country, founded in 1879, currently operates providing high-quality teaching and scientific standards, based on modern organizational management. The

vital core that supported the evolution of the University is its value system: training students in the spirit of compassion and devotion to peers, cultivating the tradition of free thinking, acceptance of diversity and multiculturalism.
So in the future, the major motivation of self-improvement and real involvement in the life of the academic community will be to coordinate young researchers in cultivating scientific careers and reputations based on the excellence of their work, which should always be a top priority.

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