The Role of Molecular Markers and Clinicopathological Features in Predicting Central Lymph Node Metastases of Papillary Thyroid Microcarcinoma

by

Dr. Orhan Ağcaoğlu

A Dissertation Submitted to the
Graduate School of Sciences and Engineering
in Partial Fulfillment of the Requirements for
the Degree of

Doctor of Philosophy

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January 12, 2024

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Dr. Zelal Adıgüzel

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ABSTRACT

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Background: Despite the favorable prognosis of papillary thyroid microcarcinoma (PTMC), the frequent occurrence (up to 60%) of central lymph node metastasis (CLNM) poses a significant challenge, influencing recurrence and survival. The limited accuracy of preoperative neck ultrasonography in detecting CLNM necessitates exploring a combination of radiologic, histopathologic, and clinical factors for effective prediction of CLNM. This study aims to evaluate the predictive value of molecular markers and clinicopathological features for CLNM in PTMC patients.

Methods: Between February 2019 and December 2023, patients who underwent thyroidectomy operations at Koç University Hospital and American Hospital were included in this study. From this group, 145 patients with tumor sizes ≤10 mm (PTMC) were included in the study. Those without prophylactic or therapeutic central neck dissection, and with <3 excised lymph nodes were excluded from the study. Patients were divided into two study groups based on CLNM. Patient demographics and clinicopathological features, $BRAF^{V600E}$ mutation, desmoplasia and Masson trichrome staining were evaluated. Both univariate and multivariate logistic regression analyses were performed.

Results: Among the 65 participants, 35 (53.8%) exhibited CLNM. Larger tumor sizes, lymphovascular invasion, aggressive subtype presence, $BRAF^{V600E}$ mutation positivity, higher extent and intensity scores of desmoplasia and trichrome staining were significant predictors of lymph node metastasis on univariate analyses ($p<0.001$). In order to predict the presence of CLNM, multivariate logistic analyses were performed. It was established that diffuse desmoplasia and trichrome extent increased the probability of lymph node metastasis by 13.6 and 9.8, respectively.

Conclusion: The findings of this study revealed significant associations between increased desmoplasia and trichrome staining scores and the probability of lymph node metastasis. However, further prospective studies with larger cohorts and comprehensive molecular analysis to validate these preliminary findings.

Keywords: Desmoplasia; Lymph node metastasis; Papillary thyroid microcarcinoma

ÖZETÇE

Papiller Tiroid Mikrokarsinomunun Santral Lenf Nodu Metastazını Öngörmede Moleküler Belirteçler ve Klinikopatolojik Özelliklerin Rolü

Arka Plan: Papiller tiroid mikrokarsinomunun (PTMK) olumlu prognozuna rağmen, santral lenf nodu metastazının (SLNM) sık görülmesi (%60'a kadar), nüks ve sağkalımı etkileyen önemli bir sorun teşkil etmektedir. Preoperatif boyun ultrasonografisinin SLNM'yi tespit etmedeki sınırlı kullanımı, SLNM'nin etkili bir şekilde öngörülmesi için radyolojik, histopatolojik ve klinik faktörlerin araştırılmasını gerektirir. Bu çalışma, PTMK hastalarında moleküler belirteçlerin ve klinikopatolojik özelliklerin SLNM'yi öngörmedeki rolünü değerlendirmeyi amaçlamaktadır.

Yöntemler: Şubat 2019 ile Aralık 2023 tarihleri arasında Koç Üniversitesi Hastanesi veya Amerikan Hastanesi'nde tiroidektomi operasyonu geçiren hastalar arasından tümör boyutu ≤10 mm (PTMK) olan 145 hasta çalışmaya dahil edildi. Profilaktik veya terapötik santral boyun diseksiyonu olmayanlar ve eksize edilen lenf nodu sayısı 3'ün altında olanlar çalışma dışı bırakılmıştır. Hastalar SLNM'ye göre iki çalışma grubuna ayrılmıştır. Hastaların demografik özellikleri ve klinikopatolojik özellikleri, $BRAF^{V600E}$ mutasyonu, dezmoplazi ve Masson trikrom boyaması değerlendirilmiştir. Hem tek değişkenli hem de çok değişkenli lojistik regresyon analizleri yapılmıştır.

Bulgular: 65 katılımcının 35'inde (%53,8) SLNM görülmüştür. Tek değişkenli analizlerde tümör boyutlarının büyük olmasının, lenfovasküler invazyonun, agresif alt tip varlığının, $BRAF^{V600E}$ mutasyon pozitifliğinin, desmoplazi ve trikrom yayılım ve yoğunluk skorlarının yüksek olmasının lenf nodu metastazı varlığını öngörmede anlamlı değişkenler olduğu gösterilmiştir ($p<0,001$). SLNM varlığını tahmin etmek için çok değişkenli lojistik analizler yapılmıştır. Diffüz desmoplazi ve trikrom yayılımının lenf nodu metastazı olasılığını sırasıyla 13,6 ve 9,8 kat arttırdığı belirlenmiştir.

Sonuç: Bu çalışmanın bulguları, artan desmoplazi ve trikrom boyama skorları ile lenf nodu metastazı olasılığı arasında anlamlı ilişkiler olduğunu ortaya koymuştur. Ancak bu ön bulguları doğrulamak için daha büyük gruplarla ve kapsamlı moleküler analizlerle daha ileri prospektif çalışmalar yapılması gerekmektedir.

Anahtar Kelimeler: Desmoplazi; Lenf nodu metastazı; Papiller tiroit mikrokarsinomu

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ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ATA	American Thyroid Association
ATC	anaplastic thyroid carcinoma
beta HCG	beta human chorionic gonadotropin
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CEA	carcinoembryonic antigen
CLN	central lymph node
CLNM	central lymph node metastasis
CPTC	classical variant papillary thyroid cancer
CS	Cowden syndrome
CT	computed tomography
D1	type 1 deiodinase
D2	type 2 deiodinase
D3	type 3 deiodinase
DSV	diffuse sclerosing variant
DTC	differentiated thyroid cancer
DITs	diiodotyrosines
ECM	extracellular matrix
EORTC Cancer	European Organisation for Research and Treatment of
ETA	European Thyroid Association
ETE	extrathyroidal extensions
FAP	familial adenomatous polyposis
^{18}F-FDG	2-[18F]-fluoro-2-deoxy-d-glucose
FMGS	familial multinodular goiter syndrome
FNA	fine needle aspiration
FNAB	fine needle aspiration biopsy
FNAC	fine needle aspiration cytology

FNMTC	familial non-medullary thyroid cancer
fPTC	familial papillary thyroid cancer
FTC	follicular thyroid carcinoma
FVPTC	follicular variant papillary thyroid carcinoma
GBDT	gradient-boosting decision tree
HCC	hepatocellular carcinoma
H&E	hematoxylin and eosin
HSP	heat shock proteins
LATS	Latin American Thyroid Society
LLNM	lateral lymph node metastasis
LMF	leptomeningeal failure
LNM	lymph node metastasis
MEN2	multiple endocrine neoplasia type 2 syndrome
mETE	micro-extra-thyroidal extension
MITs	monoiodotyrosines
ML	machine learning
MRI	magnetic resonance imaging
MTC	medullary thyroid carcinoma
NIFTP	non-invasive follicular thyroid neoplasms with papillarylike nuclear features
NIS	sodium/iodine symporters
NSCLC	non-small cell lung carcinoma
OS	overall survival
PDAC	pancreatic ductal adenocarcinoma
PET	positron emission tomography
PHTS	PTEN-hamartoma tumor syndrome
PI3K-AKT	phosphoinositide 3-kinase-Akt serine-threonine kinase
PRN	papillary renal neoplasia
PTC	papillary thyroid carcinoma
PTMC	papillary thyroid microcarcinoma
RAI	radioactive iodine
RFA	radiofrequency ablation

T3	triiodothyronine
T4	thyroxine
TBG	thyroxine-binding globulin
TBPA	thyroxine-binding prealbumin
TCV	tall cell variant
Tg	thyroglobulin
TPO	thyroperoxidase
TR	thyroid receptors
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
UICC	Union for International Cancer Control
US	ultrasound

Chapter 1

INTRODUCTION

1. Thyroid

1.1. Thyroid Gland Anatomy

The thyroid gland extends from the fifth cervical vertebrae to the first thoracic vertebrae. The gland is divided into two parts - the right and left lobe. They are connected at the midpoint by the isthmus. A pyramidal lobe may be present in up to 50% of the patients. This lobe can extend upwards from the isthmus or project from the midline sections of either the left or right lobes. Both thyroid lobes, are typically around 4 cm in length, extend into 2 cm in width and 2-3 cm in depth. Hence normally each gland weighs between 15–25 g in adults. However, it's important to note that these dimensions can vary significantly depending on gender, iodine intake, and the presence of thyroid diseases (1, 3).

To understand the anatomically complex arrangement of structures within the neck, the neck can be broken down into seven levels, each identified with important landmarks. The thyroid gland is located within Level VI. In some thyroid pathologies such as in medullary thyroid cancer, clearing the lymph nodes that drain the thyroid from various neck levels is necessary (4).

The thyroid is surrounded by a layer of pretracheal fascia, also referred to as the perithyroid sheath. On the posterior aspect of this fascia there is a thickening which enables the connection of the thyroid gland to the cricoid cartilage. This thickening of the fascia is known as the ligament of Berry, also referred to as the lateral ligament of the thyroid (1).

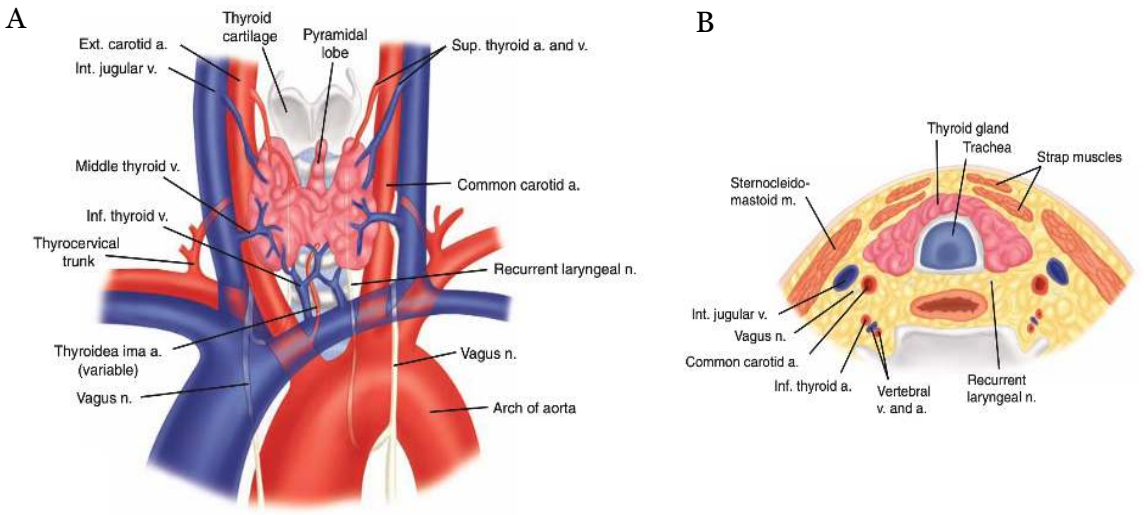

Figure 1. *The anatomy of the thyroid gland and surrounding structures (A) and cross-section (B). Ref. (2)*

The thyroid gland in adults has a firm solid texture and brown color. In their anatomical position, the thyroid lobes extend superiorly to the midthyroid cartilage and laterally are neighbor the carotid sheaths and sternocleidomastoid muscles. The gland is anteriorly related to the sternohyoid, sternothyroid, and the superior belly of the omohyoid, the strap muscles (2).

The upper parts of the thyroid gland medially neighbor the larynx and laryngopharynx, as well as the cricoid and thyroid cartilage. The inferior pharyngeal constrictor muscles are also related to this portion of the gland. The inferior part of the thyroid gland medially neighbors the trachea and esophagus. Laterally, it relates to the carotid sheath, which contains vital structures such as the common carotid artery, internal jugular vein, and vagus nerve. The superior portions of the thyroid gland, posteriorly, are related with the longus colli and longus capitis muscles. The isthmus, on the other hand, allocates itself anteriorly to the second and third tracheal rings (1).

1.1.1. Blood Supply

The thyroid gland is extremely vascular with a rich network of anastomoses. The gland receives dual blood supply by the superior thyroid artery and the inferior thyroid artery. Superior thyroid artery is the first branch of the external carotid artery, and it branches off just below the hyoid bone. However, in some individuals

it can show variations such that it emerges from the inferior part of the common carotid artery. The inferior thyroid is the third branch of the thyrocervical trunk of the subclavian system. Extending from the brachiocephalic artery, a singular thyrocervical ima artery may be present in some individuals (Figure 2 and Figure 3).

This superior thyroid artery travels together with the external laryngeal nerve to the upper pole of the thyroid. In the gland the artery divides into anterior and posterior branches. The anterior branch travels along the medial edge of the lobe and anastomoses in the middle with the anterior branch of the superior thyroid artery from the opposite side.

The inferior thyroid artery has a looping course. The artery ascends along the anteior scalene muscle and turns medially, passing posteriorly the carotid sheath and the sympathetic trunk. The artery continues to travel downwards until it reaches the lower pole of the thyroid. Once it reaches the gland it allocates itself either anteriorly or posteriorly to the recurrent laryngeal nerve. This necessitates the precise identification of the two structures before ligating the inferior thyroid artery. A thyroidea ima artery can be seen in 1-4% of the individuals. It usually arises from the aorta and enters the isthmus. In some cases, in which an inferior thyroid artery is missing, this artery can replace it.

Upon entering the thyroid, the inferior thyroid artery branches into superior and inferior branches. The superior branch climbs upwards along the posterior part of the gland and forms connections with the posterior branch of the superior thyroid artery. The inferior branch, on the other hand, supplies the lower portion of the thyroid gland, as well as the inferior parathyroid glands (1, 2).

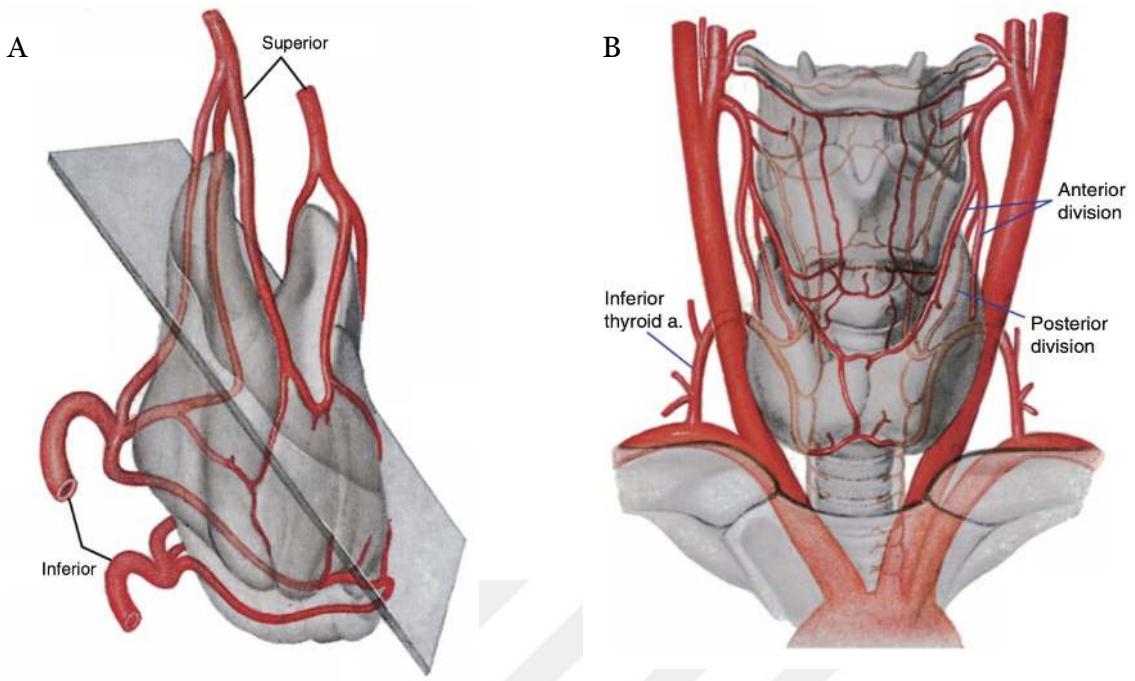

Figure 2*. Arterial supply of thyroid gland, including a scheme of the watershed areas of the superior and inferior arterial system (A). Arterial supply of the thyroid gland is derived from four vessels (B). Ref. (1)*

1.1.2. Venous Drainage

The venous drainage of the thyroid gland is achieved mainly by three sets of veins — the superior, middle, and inferior thyroid veins. These veins run alongside their arterial counterparts. On both sides, the superior thyroid veins travel alongside the superior thyroid arteries. The middle vein in some cases might be absent. If present in some individuals, they might cross the recurrent laryngeal nerve and the common carotid arteries. The superior and middle veins usually end their journey by draining directly into the internal jugular veins. The inferior vein is the one that is constantly present however tends to show the greatest variation. It typically drains into the brachiocephalic veins. It is also important to note that this vein is related to the anterior side of the trachea, which calls for attention for the surgical team (1, 5) .

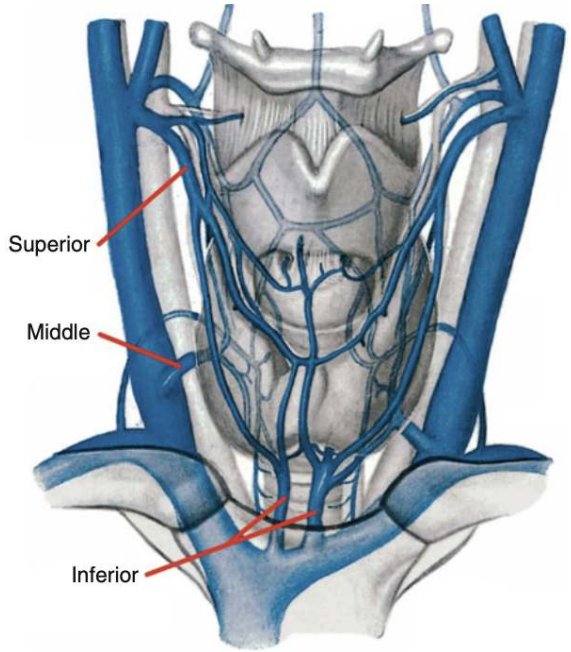

***Figure 3**. Venous drainage of the thyroid gland. Superior, medial, and inferior veins are indicated. Ref. (1)*

1.1.3. Lymphatic system

The thyroid gland is embedded in an abundant network of lymphatics. The Lymphatic system closely resembles the arterial supply. Lymphatic vessels either ascend alongside the superior thyroid artery or descend alongisde the inferior thyroid artery, both ultimately draining in the jugular chain of nodes. Medially, the lymphatics vessels climb upwards to the digastric nodes and descend to the pretracheal and brachiocephalic nodes. The lymph nodes draining the thyroid gland can be compartmentalized into several levels (Fig. 5). Metastasis of the thyroid cancer can be seen in any of the draining, lymph nodes. Nevertheless, it is important to note that metastasis to the submaxillary area is rare, accounting for less than 1%. Moreover, “skip” metastasis can be observed, which are defined by metastasis to the nodes in the lateral ipsilateral nodes while skipping the central nodes (1, 2) .

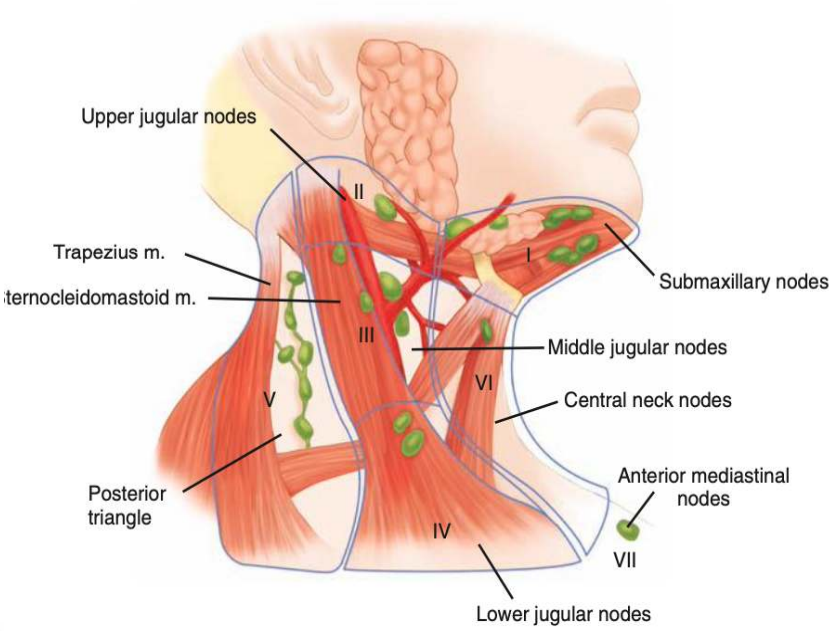

***Figure 4**. Lymph nodes in the neck are divided into six levels. Ref. (2)*

1.1.4. Nerves

The laryngeal innervation has a very close connection with the thyroid gland. There are two laryngeal nerves which are closely neighboring the thyroid gland, they are the external branch of the superior laryngeal nerve and the recurrent laryngeal nerve. It is vital to note that a mastery of the anatomical location and the course of these nerves is very important to ensure a successful thyroid surgery.

External Branch of the Superior Laryngeal Nerve

External laryngeal nerve is a branch of the superior laryngeal nerve, a branch of the vagus nerve. The external laryngeal nerve plays the important function of innervating the cricothyroid muscles and ensuring adequate vocal apparatus movements. Hence, any damage to this nerve can disrupt normal phonation. There is a risk of entrapment of the external laryngeal nerve with the superior thyroid vessels as it passes very close to the superior pole of the thyroid. As a consequence, due to the close location of the artery and nerve to one another, there is a risk of nerve injury during the ligation of these vessels (6). Recent evidence indicates that this nerve also plays a role in the proper functioning of the glottic closing force. This further highlights the importance of the mastery of the anatomic location and course of this nerve to minimize any complication (7).

Recurrent Laryngeal Nerve

The recurrent laryngeal nerve is also a branch of the vagus nerve. Its functions are to supply all of the intrinsic laryngeal muscles except the cricothyroid muscle. This nerve sends sensory fibers and provides sensory innervation to the region inferior to the glottis. On the right side, the nerve loops behind the subclavian artery and on the left side it loops behind the arch of the aorta. On the right, it climbs upwards until the tracheoesophageal groove. The left branch after looping behind the arch of the aorta ascends towards the larynx through the tracheoesophageal groove (1).

The recurrent laryngeal nerve lies very close to the inferior thyroid artery, making it essential to accurately localize these two structures for successful thyroid surgery. Research suggests that there is high variability when it comes to the relative location of the inferior thyroid artery to the recurrent laryngeal nerve, however the most common location of recurrent laryngeal is thought to be– posterior to the inferior thyroid artery (8).

In addition to this, the recurrent laryngeal nerve may be closely neighboring with or be allocated within the ligament of Berry. However, it is vital to note that, there is evidence suggesting that branching of the recurrent laryngeal nerve is very common. This seems to increase the possibility of entrapment of the nerve at the region of ligament of Berry (9).

It is very important to identify the nerve and to get familiar with its anatomical variations. Damage to the recurrent laryngeal nerve as a thyroid surgery complication has been reported as 1-2 % in the hands of an experienced surgeon. However, when risk factors such as thyroid carcinoma, re-operation, and failure to identify the nerve, are present, the incidence seems to increase. Hence a mastery of the anatomy of the course, function and location of the recurrent laryngeal nerve is of vital importance (10).

Landmarks of the Laryngeal Nerves

The accepted landmarks for the identification of the external laryngeal nerve are Joll's triangle and Reeve's Space. For the recurrent laryngeal nerve there are more landmarks including Berry's ligament, Cricothyroid joint, Lore's triangle, and Nodule of Zuckerkandl (11).

1.2. Thyroid Gland Physiology

Follicle is the basic functional unit of the thyroid. The adult thyroid gland typically consists of around 3 million follicles and clusters of 20-40 follicles form lobules. The follicles have a spherical shape with an average dimension of 30 μm in diameter. Each follicle is lined by cuboidal epithelial cells, often referred to as thyrocytes or follicular cells. They form the frame of a central reservoir of colloid. This colloid is secreted by the epithelial cells stimulated by the pituitary hormone - Thyroid Stimulating Hormone (TSH). Colloid contains thyroglobulin (Tg), a large glycoportein and the primary storage form of thyroid hormone. Another group of cells which are important in the secretion of thyroid hormones, are the C cells, also known as parafollicular cells. The function of these cells is to contain and release the hormone calcitonin. They are located either individually or in small clusters within the interfollicular stroma (12).

TSH binds to its specific receptor on thyrocytes, the TSH receptor (TSHR). These receptors are located on the basolateral membrane of the cells. This receptor is a 7-transmembrane G-coupled protein. This protein is studied for its structure and role in the development of various thyroid pathologies. Stimulation of the TSHR (Thyroid-Stimulating Hormone Receptor) plays a central role in the development of Graves' disease, the primary cause of hyperthyroidism. It is one of the diseases of thyroid autoimmunity and is characterized by hyperthyroidism and diffuse goiter. TSH receptor antibodies bind to TSHR leading to overstimulation (13).

Once activated, TSHR leads to intracellular effects primarily mediated by the stimulation of adenylyl cyclase, which results in an increase in intracellular cyclic adenosine monophosphate (cAMP) concentrations. TSH stimulates thyrocytes to enhance uptake and produce thyroid hormone. TSH levels play a vital role in the differentiation of the thyroid diseases. As observed in overt primary hypothyroidism, TSH levels are increased in combination with lower than baseline levels of free thyroxine. Subclinical hypothyroidisms, on the other hand, is marked by elevated TSH and free thyroxine levels within the reference range (14).

There are two main types of thyroid hormones: thyroxine (T4, carrying 4 iodine atoms) and triiodothyronine (T3, carrying 3 iodine atoms). Normally, about 90% of the synthesized thyroid hormones is in the form of T4, and approximately 10% is T3. Iodine is central to the function of the thyroid gland. Iodine is

concentrated within the thyrocytes against a concentration gradient which means that a complicated molecular mechanism is necessary in order to facilitate this synthesis (15).

This complex molecular mechanism is orchestrated by an energy-consuming process, with the sodium/iodine symporters (NIS). After the binding of TSH to its receptor and the activation of cAMP, a cascade of several steps occurs in the pathway of thyroid hormone synthesis. The initial step in the synthesis of the thyroid hormones, involves the transport of iodide across the basal membrane through the NIS. The enzyme thyroperoxidase (TPO) together with hydrogen peroxidase, covalently attaches iodine to thyroglobulin, the precursor of thyroid hormone.

TPO is a protein which has a selenium incorporated into its tertiary structure, hence it is known as selenoprotein. It is well established that thyroid gland has a mechanism for detecting iodine excess and iodine scarcity and feedback loops are used to ensure homeostasis. Excessive concentrations of iodine are known to block TPO, through iodopeptides that inhibit TPO mRNA and protein synthesis, subsequently limiting the thyroglobulin iodination. This is known as Wolff-Chaikoff Effect. This can manifest itself as clinical hypothyroidism (16).

Within the thyroglobulin the iodine gets attached to form monoiodotyrosines (MITs) and diiodotyrosines (DITs). TPO further couples two iodotyrosines to produce the bioactive thyroid hormone, T4 and T3. T3 and T4 remain as parts of the thyroglobulin and get stored in the colloid. This underlines the unique ability of the gland to store thyroid hormone in vast quantities as colloid and releasing it as needed upon stimulation by TSH. When stimulated by TSH colloid uptake into the cytoplasm is accomplished through pinocytosis, by forming a cytoplasmic vesicle. These vesicles fuse with lysosomes packed with proteases which hydrolyze the peptide bonds of thyroglobulin, liberating T4 and T3 into the cytoplasm. Following this, the hormones enter the bloodstream (17).

Once released into the bloodstream, only a fraction of the T3 and T4 remain free or in unbound form. There are several major thyroid hormone binding proteins such as thyroxine-binding globulin (TBG or thyropexin), transthyretin, also known as thyroxine-binding prealbumin (TBPA) and human serum albumin (18).

T4 is a prohormone meaning that it needs to be converted to its active form to exerts it actions. The active form is T3. This conversion happens through the

action of selenoproteins called deiodinases. Type 1 deiodinase (D1) and type 2 deiodinase (D2) have the function of activating the thyroid hormone, in other words converting T4 into T3. Type 3 deiodinases (D3), on the other hand, inactivates both T4 and T3 (19).

D2 is the primary activator of the thyroid hormone, converting T4 to T3, the active form. D2 is considerably more efficient in activating the thyroid hormone compared to D1. Hence the primary regulator of intracellular T3 concentration, controlling the availability of T3 to the nuclear and saturation of T3 receptors in target tissues, is D2. Acting together D2 and D3 regulate the availability of T3 to the target tissues regardless of serum thyroid hormone concentrations (20).

Thyroid hormone exerts it their action by binding to specific nuclear receptors known as thyroid receptors (TR). They are transcriptionally active proteins which result in expression of genes responsive to the thyroid hormone. Two main types of thyroid receptors have been reported TRa and TRb. Isoforms of these receptors are also reported, which seem to have tissue specific functions (15).

1.3. Thyroid Nodules

Thyroid nodules are defined as lesions within the thyroid gland that exhibit radiological differences from the surrounding thyroid parenchyma (21). Thyroid nodules can be singular or multiple, also referred to as multinodular gland or cystic lesions. With an increase of the median age of industrialized segments of the population, the incidence of thyroid nodule also increases. Moreover, with the advancement of various technological methods, management and detection of thyroid nodules has also increased considerably (22). Computer assisted tomography and magnetic resonance imaging of the head, neck, and spine, frequently result in the incidental detection of thyroid nodules. Moreover, including thyroid ultrasound in a regular diagnostic workup contributes to the increased numbers of detected thyroid nodules (23, 24).

1.3.1. Thyroid Nodule Histology

Most thyroid nodules originate from follicular cells. They can be classified into benign, low risk, or malignant processes. Benign thyroid nodules can include follicular adenomas, hyperplastic nodules and oncocytic, also known as Hürthle

cell, adenomas. Hyperplastic nodules are typically without capsules. Their architecture is mixed macrofollicular, normofollicular, and microfollicular. They are formed by follicular cells without known nuclear features of papillary thyroid carcinoma (PTC). Follicular adenomas, on the other hand, are encapsulated and most typically have a microfollicular architecture. These are known as clonal neoplasms without any vascular or capsular invasions and without any nuclear features of PTC. It is vital to perform a histological examination of the capsule to differentiate between follicular adenoma or carcinoma. The same principle applies to the oncocytic adenomas. Oncocytic adenomas, contrary to other benign nodules, are comprised of more than 75% oncocytes. These cells have a cytoplasm, rich with eosinophilic granules and lack nuclear features of PTC (25).

The low-risk category of benign thyroid nodules consists of non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP), thyroid tumors of uncertain malignant potential, and hyalinizing trabecular tumor. Due to the metastasis rate of these tumors being very low, the current management of resection alone is sufficient. NIFTP histologically is defined by encapsulation, having a follicular architecture and having nuclear features of PTC. This enables NIFTP to be differentiated from follicular adenoma. Nevertheless, invasion, a great solid growth pattern, or high-grade features such as mitotic activities, should not be present. Tumors of uncertain malignant potential are encapsulated, have a follicular architecture and demonstrate a point of questionable invasion to either the capsule or vascular structures. Hyalinizing trabecular tumor, on the other hand, is one of the rare thyroid neoplasms. It is characterized by well circumscribed architecture and consists of cells which have nuclear changes similar to those of PTC. The cells are arranged in trabecular structures and have hyaline material in between the trabecula (25).

1.4. Epidemiology of Thyroid Nodules

The prevalence of thyroid nodules within the population has been a research topic for a long time. Evidence has suggested that the prevalence can range between 13-46% within the general population (21, 26). Some pooled analysis data suggests that discrepancies between countries can also be observed. Prevalence of thyroid nodules in Hungary, Cyprus, and China are 4.75%, 46.72%, and 30.23%,

respectively (26). There is also evidence suggesting that developing countries have a slightly higher prevalence of nodules compared to developed countries, however without significant difference. Nevertheless, this discrepancy could be explained by the sensitivity of the imaging techniques used and the number of studies that have focused on this matter (21). Using a more sensitive imaging yields a higher incidence of thyroid nodules. Recent data from one such study using a 13 MHz transducer showed that the prevalence of thyroid nodules within the general population can be up to 68% (27).

Prevalence of thyroid nodules can show differences from one geographic location to another one even within the same country. One study aiming to compare thyroid disease from northern and southern Germany has shown that there is a greater prevalence of thyroid nodules in the southern part compared to northern, 59.3% and 36,3%, respectively. One possible explanation for these discrepancies is current and historical differences in iodine uptake (28). As mentioned above increased used of diagnostic techniques is another major contributor in the incidental detection of thyroid nodules. The majority out of these asymptomatic thyroid nodules are shown to be singular and within a follow up of 5 years, vast majority indicate no change in size following initial detection (29).

The prevalence of thyroid nodules has been shown to increase with age. Evidence suggests that that annual increase in the number of thyroid nodules can be up to 1.6%. However, similar data seems to suggest that malignancy rate is declining with age (30). Females in every age group have been shown to have a higher prevalence of thyroid nodules. Amongst females the nodules seem to be larger in size. Increased BMI has also been associated with an increased incidence of thyroid nodules (28, 31).

Between 7-15% of thyroid nodules are thought to have malignancy (32, 33). However, there is also evidence suggesting a much lower rate of malignancy, notably around 1.1% (34). Nevertheless, current guidelines highlight that upon detection of thyroid nodules further assessments are necessitated due to malignancy concerns.

1.5. Screening of Thyroid Nodules

According to current guidelines, once a thyroid nodule is identified a thorough

history, physical examination should be performed followed by evaluating the TSH levels and performing a neck ultrasound (US). US risk stratification should be performed, analyzing the size, surrounding lymph node, consistency, and borders. Thyroid nodules with size larger than or equal to 5 mm and with ultrasound features suggesting thyroid cancer should be nodules deemed assessed by fine needle aspiration (FNA). Thyroid nodules with a size larger than 10-15mm or size larger than or equal to 20mm with a cystic solid consistence should be assessed with FNA independent of US risk stratification (35).

2. Thyroid Cancer

2.1. Epidemiology of Thyroid Cancer

Thyroid cancer is the most common endocrine malignancy with its incidence rising steadily over the past four decades. Thyroid cancer can occur across various age groups, with the median age of diagnosis in the early 50s. Nevertheless, it remains the most common malignancy among individuals aged 16 to 33.(36). This rise in incidence was initially noted in high-income countries and is currently also seen in middle-income countries. It is thought that a major contributor to the increased thyroid cancer incidence is overdiagnosis. In an up-to-date global analysis for the year 2020, incidence rate, as well as the global increase in thyroid cancer, was five times higher in countries which have scored high or very high in the human developmental index (37).

For instance, in South Korea, the incidence of thyroid cancer has increased by 25% annually over the past 10 years. Between the years 1996 and 2010, thyroid cancer per 100,000 people saw a dramatic increase from just 10.6 to 111.3 in females. In males an increase from 1.9 to 27 was noted. (24) A similar pattern has been observed in data from the US – a large analysis of thyroid cancer trends suggests that there is a threefold increase in the incidence of thyroid cancer between the years 1975 and 2009: from 4.9 to 14.3 cases per 100,000 people. Moreover, this thorough analysis has highlighted that virtually all the increased diagnosis of thyroid cancer can be attributed to an increased diagnosis of PTC (38).

Hence one of the main contributors to the increased incidence of thyroid cancer seems to be the rapid increase in the detection of PTC. The highest increase

in PTC incidence has been detected in high income countries, closely followed by middle-income countries. PTC has displayed an opposite age-specific trend to anaplastic thyroid cancer. While anaplastic thyroid cancer incidence increases with age, PTC incidence peaks in middle age. Researchers suggests that an explanation for this is the increased use of sensitive imaging techniques in middle-aged individuals (39). However, it is vital to note that this increased incidence of thyroid cancer is not correlated to an increase in mortality; mortality rates have remained stable (24, 37, 40). It is also important to note that alongside stable mortality rates, evidence suggests that the absolute number of large tumors has also not changed, while the detection of microcarcinomas is responsible for the increase in new thyroid cancer diagnoses (41).

Thyroid cancer exhibits female dominancy, incidence showing that it occurs three times more in women than in men (37, 42). However, following research on autopsies, the prevalence of thyroid cancer seems to be equal between the two genders. Hence it is suggested that healthcare utilization discrepancies between the genders could be a potential explanation for this phenomenon (42).

2.2. Tumor Classification

"No classification is more difficult to establish than that of thyroid carcinomas, of all cancers they teach, perhaps, the greatest lessons of humility to histopathologists."

- **From 'Human Tumors' by Pierre Masson**

Thyroid cancers can be classified into three main histological categories: differentiated thyroid cancer (DTC) including papillary, oncocytic, follicular thyroid carcinoma, and Hürthle cell cancer, medullary thyroid carcinoma (MTC), which can be observed in multiple endocrine neoplasia type 2 syndrome (MEN 2), and anaplastic thyroid cancer, which arises from DTC and has a high mortality rate (36). Out of all the diagnosed thyroid carcinomas about 90% are PTC, 4 % are follicular thyroid carcinomas (FTC), 2% are Hürthle cell carcinomas, 2% are MTC, and 1% is anaplastic thyroid carcinoma (ATC) (43).

2.3. Differentiated Thyroid *Carcinoma*

Histologically, DTC are classified into 4 main subtypes: PTC which is the most prevalent thyroid cancer type, FTC, which is further divided into three variants that have increasing aggressiveness – minimally invasive with capsular invasion only, encapsulated with angioinvasion, and widely invasive, Hürthle cell cancer, and poorly differentiated thyroid cancer (44).

2.3.1. Etiology of DTC

DTC have been characterized by some genomic alterations. After an analysis of 496 tissue samples of PTC, it has been shown that a driver alteration is found in 96.5% of these tumors (45). $BRAF^{V600E}$ is the most frequently found point alteration in PTC, so frequent that it can be found in 45-60% of the patients. The other common mutations, respectively, are RAS and TERT promoter point mutation. Additionally, after an analysis of 125 PTC samples, it was found that RET, NTRK1, NTRK2, NTRK3, BRAF, and ALK gene fusions were involved in 15% of the patients' samples. In 15% of the sample's gene fusions involving RET, NTRK1, NTRK2, NTRK3, BRAF, and ALK were found. The most common mutations associated with FTC, are RAS mutations, which are found in 25% of the patients, followed by alterations in EIF1AX and PTEN point mutations (46). Hürthle cell cancer, on the other hand, has a different spectrum of mutations mainly involving complex I mitochondrial DNA mutations. Lastly, in poorly differentiated thyroid cancers, a greater density of mutations is seen with the most frequent point mutations being in BRAF and RAS (44).

2.3.2. Medullary and Anaplastic Thyroid Cancer

When it comes to the genetic variability of MTC it is vital to note that, this cancer can be seen as a part of MEN2 syndrome, both MEN2A and MEN2B, which are defined by mutations in RET proto-oncogene on chromosome 10q11.2. Knowing specific RET mutations allows clinicians to calculate the risk groups of aggressiveness which is stratified into three levels. MEN2B typically presents as the most aggressive one, level III, with invasive carcinoma and lymph node metastasis (LNM) seen during the first years of life. MEN2A, on the hand, has a

more variable course which depends on codon mutations (47).

ATC etiology and the influence of genetic factors are yet to be recognized, however, there are some theories suggesting that ATC might be developing from DTC as a result of acquiring more mutations. The evidence for this is the frequent co-morbidity of ATC with another thyroid disease or surgical history of DTC. However, this is a phenomenon that is yet to be established. On a molecular level, very frequently mutations or overstimulation of p53 gene are detected in ATC (48).

2.3.3. Prognosis of Thyroid Cancers

The prognosis of thyroid cancer is generally very well as most thyroid cancers are PTC and are localized within the gland at diagnosis. Data from U.S. indicates that the 5-year survival rate is about 98.6-99.9% for localized and 98.35% for regionally spread carcinomas. The survival rate for distant metastatic disease is around 54.9% (43). Survival rates are highest for PTC and FTC compared to the other histological types, owing to the present effective therapies and the slow-growing nature of these tumors.

The prognosis of PTC is nearly excellent with a 5-year survival rate between 98.6 – 99.9% for localized cancers (43). Nevertheless, assessing for LMN is vital to determine the prognosis and surgical treatment strategy. Although the prevalence of lymph node metastasis in PTC can show great variations, a meta-analysis from 2020 showed that out of 9369 PTC cases 3482 out of them had a lymph node metastasis, accounting for 37.17% out of all the cases (49). Additionally, the same meta-analysis highlighted that the risk factors for lymph node metastasis were male gender, age of patients <45 years, tumor size being greater than 1 cm, presence of capsular invasion. Analysis of the data from Surveillance, Epidemiology, and End Results cancer registry showed that PTC lymph node metastasis was also shown to be correlated with younger age with lymph node metastasis rates being inversely associated with age at diagnosis (50).

MTC, on the other hand, has a less favorable prognosis than DTC. The 5-year survival rate is estimated to be 80-97% and the 1- year survival rate is between 75-88%. However, recurrence can occur in up to 50% of cases and LNM are already present in 30-60% of the patient upon diagnosis. There are two important biomarkers which can indicate the course of the disease – basal serum calcitonin

and carcinoembryonic antigen (CEA) doubling times. When these levels are considered, a patient can be classified into stable or progressive course. Histologically, poorly differentiated tumor histology is correlated by a more rapid progression. Moreover, high expression of Ki-67 has been shown to result in less favorable prognosis (51).

ATC, on the other hand, is a lethal carcinoma with the medial survival ranging from 2 to 12 months. Between the years 1973 and 2012, ATC was responsible for only 1.3% of thyroid cancers diagnoses out of 77,276 total diagnoses, however, it accounted for 19,9% of deaths due to thyroid cancer out of total death total 2,371 deaths in the US (52). It is aggressively growing and expanding; hence, long-term survival or cure is possible mostly in young patients with resectable tumors without any distant metastases at presentation. Favorable prognostic factors for ATC are young age, small tumor size (<5 cm), resectable tumor, and focal anaplastic transformation in DTC. A prognostic index containing four elements was developed for ATC, including the acute onset of symptoms, tumor diameter >5cm, white blood cell count >10,000/mm^3, and distant metastases at presentation. For patients with a high prognostic index, palliative therapy is initiated and for low prognostic index, a multimodal therapy including surgery, chemotherapy, and radiotherapy is initiated (48).

2.4. Diagnostic Methods

2.4.1. Ultrasound Imaging

Ultrasound (US) has been the primary diagnostic tool for the thyroid gland. It is easy to use, cost effective, and provides rapid information without exposing the patient to radiation. The anatomical location of the thyroid gland is very suitable for the US evaluation as the gland is located in a superficial position providing easy access to imaging. Moreover, thyroid gland has a distinctive echotexture and different disorders manifest with characteristic sonographic features. Thyroid US can provide more detailed anatomic features than magnetic resonance imaging (MRI), Computed tomography (CT) or radionuclide studies. With the recent technological advancements, even small ultrasound machines can be employed for valuable thyroid imaging (53). Thus, US is the primary imaging modality to assess thyroid nodules. Nevertheless, the expertise of the examiner plays an important role

in evaluating imaging findings (53).

Ultrasound Characteristics of Thyroid Nodules

There are several ultrasound features that are associated with a risk of malignancy, hence careful investigation is necessary. Evidence suggests that adopting two instead of one abnormal characteristic on nodules greater than 5 mm to warrant biopsy would result in lower false positive rates and reduce unnecessary biopsies by 90% (54). There are several ultrasonographic findings that play a critical role in predicting malignancy and indicate the need for further investigation. The most important ones out these findings are hypoechogenicity, presence of irregular margins, presence of microcalcifications, consistency, multinodularity, and vascularity of the nodule (55). Moreover, the same study suggests that three ultrasound characteristics showed a statistically significant association with thyroid cancer risk and should prompt biopsy investigations. These findings are the presence of micro-calcification, size greater than or equal to 2 cm, and solid composition (54). US is also a valuable tool for assessing for LNM. Out of all US characteristics, microcalcification and irregular shape are regarded as meaningful features. It is also suggested that radiomic analysis can further aid in more specific LNM diagnosis (56).

2.4.2. Fine Needle Aspiration of the Thyroid

Fine needle aspiration biopsy (FNAB) plays an integral part in the management of thyroid nodules. Its purpose is to evaluate surgical suitability, determine appropriate surgical plans, distinguish between malignant and inflammatory diseases. With the inclusion of FNAB into guidelines, the number of patients requiring surgery has declined by 50%, thereby lowering costs associated with the management of thyroid disease, and the rate of detecting the malignancies has increased (57). FNAB is commonly performed using a thin needle with sizes from 22 to 27 – gauge. This technique involves aspiration to obtain a tissue specimen and no histological fixation is needed as seen in percutaneous large-needle biopsies. Aspiration biopsy offers cytological examination of the specimen. US-guided FNAB has been shown to increase the sensitivity and specificity of the FNAB procedure (57).

Fine needle aspiration cytology (FNAC) of the thyroid gland has been

shown to be highly accurate with a small chance of false-negative and false-positive diagnoses. One study suggests that the rate of false-negative findings is less than <1% in an analysis of 6226 FNA samples, in addition to this, the same study suggests a rate of 2% for false-positive findings after an analysis of the same data set (58). Evidence suggests that the sensitivity and specificity of FNAC in diagnosis ranges from 65% to 98%. One explanation for this wide range could be explained by variabilities in pathologist approach to suspicious tissue samples, and disparities in definitions of false positive and false negatives (58, 59).

2.4.3. Computed Tomography

CT imaging of the head and the neck is often used to detect masses and nodal metastasis. CT is not operator-dependent contrary to US and produces a very detailed image. CT is the test of choice when thyroid cancer invasion to the laryngeal and tracheal cartilage is suspected (60). Position emission tomography (PET)/CT, CT, and US have comparable abilities for detection of positive lymph nodes pre-operatively (61). However, researchers remain divided on the question of which imaging technique, US or CT, can better detect positive lymph node as some evidence has been shown that combining CT with US is superior to using these imaging techniques alone (62). It is important to note the downside of CT imaging is that when CT with iodinated contrast is done, patients will not be able to receive their post-operative radioactive iodine (RAI) treatment for nearly 2 months. Typically, radioactive iodide is given 6-8 weeks post-thyroidectomy, hence the delay due to CT imaging might not be significant (60).

2.4.4. PET/CT

PET/CT is a vital imaging technique for patients diagnosed with cancer. An important marker showing the metabolism rate in cells is 2-[18F]-fluoro-2-deoxy-d-glucose (^{18}F-FDG). In poorly differentiated cells, there is an over-expression of regulatory glycolytic enzyme and transporters (63). However, in the most recent (2015) American Thyroid Association (ATA) guidelines, the utility of ^{18}F-FDG PET/CT in the evaluation of thyroid nodules scan is not recommended. Moreover, this imaging technique is also not recommended for routine pre-operative assessments (64). Nevertheless, the real utility of ^{18}F-FDG PET/CT lies in the

follow up of high-risk patients with elevated serum Tg and negative ^{131}I imaging. This imaging is also highly recommended to assess the response of patients to the given therapy, particularly for the detection of lesions in metastatic patients, which can enable accurate predication of outcomes or prognoses in high-risk patients (63).

3. Papillary Thyroid Cancer

3.1. Histological Subtypes of Papillary Thyroid Cancer

PTC are classified into various histological subtypes based on the characteristic histopathological features (including growth pattern, cell type, nuclear or stromal changes) they exhibit (65, 66). Some of the most commonly assessed histopathological features are papillary and follicular growth patterns, presence of a capsule, invasion characteristics (capsular or vascular), and nuclear morphology (67). PTC subtypes include but are not limited to classic (or encapsulated classic), follicular (including infiltrative follicular), macrofollicular, tall cell, diffuse sclerosing, solid, oncocytic, warthin-like, columnar cell, clear cell, spindle cell, cribriform morular, hobnail, and PTC with fibromatosis variants. However, a wide range of histopathological features can be observed for each subtype and therefore, the variability of pathological evaluation can lead to differences in subtype interpretation by clinicians (65).

3.1.1. Classical Variant Papillary Thyroid Carcinoma (CPTC)

The classic variant PTC (CPTC) is the most commonly diagnosed subtype of PTC, comprising 55-65% of PTCs (68). CPTC often have distinctive nuclear features including ground glass appearing nuclei, chromatin clearing with peripheral margination of chromatin (Orphan Annie nuclei), nuclear grooves, intranuclear cytoplasmic pseudoinclusions, irregular nuclear contour, and enlarged and elongated nuclei (69). In addition, complex papillae, neoplastic lesions with fibrovascular core components, dystrophic calcifications, psammoma bodies can be observed in CPTC.

3.1.2. Follicular Variant Papillary Thyroid Carcinoma (FVPTC)

It has been estimated that the global yearly incidence of follicular variant papillary thyroid carcinoma (FVPTC) is 45,000 and that FVPTC represents 21-45% of PTC (67, 68). Characteristic features of PVPTC include a multitude of follicles surrounded or interspersed by cells resembling histological features of papillary carcinoma (70). In addition to aforementioned histological characteristics, FVPTC assessment relies on the following: (i) the presence or lack of capsule, (ii) capsular or vascular invasion, and (iii) diffuse versus multifocal tumor. Various degrees of intratumoral fibrosis, peripheral scalloping, and multinucleated giant cells within follicular cells can also be observed in FVPTC (65). Immunohistochemical staining can be employed to differentiate FVPTC from follicular adenomas and carcinomas. Encapsulated and noninvasive FVPTC have recently been termed noninvasive follicular thyroid neoplasm with papillary-like nuclear features following the discovery that the variant behaved more similar to follicular thyroid adenomas and carcinomas than PTC (71). Molecular studies have also supported these findings, demonstrating a lack of $BRAF^{V600E}$ mutations which are commonly associated with aggressive PTC (72). Thus, treatment strategies for FVPTC have been largely debated. It has also been argued that FVPTC is often overtreated despite having a low risk of metastasis to lymph nodes or distant sites (67). However, encapsulated FVPTC with high mitotic rate and tumor necrosis have been associated with aggressive progression (72).

3.1.3. Tall Cell Variant (TCV)

Approximately 10% of PTC are classified under the tall cell variant (TCV) which is characterized by cells that are twice as tall as they are wide (66). TCV has been associated with aggressive features including old age at diagnosis, high mitotic rate, necrotic components, extrathyroidal extensions (ETE), and high metastatic potential. It has been demonstrated that TCV without ETE displays a higher rate of vascular and capsular invasion and LNM compared to CPTC (73). TCV has also been associated with increased stage, higher grade, a lower 10-year survival rate (79%), and a lower 10-year event-free survival rate (67%) compared to CPTC (74).

3.1.4. Diffuse Sclerosing Variant (DSV)

Comprising approximately 3% of all PTC, diffuse sclerosing variant (DSV) is characterized histologically by squamous metaplasia, presence of psammoma bodies, extensive calcification, and extensive fibrosis (66, 75). Lymphocytic infiltration and germinal center formation can be observed. Typically, DSV presents with goiter and diffuse infiltrations. A higher incidence of DSV has been reported in the pediatric population and particularly in patients who have been exposed to radiation (76, 77).

3.1.5. Solid Variant

Another subtype of PTC commonly observed in pediatric and adolescent populations is the solid variant which is characterized by solid nests of cells comprising over 70% of tumoral tissue (78). Predominantly solid, trabecular, or insular growth patterns are observed in solid variants. The solid variant has been associated with radiation exposure and specifically with nuclear events (77, 79). A meta-analysis of 205 solid variant patients has demonstrated higher risk for vascular invasion, and therefore a higher risk for recurrence, compared to CPTC and has classified the solid variant as an aggressive subtype of PTC (80).

3.2. Role of Genetic Factors in Papillary Thyroid Cancer

The role of genetic factors in the carcinogenesis of thyroid cancers has been an area of active research for the past few decades. Although some somatic and germline mutations as well as epigenetic mechanisms have been identified, establishing the relationship between genetic factors and prognosis or clinical progression has proved challenging.

3.2.1. Somatic Mutations

BRAF mutations are observed in 36-83% of PTC cases and are associated with papillary or follicular/papillary mixed growth patterns (81). The development and progression of PTC are significantly impacted by certain gene mutations that are indicative of its molecular pathogenesis. Several studies have evaluated the impact of $BRAF^{V600E}$ mutations, which is the most prevalent mutation in PTC. The

$BRAF^{V600E}$ mutation is observed in around 40–45% of PTC cases and is strongly associated with aggressive tumor activity and poor clinical outcomes (82).

RAS mutations and RET/PTC rearrangements are two other prominent genetic mutations that are related to carcinogenic signaling pathways that cause uncontrolled cell proliferation and tumor formation. A meta-analysis that included 2395 patients evaluated the prognostic and clinical role of RET/PTC1 and RET/PTC3 in PTC and demonstrated a correlation between radiation exposure and RET/PTC3 mutations (83). An association between young age and RET/PTC3 was also established. Similarly, ETV6-NTRK3 translocations in PTC have also been linked to increased radiation exposure (84). In addition, a study demonstrated a correlation between ETV6-NTRK3 translocations and locoregional aggressiveness and high metastatic potential in PTC patients with no radiation exposure (85).

Cells produce the heat shock protein (HSP) family of proteins in response to stressful situations. As molecular chaperones, they facilitate the stability of membranes and proteins as well as the refolding of misfolded or denatured proteins, which can be crucial in stressful situations (86). In addition to their functions in normal cellular homeostasis and the stress response, HSPs are also involved in cell cycle regulation and signaling (87). HSPs can be overexpressed in a variety of cancer types, including thyroid carcinoma. The overexpression may contribute to cancer cell survival, proliferation, and medication resistance due to HSPs' ability to protect cancer cells from the stressful circumstances of the tumor microenvironment and the cancer therapies. Nuclear translocation of Hsp70 has been related to advanced tumor stage and poor prognosis (88). Moreover, HSPs, which are conserved protein family protecting cells from stress and sustaining proteostasis in the cell, are overexpressed in many types of cancer like breast, lung and gastric as well as thyroid. Its overexpression is evaluated as a sign of poor prognosis (86).

Likewise, mutations in the TP53 gene and the TERT promoter are associated with aggressive types of PTC (82). TP53 mutation, which is a tumor suppressor gene mutated in many types of cancer, is observed in 85% of PTC and has been associated with larger tumor size and tumor stage (89). TERT promoter mutations are observed in approximately 8% of PTC and have been associated with a poor prognosis, large tumor sizes, distant metastases, male gender, and older age (>50 years) (90). TERT mutations are also commonly observed in FTC and have

been linked to aggressive disease progression and poor prognosis (91). Moreover, the utilization of TERT promoter mutations along with BRAF mutations and Ki-67 labelling index has shown great promise in predicting disease recurrence in PTC (92). RAS mutations are detected in approximately 10% of PTC and are commonly linked to FVPTC and FTC. Similarly, PAX8-PPARγ have also been associated with FVPTC and FTC (93, 94). Studies have demonstrated conflicting results regarding the prognostic role of RAS and PAX8-PPARγ mutations in PTC.

3.2.2. Germline Mutations

It has been approximated that 5-15% of non-medullary thyroid cancers (including FTC and PTC) are linked to familial tumor syndromes (95). PTC caused by familial syndromes often present as multinodular, multifocal, and bilateral lesions. 5-10% of FTC have been associated with familial non-medullary thyroid cancer (FNMTC) syndrome (96, 97). FNMTC inheritance has not been well established, however, MNG1, TCO, PRN, NMTC1, FTEN, and telomere-telomerase gene regions are thought to play a role in its development. It has been speculated that FNMTC follows an autosomal dominant pattern and demonstrates incomplete dominance and variable expressivity. Furthermore, FNMTC has been associated with more aggressive disease progression unless detected early through familial screening programs. FNMTC type 1 typically presents with CPTC and has been associated with the 2q21 locus (98). In addition to FNMTC, other familial thyroid cancer syndromes have been described. Familial PTC (fPTC) syndrome has been linked to the chromosomal region 19p13 and has been associated with the development of bilateral multifocal PTC as well as increased incidence of thyroid nodules (96). However, no germline mutations have been identified yet. Although rare, fPTC has also been associated with the occurrence of papillary renal neoplasia (PRN). While the genetics background of fPTC/PRN phenotype has not been fully described, the 1q21 locus and the protooncogene of isolated familial PRN are thought to play a role in its pathogenesis (99). Familial multinodular goiter syndrome (FMGS) which has been linked to PTCSC3 tumor suppressor gene mutations can also result in an increased risk for the development of PTC (96).

Autosomal dominant syndromes that can lead to the development of PTC include familial adenomatous polyposis (FAP) and PTEN-hamartoma tumor

syndrome (PHTS). Characterized by hundreds of colorectal polyps, FAP is caused by inherited APC gene mutations. In addition to colorectal adenomas, in approximately 2% of cases, FAP can present with various extraintestinal manifestations which include desmoid tumors, osteomas, upper gastrointestinal adenomas, hamartomas, congenital hypertrophy of retinal pigmented epithelium, epidermoid cysts, and dental abnormalities (100). Patients with FAP, particularly females (<35 years of age) are estimated to be 160 times more likely to develop PTC compared to healthy individuals (101). Furthermore, FAP has been associated with the sporadic development of well-differentiated and multifocal cribriform-morular PTC characterized by morular areas of biotin accumulation, cribriform structure, focal trabecular areas, nuclear pseudostratification, and encapsulation (102). PHTS is an overarching term used to describe genetic syndromes (Cowden syndrome, Proteus syndrome, Proteus-like syndrome, and Bannayan-Riley-Ruvalcaba syndrome) caused by inactivating germline mutations on the PTEN gene (103). Cowden syndrome (CS) is associated with mucocutaneous and breast lesions, thyroid-related issues (i.e. goiter, adenoma, or carcinoma), and genitourinary abnormalities (104). A meta-analysis of 181 CS patients has estimated that the incidence of FTC or PTC observed in CS patients is 3-7%. Other PHTS have been associated with various thyroid disorders such as thyroid adenomas or Hashimato's thyroiditis but have not been linked to PTC. In addition to FAP and CS, PTC has been associated with autosomal dominant Camey's complex syndrome and autosomal recessive Werner's syndrome which are caused by gene mutations in PRKAR1-x and WRN, respectively. The incidence of FTC and PTC in Camey's complex is 4-60% while 18% of Werner's syndrome patients are estimated to have follicular anaplastic PTC (95). PTC incidence in McCune Albright syndrome which is caused by GNAS gene mutations has also been reported (96).

3.2.3. Epigenetic Alterations

In addition to somatic and germline mutations, the role of epigenetic alterations (i.e. methylation of DNA or miRNA) in the development and characterization of thyroid cancer has also been an area of active research (105). Post-transcriptional regulation of BRAF genes in PTC models as well as the potential use of DNA methylation

markers and BRAF mutations in differentiating PTC on FNAB has been demonstrated (106, 107). Similarly, it has been suggested that miRNA deregulation analysis can support clinicians in distinguishing between PTC and poorly differentiated thyroid carcinoma (108). Although evidence on the prognostic or diagnostic role of epigenetic alterations has not been produced yet, studies focusing on the role of epigenetic alterations on thyroid tumor carcinogenesis are ongoing. Besides genetic and environmental factors several other mechanisms (i.e. desmoplasia, which is the growth of fibrotic connective tissue, can be observed around tumor) can appear as a significant determinant for poor prognosis (109). To enable accurate molecular diagnosis, prognostication, and development of individualized therapy plans for PTC patients, an understanding of these genetic mutations and histopathological characteristics of tumor are essential.

3.3. Role of Environmental Factors in Papillary Thyroid Cancer

The role of radiation carcinogenesis in the development of PTC has been one of the most widely studied predisposing environmental factors. Following several studies demonstrating a link between pediatric thyroid cancer development and exposure to radiation through x-ray therapy, a meta-analysis conducted in the 1970s established that 76% of pediatric patients (n=878) who developed thyroid cancer had received x-ray therapy for various indications (110). In the 1990s, a correlation between radiation dose and pediatric thyroid cancer development was established (111). Although clinical practice has changed drastically since then (i.e. radiation therapy use for thymic enlargement or acne has been abandoned), several indications such as pediatric malignancies still require radiation based therapy, leading to DNA damage and subsequent neoplasm development risk (112). Moreover, it has been demonstrated that acute lymphoblastic leukemia patients treated with radiation were at an increased risk for thyroid cancer development (113). In addition to clinical exposure to radiation, studies have also investigated the impact of radioactive iodine exposure on thyroid cancer development. 95% of Chernobyl-associated thyroid cancer were PTC and histopathological features and tumor morphology of PTC differed depending on the age at presentation with aggressive solid variants commonly observed in pediatric patients (114). Particularly for radiation exposed children, robust genomic alterations, particularly

PTC drivers, have been established, leading to PTC carcinogenesis (115). An increased incidence in germline mutations were not detected following the Chernobyl accident (116). Although the carcinogenesis of radiation induced PTC development has not yet been well-established, in atomic bomb survivors diagnosed with PTC, the predominantly expressed genomic changes were RET/PTC rearrangements, suggesting RET/PTC influence on radiation driven PTC carcinogenesis (117). In addition to radiation, the role of increased iodine intake and exposure to chemical pollutants (i.e. lead) in the development of PTC has been studied (118, 119).

3.4. Prognostic Factors in Papillary Thyroid Cancer

As the most common subtype of thyroid cancer, PTC involves a systematic investigation to fully comprehend the factors that influence its prognosis. Prognostic factors are essential elements that enable an accurate prediction and, consequently, affect clinical decision-making. These parameters inform decisions about the necessity of surgery, radiation therapy, or targeted therapies, as well as the aggressiveness of cancer and its propensity to recur or spread. PTC has an exceptional prognosis, with over 90% of individuals surviving over the long term, particularly those under 45 (120). When treated with a hemithyroidectomy or a complete thyroidectomy, PTC of any size that is limited to the thyroid gland has a 99% 10-year survival rate (121). However, in certain cases, postoperative recurrence or metastasis cannot be predicted or managed with the current guides. Consequently, the 10-year survival without recurrence was reported as only 80% (122). The prognosis of PTC is determined by a complex network of genetic, clinical, and histological factors that have been thoroughly researched in the scientific literature recently. Age, sex, tumor size, invasion of lymph nodes, ETE, distant metastasis, multifocality, histological subtype, and BRAF mutant status are some of the frequently researched prognostic variables in papillary thyroid cancer. Tumor angiogenesis, blood parameters, and serum Tg level are other possible prognostic variables that have been discussed in the conext of PTC.

Age has a significant role in the prognosis of PTC. Its importance in determining patients' prognosis has been highlighted in several studies. According to recent research, age is an independent parameter determining the prognosis of

PTC, especially recurrence to thyroid, lymph nodes, lung and bones (122, 123). Studies have indicated that PTC patients who are older have a higher mortality rate if they have high-risk characteristics of the tumor (124). Nevertheless, it does not have much value as a determinant for the overall survival (OS) of PTC except in patients classified as high-risk (122). Furthermore, the prognosis of PTC is often worse in those over 60 (125). However, there is no consensus on age cutoff as an indicator of prognosis. Previous guidelines have indicated that patients above 45 years of age were more likely to encounter adverse prognostic outcomes. The 8th edition of American Joint Committee on Cancer (AJCC) has formed different risk evaluation guidelines based on the TNM staging system for patients under and over the age of 55 (126). Studies have revealed that patients over 55 years of age were more likely than those under 55 to have lower disease-free survival (122, 127-129). Moreover, the prognostic impact of age is highly related to the risk classification of the patient. *Ito Y. et al.* indicated that if a patient is diagnosed with very low-risk PTC, young age can be an essential predictor for local recurrence because the rapid growth of PTC in the early phase is more likely observed at a younger age. Nonetheless, recurrence rate is strongly correlated with age in low and intermediate-risk patient groups (129). Accordingly, advanced age is associated with an adverse prognosis characterized by decreased disease-free survival rates, while it exerts no discernible effect on overall survival outcomes.

One known prognostic factor for PTC is male sex. Male sex is related to some adverse clinicopathological aspects of PTC, which may impact response to early therapy or the overall state of the illness according to several studies that have examined the relationship between aggressive PTC and male sex (130, 131). Although it is often considered that males with thyroid cancer have a poorer outcome than women, recent research findings indicate that despite being linked to more aggressive and advanced illness, male sex is not an independent predictor of survival (132). Moreover, most male patients are diagnosed with papillary thyroid carcinoma at older age compared to women (130). Therefore, tumor features and risk stratification should be carefully evaluated in male patients diagnosed with PTC to determine prognosis and manage properly the treatment.

Tumor size is a major determinant of the prognosis of PTC, and current treatment guidelines are mostly focused on tumor size. Studies have demonstrated that a solitary 2 cm tumor size optimizes prognostic categorization, wherein tumors

above 2 cm are related to an increased risk of recurrence in comparison to those that are 2 cm or smaller (133). Ito Y. et al. divided 3,965 low-risk PTC patients into three groups according to the size of their tumors: T-1 (tumor < 2 cm), T-2 (tumor 2.1-4 cm), and T-3 (tumor > 4 cm) to determine the effect of tumor size on the prognosis. The data unequivocally indicate that larger tumor sizes are significantly associated with a higher recurrence rate for the thyroid, lymph nodes, and distant organs within ten years (123, 134, 135). Therefore, it is critical to consider tumor size when deciding treatment options to reduce the risk of cancer recurrence.

Multiple tumor foci are another prognostic factor for PTC. Some publications attribute multifocality to a poor prognosis for PTC, whereas other studies reported no statistical correlation between multifocality and prognosis. Multifocality was statistically related to high risk of tumor recurrence in patients with PTC, according to review by *Kim H. et al*. that involved a systematic review and meta-analysis of 26 studies including 33,976 individuals with PTC. Despite the increased risk of tumor recurrence, there was no discernible difference in cancer-specific survival between patients with multifocal and unifocal PTC (136). Patients diagnosed with multifocal PTC are more likely to have larger tumors or tumors that have invaded the capsule, vascular structures, and lymph nodes than those with unifocal PTC. Therefore, multifocal PTC has higher rate of recurrence and worse treatment response (137, 138). However, some studies have revealed that multifocality is not a statistically independent determinant for the prognosis of PTC and overall survival or disease-specific survival, although multifocal PTC patients have higher incidence of poor prognostic features such as LNM or postoperative disease progression (138, 139). As a result, there is ongoing debate over the impact of multifocality on the prognosis of PTC and further research is needed to comprehend its significance.

ETE, LNM, and distant metastasis represent pivotal prognostic determinants concerning the tumor aggressiveness in PTC. ETE denotes the infiltration of adjacent tissues beyond the thyroid capsule, indicating the tumor's capability to breach histogenetic and anatomical confines. Its significance as a risk factor predisposing patients to recurrence and unfavorable prognosis is well-established. Notably, a study by *Bouzehouane et al*., including 1000 PTC patients, has underscored ETE as an independent predictor for increased recurrence risk, diminished disease-free survival, and elevated disease-specific mortality (140).

Specifically, extension into or beyond the strap muscles has emerged as statistically significant for overall and disease-specific survival, as evidenced by an analysis encompassing data from 108,426 papillary thyroid cancer patients (141, 142). Additionally, ETE stands as an independent risk factor for LNM (143, 144). The adverse prognostic impact of ETE is attributed to tumor aggressiveness, diagnostic challenges, and intricate surgical excision (142). Incomplete surgical resection due to ETE may yield excessive morbidities and escalate mortality risks. Conversely, misdiagnosis of ETE in preoperative assessment may prompt a more extensive surgical approach, entailing increased surgical complexity and attendant complications (144). Current literature has delved into the prognostic implications of micro-extra-thyroidal extension (mETE) diagnosed via microscopy (145). Although mETE was omitted from the eighth edition of the AJCC cancer staging manual, previous studies highlight its association with higher recurrence rates, LNM, and vascular, lymphatic, and perineural invasion (140, 143, 146). Consequently, the presence of ETE, even at the microscopic level, augments the risk of recurrence, metastasis, and diminished disease-free survival in PTC patients (142, 143).

PTC manifests two types of LNM: central lymph node metastasis (CLNM) and lateral lymph node metastasis (LLNM). Typically, PTC disseminates to the CLN before involving the lateral cervical lymph nodes. Notably, metastasis to lateral cervical lymph nodes typically occurs ipsilaterally to the primary tumor. The progression of PTC from central to lateral lymph nodes correlates with worsened prognosis, marked by increased recurrence risk and poorer long-term outcomes. A study involving 2,418 PTC patients underscores the adverse prognostic impact of LNM, with a heightened risk of recurrence and diminished disease-free survival (50). Additionally, LNM is identified as an independent determinant for recurrence and disease-specific mortality (134). Moreover, the number of metastatic lymph nodes inversely correlates with overall survival in PTC patients under the age of 45 (147). While LNM impairs disease-free survival and predisposes to recurrence, its effect on overall survival remains controversial. Furthermore, distant organ metastasis in PTC predominantly affects the lungs and bones, albeit its incidence is rare (148). Detection of distant organ metastasis at the time of PTC diagnosis constitutes a significant poor prognostic indicator, precipitating a dramatic reduction in overall survival (149). Particularly, symptomatic metastatic lesions

exacerbate the prognosis. Large-scale studies affirm that multi-organ and brain involvement portend the poorest overall survival (148). In summary, LNM and distant organ metastasis independently contribute to unfavorable prognoses, culminating in compromised disease-free survival and OS.

Histological subtypes of PTC have been extensively studied for their implications on prognosis, with findings suggesting significant variations in clinical outcomes based on tumor histology. The comparative study emphasizes the prognostic significance of the tall cell variant, indicating its association with advanced disease characteristics, such as extrathyroidal extension and LNM, leading to poorer outcomes compared to classic PTC subtypes (150). Furthermore, another study provides insights into the aggressive nature of the tall cell variant, highlighting its correlation with increased rates of recurrence and disease persistence (151). Moreover, the classic variant of PTC, as elucidated by a study by *Sebastian S.O. et al.*, generally demonstrates a more favorable prognosis, characterized by lower rates of extrathyroidal extension and LNM (151, 152). Moreover, the review highlights the importance of histological subtyping for predicting behavior and guiding management decisions in PTC with various histological patterns (150, 153). Collectively, these studies underscore the importance of considering histological subtypes in prognostic evaluation and treatment planning for patients with PTC, as they provide crucial insights into the disease's clinical behavior and outcomes, ultimately influencing therapeutic strategies to mitigate recurrence risk and improve overall survival.

Angiogenesis, the formation of new blood vessels, plays a crucial role in tumor growth, invasion, and metastasis, thus influencing the prognosis of PTC (154-157). While the direct impact of angiogenesis on the prognosis of PTC is not fully established, its potential as an indicator of metastatic potential and a prognostic marker has been recognized. Studies such as the research conducted by *Brittelle et al*. have shed light on the molecular mechanisms underlying thyroid tumorigenesis and its relationship with angiogenesis (154). Elevated levels of angiogenic factors such as vascular endothelial growth factor (VEGF) have been consistently observed in PTC tissues and are correlated with higher rates of tumor recurrence, LNM, and disease-specific mortality (155, 157). Furthermore, microvessel density, a histological marker of angiogenesis, has been identified as an independent prognostic factor for PTC, with higher microvessel density values

being predictive of shorter disease-free survival and overall survival (155). Further research and clinical implications of angiogenesis in PTC are areas of ongoing investigation, which may lead to the identification of novel prognostic biomarkers and the refinement of prognostic models for PTC patients.

The prognosis of PTC is influenced by TSH levels and pregnancy in a complex interaction between endocrine physiology and tumor biology. TSH suppression therapy is essential in managing PTC. Its goal is to reduce the stimulatory effects on thyroid cancer cells, resulting in lower recurrence risks and better outcomes. The similarity between TSH and beta human chorionic gonadotropin (beta hCG), a hormone elevated during pregnancy, is particularly noteworthy, as beta hCG can mimic TSH's effects, potentially influencing thyroid function and cancer dynamics during pregnancy (158, 159). Studies have demonstrated that, despite complex interactions, pregnancy is not associated with adverse effects on the prognosis of PTC. In fact, pregnant patients with PTC frequently experience favorable outcomes, indicating that pregnancy does not negatively impact the course of the disease (159-161). This emphasizes the significance of individualized treatment and close observation of PTC in expectant mothers, considering the complex interaction of TSH levels, beta hCG, and the molecular characteristics of the malignancy. Recent studies exploring the clinical and molecular aspects of PTC during pregnancy highlight the need for a comprehensive approach that addresses the unique challenges posed by the hormonal milieu of pregnancy (159).

3.5. Prognostic Scoring Systems

Prognostic scoring systems play a critical role in the healthcare industry, supporting quality improvement initiatives, guiding treatment decisions, simplifying patient communication, improving clinical trials, and risk classification. These methods allow medical professionals to customize therapies to each patient's risk profile, enhancing the efficacy of interventions while reducing needless side effects. Patients are categorized depending on the likelihood of different outcomes (162, 163). Informed and collaborative decision-making is facilitated by the organized framework they offer for discussion with patients about their prognosis and available treatments. Additionally, these scoring systems assist in allocating

treatment for individuals who are most likely to benefit in situations where resources are scarce, guaranteeing effective utilization of healthcare services (164). In conclusion, healthcare institutions may drive initiatives to improve the quality of patient care by identifying areas for improvement by evaluating patient outcomes against prognostic predictions (165). In this regard, many scoring systems are designed to categorize PTC patients and predict the prognosis of PTC, which are TNM, AGES, MACIS, AMES and EORTC scoring system. (166)

PTC is elaborately categorized using the TNM staging system, which informs prognosis and treatment decisions. Importantly, the "T" category describes the size and extent of the main tumor. Tumors are categorized from T1, for those 2 cm or less in diameter, to T4b, indicating extensive invasion into critical structures like the prevertebral fascia or mediastinal vessels. (167) T1 and T2 are confined to the thyroid, while T3 is differentiated into T3a for tumors larger than 4 cm but still limited or minimally extending beyond the thyroid, and T3b for those with significant extrathyroidal invasion. T4 is subdivided into T4a, signifying invasion into nearby tissues such as the larynx or esophagus, and T4b, for tumors invading into more critical areas, often rendering them unresectable (167). The "N" category represents lymph node involvement, which is an important prognostic factor. While N1 is divided into N1a, which suggests metastasis to central neck lymph nodes, which is more common and less severe, and N1b, which indicates metastasis to lateral or mediastinal nodes, suggesting a more advanced disease state, N0 indicates no regional LNM. It is critical to differentiate between N1a and N1b when deciding a treatment plan, with N1b typically needing more aggressive approaches (167). The "M" category, which denotes the presence or absence of distant metastases, is a crucial determinant of prognosis. It's critical to differentiate between N1a and N1b when deciding a treatment plan, with N1b typically needing more aggressive approaches. The "M" category, which denotes the presence or absence of distant metastases, is a crucial determinant of prognosis. Whereas M1, which indicates distant dissemination, strongly affects survival and treatment plans and frequently calls for systemic medicines, M0 indicates the lack of such spread (126, 167). The comprehensive TNM classification thus facilitates a tailored approach to managing PTC, ranging from localized surgical interventions to more complex treatments for advanced stages, and emphasizes the importance of follow-up to monitor for recurrence or progression (168). TNM stage at diagnosis is a strong predictor of 5-

year survival rates for PTC, with generally positive outcomes. Early in the disease's course, throughout stages I and II, the survival rate is over 100%, which is indicative of the tumor's small size and localized confinement to the thyroid gland, free of lymph node involvement or distant metastases (166). The survival rate somewhat declines but usually stays around 90% when the cancer advances to stage III, with bigger tumors or modest extrathyroidal spread and potential lymph node involvement. The most significant variation in survival rates is observed in stage IV, which is subdivided into IVA, IVB, and IVC. While stages IVA and IVB, characterized by extensive local invasion or significant lymph node involvement, still exhibit relatively high survival rates, often in the 70-80% range, stage IVC, marked by distant metastasis, sees a substantial drop, with 5-year survival rates potentially falling below 50% (166). Even though therapeutic advances are continuously improving outcomes for PTC patients at all stages, these prognostic data highlight the significance of early identification and the role of disease progression on prognosis.

The AGES scoring system for papillary thyroid cancer incorporates factors such as age, grade of the tumor, extent of tumor spread, and size of the primary tumor (169). Each of these components contributes to a score that helps predict the prognosis for patients with papillary thyroid cancer. Scores are allotted by the system according to the patient's age, the tumor's histological grade, whether the cancer has progressed outside of the thyroid, and the tumor's size; larger tumors, higher tumor grades, and more extensive dissemination are indicative of a poorer prognosis. The scoring method adds 0.05 points for every year of age for patients who are 40 years of age or older. Another important factor is the tumor grade, which adds one point for grade 2 tumors and three points for more aggressive grade 3 or 4 cancers. An additional 3 points are added to the score in the event of distant metastases, reflecting a more advanced stage of the illness. Finally, 0.2 points are added for every centimeter to account for the tumor's growth, representing the effect of tumor burden on prognosis (169). In order to evaluate patient outcomes and guide treatment decisions, this score system is utilized in combination with other prognostic techniques. The 20-year survival rate for papillary thyroid carcinoma is 99 % for scores below 4, according to the AGES grading system. The survival rate is 80% for scores between 4 and 4.99. The survival rate drops to 67% when the score is between 5 and 5.99. The 20-year survival rate dramatically decreases to

13% with scores higher than 6 (166). In order to provide a standardized framework for assessing prognosis in patients with papillary thyroid cancer, the AGES scoring system evaluates age, tumor grade, amount of spread, and size. Nevertheless, the prognostic accuracy of this system varies based on the patient group and treatment approaches (166). The AGES methodology should thus be combined with other prognostic factors and clinical judgments in order to provide a more thorough and customized method of patient care and outcome prediction (163, 166).

The size of the tumor, invasion, age, completeness of resection, and metastases are all evaluated by the MACIS scoring system for papillary thyroid cancer. Different scales are applied to each component to get a total score that aids in prognosticating the patient. In order to determine the prognosis of papillary thyroid carcinoma, the MACIS score is calculated by examining many critical factors: A base score of 3.1 is applied to patients under 40, and 0.08 is multiplied by the patient's age in years for patients 40 and above. The score is increased by multiplying the tumor size in centimeters by 0.3. Another point is added if the tumor is not completely removed. Another point is added by the existence of a local invasion. The score rises by three if distant metastases are diagnosed (170). Based on the MACIS score, the 20-year survival rates for papillary thyroid carcinoma indicate a significant positive correlation between the score and the prognosis over the long term. A 99% survival rate over 20 years is expected for patients with a MACIS score of less than 6. The survival rate is 89% for those with scores between 6 and 6.99. Nevertheless, the prognosis becomes more meticulous with higher scores. A score of 8 or above implies a much reduced 20-year survival chance of 24%, whereas a score between 7 and 7.99 is related to a 56% survival rate (170). These survival statistics demonstrate how useful the MACIS score is for classifying patients based on their long-term risk and appropriately adjusting therapy and follow-up (166, 170).

Age, metastasis, tumor size, and sex are used by the AMES score system, a predictive tool for thyroid cancer, to divide patients into low- and high-risk categories (171). With particular thresholds and parameters for each aspect, the criteria take into account factors including the patient's age at diagnosis, the size of the primary tumor, the existence or absence of distant metastases, and the patient's sex. Men and women who are 40 years of age or younger and who do not have any distant metastases are considered low-risk patients. This group also includes elderly

patients who come with intrathyroidal tumors that are less than 5 cm in size, do not have distant metastases, and exhibit little capsular invasion (171). Conversely, the high-risk group encompasses all patients with distant metastases, extrathyroidal lesions, significant capsular invasion, or tumors 5 cm or larger in older patients (171). This system aids in the prognosis prediction and treatment decision-making process for patients with thyroid cancer (166, 171). According to the AMES scoring system, patients with thyroid cancer who are classed as low-risk have an estimated 20-year survival probability of 99 percent, whereas those who are classified as high-risk have a much lower survival rate of 61 percent (171). In conclusion, the AMES score system is a useful tool for enabling personalized prognosis evaluations and well-informed treatment strategies when it comes to risk-based patient stratification for thyroid cancer.

The European Organisation for Research and Treatment of Cancer (EORTC) score system for thyroid cancer provides a distinct method for evaluating patient prognosis (172). This method divides patients into many risk groups based on a variety of clinical and pathological variables. In particular, it grades patients according to variables such as age, gender, type of tumor, and metastasis presence, which helps forecast survival rates in the long term. In light of the noted variations in prognosis between genders, male patients are scored with an additional 12 points. An additional 10 points are added to the score in the event of poorly differentiated follicular lesions, which is indicative of a more malignant type of malignancy. Additionally, 10 points are given to tumors that are graded as T3 or greater, indicating a larger and possibly more invasive tumor. One important component of the score is distant metastases: 15 points are added for the first metastasis and another 15 for each one afterwards (172). This emphasizes how much metastatic spread affects prognosis. Patients are then stratified into various risk categories based on the cumulative score that is generated from these parameters; as the scores rise, the 5-year survival rates decrease. 5-year survival probability of only 5% is associated with scores above 108, indicating a worse prognosis. Scores below 50 are mainly attributed to a 95% 5-year survival rate (166, 172). Such a methodical grading system enables treatment planning to become more individualized and efficient despite the variety and complexity of thyroid cancer presentations.

It is difficult to compare prognostic scoring systems in terms of predictive val ue because of the inherent differences in their designs, the distinct patient groups f

or which they were developed, and the particular outcomes that they are meant to predict (166). The effectiveness of these systems varies depending on how well they include relevant prognostic data, adapt to changing patient conditions over time, and function in different clinical settings (173). Furthermore, the efficacy of a scoring system in one setting does not necessarily correlate to another because variables like patient demographics, therapeutic developments, and genetic diversity can have a substantial impact on results (173, 174). In contrast, the comprehensive study shows that the MACIS scoring system performs more accurately than other systems such as AGES, EORTC, AMES, and TNM when determining prognosis in cases with papillary thyroid cancer (166). The superiority of MACIS lies in its capacity to guide management decisions more effectively in different conditions and better reflect for variations in global clinical practice.

4. Papillary Thyroid Microcarcinomas

4.1. Epidemiology of Papillary Thyroid Microcarcinomas

Papillary thyroid microcarcinoma (PTMC), formerly known as occult papillary thyroid carcinoma, is defined as a well differentiated papillary carcinoma of the thyroid with a diameter ≤10mm (175, 176). Some studies have expanded the definition of PTMC to include papillary carcinomas with a diameter ≤15mm (177). The early detection or diagnosis of PTMC has remained challenging as disease progression is often clinically occult. Typically, PTMC have either been identified incidentally on biopsies or thyroidectomies conducted for other indications, have presented with cervical LNM, or have been detected post-mortem (178, 179). Moreover, 71% PTMC have been diagnosed incidentally following thyroidectomies performed to manage benign thyroid or parathyroid disorders (175). Therefore, historically, PTMC have long been considered insignificant due to the lack of associated clinical manifestations. Symptomatic presentation of PTMC has been correlated to locoregional or distant metastases. It has been demonstrated that a majority of thyroid microcarcinomas (65-99% of cases) are of papillary histology and the median size of PTMC at diagnosis has been recorded as 4.1-8mm, with 35.2-79% of PTMCs appearing larger than 5mm (175). Mean age at diagnosis was between 41.9 and 55 years and although autopsy-based studies have not reported a significant difference in age or sex-based prevalence of PTMC, of

6653 patients diagnosed with PTMC, 82.9% were women and 17% were men. This striking difference could be attributed to the increased incidental detection of PTMC in women as the prevalence of thyroid disorders is higher in women, potentially leading to an increased number of diagnostic or therapeutic procedures, and thereby to the incidental diagnosis of PTMC.

Radiographic imaging techniques have improved remarkably over the past few decades and US can currently be used to reliably identify thyroid lesions ≤10mm, enabling the detection of PTMC. Following the detection of a suspected PTMC, FNAC or FNAB can be used to confirm the diagnosis. The incidence of PTMC has increased significantly in developed countries owing to the availability of US imaging and implementation of better thyroid screening practices (180). In the United States, PTMC has been identified as the most commonly occurring type of papillary thyroid carcinoma in patients over the age of 45 (181). The increased incidence has not been linked to an increase in mortality. Similar trends have been demonstrated across the globe with the prevalence of PTMC among all thyroid cancers gradually increasing in France, Geneva, and Hong Kong (113, 182, 183). Furthermore, PTMCs have been detected in 28.8% of PTC, 24.8% of DTC, 22.9% of all thyroid cancer, 7.7% of nodules or nodular goiters, 4.1% of hyperthyroidism cases (including Graves' disease), and 3.8% of all thyroidectomies (175).

Although often considered indolent lesions with low metastatic potential, metastasis of PTMC to the central lymph nodes (CLN) are detected in 21.2-64.1% of cases (184-187). In addition to these imaging findings, it has been suggested that several molecular mechanisms (such as the presence of BRAF mutations) and distinct histopathological features, could explain aggressive progression and metastatic potential of PTMCs.

4.2. Histological Classification of PTMCs

Thyroid microcarcinomas can be classified based on their histopathological features. Following FNAC, most microcarcinomas are diagnosed as PTMC. Classical subtypes of PTMC characteristically show features of papillary carcinomas including nuclear grooves, nuclear pseudo-inclusions, irregular nuclear contours, and intranuclear intracytoplasmic inclusions (66). PTMCs can appear encapsulated or nonencapsulated and can include sclerotic components (188).

Reactive changes of follicular cells in chronic lymphocytic thyroiditis can sometimes exhibit similar features but often lack sclerosis, yet, in PTMC of thyroiditis, sclerosis and papillary carcinoma-like features are commonly observed (188). Psammoma bodies can also be seen, particularly in cases of micro-metastases to lymph nodes. In a study that included 745 patients with PTMC, the observation of intra-lymphatic psammoma bodies was reported in 29% of cases with LNM (189). Although a majority of microcarcinomas are of classical PTMC subtype, some show distinct histopathological features and are classified under follicular, tall cell, encapsulated, diffuse sclerosing, solid, columnar cell, Warthin-like, and hobnail variants.

4.3. Diagnosis of PTMC

Definitive diagnosis of PTMCs can be achieved using US-guided FNAB/FNAC. However, the diagnostic work-up and management of PTMC has remained controversial as most PTMCs remain subclinical while some are associated with aggressive progression and micro-metastases.

4.4. Pathological Classification, Staging, and Risk Stratification

4.4.1. Pathological Classification

Several histological and pathological features including tumor size, histological subtype, lymphovascular invasion (LVI), laterality, multifocality, tumor border pattern, tumor capsule invasion, tumor burden, nodal and metastatic evaluation, and ETE are commonly assessed to enable the pathological classification of PTMCs (190).

4.4.2. AJCC/UICC Staging

AJCC / Union for International Cancer Control (UICC) staging is based on the TNM classification where tumor size (T), nodal status (N), and metastasis (M) are evaluated. 8th edition AJCC/UICC defines TNM staging for papillary thyroid cancer as follows (191, 192):

T0 – no primary tumor

T1a – size ≤1cm and intrathyroidal

T1b – 1cm<size ≤2cm and intrathyroidal

T2 – 2cm<size ≤4cm and intrathyroidal

T3a – size <4cm and intrathyroidal

T3b – ETE (sternohyoid, sternothyroid, or omohyoid muscles)

T4a – ETE (subcutaneous soft tissue, larynx, trachea, esophagus, recurrent laryngeal nerve)

T4b – ETE (prevertebral fascia or carotid artery / mediastinal vessels)

N0 – no locoregional LNM

N1a – lymph node involvement at level VI or VII

N1b – lymph node involvement at level I, II, III, IV, V, or at retropharyngeal lymph nodes

M0 – no distant metastasis

M1 – distant metastasis

8th edition AJCC/UICC staging is categorized into two groups (192). For patients younger than 55 years of age, Stage I refers to M0 disease without distant metastasis (regardless of T and N stage) while Stage II refers to M1 disease (regardless of T and N stage). For patients older than 55 years of age: Stage I refers to T1a, T1b, or T2 disease without nodal involvement (N0) and distant metastasis (M0); Stage II refers to T1, T2, or T3 disease with lymph node involvement at level VI or VII (N1) but without distant metastasis (M0) and to T3 disease without nodal involvement (N0) and distant metastasis (M0); Stage III refers to T4a disease (regardless of N) without distant metastasis (M0); Stage IVa refers to T4b disease (regardless of N) without distant metastasis (M0); and Stage IVb refers to disease with distant metastases (M1).

4.4.3. ATA, ETA, LATS Risk Stratification

The American Thyroid Association (ATA) (193), the European Thyroid Association (ETA) (194), and the Latin American Thyroid Society (LATS) (195) have each developed risk stratification guidelines to better evaluate thyroid cancer patients (196). According to the risk stratification system, thyroid cancers are classified under very-low-risk, low-risk, intermediate-risk, and high-risk categories which informs management guidelines.

Very-Low-Risk. Although the ATA does not have a separate very-low-risk classification, both the ETA and the LATS have described unifocal microcarcinomas without nodal involvement, distant metastases, or ETE under the very-low-risk category.

Low-Risk. According to the ATA, intrathyroidal (N0M0), unifocal or multifocal microcarcinomas (tumor size <10mm) without aggressive histology or ETE, with no or minimal vascular invasion (<4 foci for well differentiated follicular thyroid cancer with capsular invasion), should be classified under the low-risk category. Tumors with clinical N0 or pathological N1 with 5 or less micrometastases (<2mm) have also been categorized as low risk. Similarly, the ETA has classified intrathyroidal (N0M0), unifocal or multifocal tumors (1cm<size<4cm), without aggressive histology, ETE, or vascular invasion under the low-risk category. The LATS classification also groups intrathyroidal (N0M0) tumors (1cm<size<4cm) under the low-risk category.

Intermediate Risk. While the ETA and the LATS have not defined an intermediate-risk category, the ATA has defined tumors with microscopic soft tissue invasion or aggressive histology, papillary carcinoma with vascular invasion, multifocal PTMC with ETE and $BRAF^{V600E}$ mutations as intermediate risk. Tumors with clinical N1 or pathological N1 with over 5 metastases (<3cm) have also been categorized as intermediate risk.

High Risk. Tumors with any of the following have been classified as high risk by the ATA: macroscopic ETE, distant metastases, pathologic N1 (≥3cm), or follicular thyroid cancer with vascular invasion (>4 foci). Similarly, according to the ETA, tumors with any of the following are considered high risk: macroscopic ETA, large tumor size (>4cm), nodal involvement (N1), or distant metastases (M1). The LATS classifies tumors of aggressive histology, nodal involvement (N1), distant metastases (M1), large tumor size (>4cm) (for patients under 45 years of age) and macroscopic ETE (for patients under 45 years of age) under the high-risk category. Both the ATA and LATS also classify cases in which the presence of residual disease was observed under the high-risk category.

4.5. Treatment Strategies for PTMC

Similar to the ongoing debates around diagnostic algorithms, there has also been a lack of consensus regarding PTMC treatment. Thus, treatment strategies for PTMCs encompass a wide range including surgical interventions, radioactive iodine (RAI), radiofrequency ablation (RFA), and active surveillance. Current ATA guidelines (2015) explain that for FNAC-confirmed asymptomatic PTMC without nodal involvement or metastasis, further treatment is not indicated unless strong cytological evidence indicating aggressive disease is present (193).

4.5.1. Surgical Interventions

Upon assessment of patient risk factors and clinical evaluation, surgery can be performed to treat PTMCs. Even though most PTMCs have favorable prognosis even when no or minimal interventions are performed, other PTMCs can result in LNM. Factors that directly correlate to PTMC metastasis have not yet been well established. As such, several surgical strategies can be employed in managing PTMC. Given that lymph node involvement is not suspected at preoperative evaluation, a lobectomy is often considered adequate for a unifocal PTMC while total thyroidectomy is preferred when managing multifocal PTMCs. Depending on preoperative or intraoperative evaluation of lymph nodes and surgeon experience, therapeutic total central lymph node dissection can be performed to prevent metastasis or recurrence. When patients present with palpable lymphadenopathy and PTMC is confirmed on US, therapeutic node dissection is preferred (197). Similarly, therapeutic lateral compartment lymph node dissection should be performed if palpable lateral lymph nodes are observed, or if suspicious lateral lymph nodes are detected pre- or intraoperatively (198).

4.5.2. Radioactive Iodine Ablation

Since its initial therapeutic use in the 1940s, RAI therapy has become an additional therapeutic option for thyroid cancer patients and has been primarily used (i) for remnant ablation post-thyroidectomy in which residual tissue persists, (ii) as adjuvant therapy to prevent recurrence, and (iii) as definitive therapy in cases where locoregional or distant metastasis is detected (199). Over the past two decades, guidelines for RAI use have been updated to reflect risk stratification criteria.

According to ATA guidelines, RAI is not routinely recommended for low-risk thyroid cancers (including clinical N0 stage tumors, ≤5 N1 stage micrometastases (<2mm), unifocal or multifocal intrathyroidal PTMC). RAI can be considered for intermediate-risk thyroid cancers (i.e. clinical N1 stage tumors, multifocal PTMCs with ETE and BRAF mutations) and is routinely recommended for high-risk thyroid cancers. Furthermore, RAI is not indicated post-thyroidectomy for multifocal PTMC unless red-flag features have been detected as RAI is unlikely to prolong survival or offer significant therapeutic improvement (193). A study of 900 patients has demonstrated no significant benefits of RAI post-thyroidectomy (200). Similarly, in another study that included 2579 patients, no benefits of post-operative RAI therapy in preventing disease recurrence was observed in low- and intermediate-risk patients (201). Recent ATA guidelines reflect findings of such studies and not only recommend lower doses of RAI, but also promote selective use of RAI determined on a case-by-case basis. Thyroglobulin (Tg) monitoring can also be employed to support clinician decision making processes for post-operative RAI use (199).

4.5.3. Radiofrequency Ablation (RFA)

In addition to surgical approaches and RAI, minimally invasive strategies more commonly used to treat benign nodules such as RFA have been used in the management of PTMC (202). A meta-analysis that included 15 studies and 1770 patients evaluated the efficacy and safety of RFA for low-risk PTMC (203). The complete disappearance frequency 12 months post-intervention ranged from 27.8-91% and repeat ablations were performed at follow-up for 24 patients, demonstrating high variability between studies. Residual PTMC, newly appearing PTMC, and LNM was observed for 0.4%, 0.9%, and 0.2% of patients, respectively. 2.5% of patients experienced minor complications (i.e. postoperative pain, transient voice changes (<1month duration), skin burns, hematomas, transient hypoparathyroidism, and fever). A single case of intra-operative cardiac arrythmia was recorded and 2 cases of voice changes (<2months duration) were also reported. Although not yet included in guidelines for the treatment of PTMC, the use of RFA has also been suggested for patients with high surgical risk (193).

4.5.4. Active Surveillance

Contrary to interventional strategies, active surveillance has also been employed in the management of PTMC owing to the indolent nature of microcarcinomas. In a study in which 1235 PTMC patients (confirmed with biopsy) did not receive surgical treatment and were followed under active surveillance for 15 years, tumor enlargement (<3cm) was detected for 5% and 8% at 5-year and 10-year follow-ups, respectively (204). All 1235 patients who were offered active surveillance were initially classified as low-risk patients with asymptomatic PTMC and surgery was performed for 191 patients during the active surveillance period. A higher rate of disease progression (8.9%) was observed for patients <40 years compared to their older counterparts. Following surgery, recurrence was observed for a single patient. Similar findings were described in another study that followed 230 asymptomatic PTMC patients (205). Tumor progression and LNM were detected for 7% and 1% of patients, respectively. Such studies have revealed that non-invasive strategies such as active surveillance can play an important role in PTMC management. Moreover, it has been suggested that while active surveillance could remain a feasible option for low-risk patients, adequate management of high-risk patients demands a higher degree of intervention. Of 18,845 PTMC cases, the risk of disease-specific mortality was calculated as 0.5% (206). 92% of patient mortality was associated with having at least two risk factors (age >45 years, male sex, African American or minority race, node metastases, distant metastases, ETE).

4.6. Monitoring

Monitoring for well differentiated thyroid carcinoma is essential in the early detection of recurrence and disease progression tracking. ATA guidelines recommend US imaging of the neck and serum Tg measurement post-operatively following complete thyroid ablation (193). Moreover, serum Tg level increase has been strongly correlated to persistent or recurrent thyroid disease after total thyroidectomy (207). Typically, serum Tg levels <0.1 ng/ml in the absence of Tg autoantibodies post-thyroidectomy or thyroid ablation is strongly indicative of complete treatment of disease and complete ablation of thyroid tissue, whereas a serum Tg concentration of >2 ng/ml is suggestive of the presence of thyroid remnants or recurrence of disease (208). A study that included 150 patients with

DTC, 54 of which were microcarcinomas, demonstrated a correlation between tumor persistence or recurrence and initial post-operative stimulated serum Tg value (209). However, the role of Tg in initial evaluation is limited.

4.7. Prognostic Factors for PTMC

The prognostic factors for PTMC involve a complex interplay of demographic characteristics, ultrasound features, and pathological results. Age is a pivotal factor, with younger patients generally exhibiting a higher rate of LNM, but not necessarily correlating with a poorer prognosis, suggesting that while young age is a risk factor for LNM, older age is associated with worse outcomes due to age-dependent physiological changes and comorbidities. A study by *Ito et al*. has provided a comprehensive analysis of prognostic factor, including those relevant to PTMC. It has been emphasized that older age and the presence of distant metastases at diagnosis (M1) significantly affect both OS and cause-specific survival of PTC patients (210). It has also been demonstrated that gender plays a role in the prognosis of PTC; males have a higher rate of LNM compared to females, possibly due to hormonal differences and metabolic rates, although the prognostic impact of gender on PTMC remains controversial. Multifocality and bilaterality in PTMC have been linked to an increased risk of LNM, yet the direct relationship with tumor recurrence is still under investigation (211). ETE signifies a higher likelihood of LNM, indicating a more aggressive disease course (212). Additionally, molecular markers such as BRAF and TERT promoter mutations, intraglandular dissemination, psammoma bodies in normal thyroid tissue, and a high Ki-67 labeling index have been identified as indicators of more aggressive PMCs, potentially aiding in the prognostication and management of patients with PTMC (210, 212, 213).

4.8. Prognostic Molecular Biomarkers in PTMC

The mutations in BRAF gene are remarkable genetic alterations at the molecular level of PTMC, which are associated with poor prognosis. BRAF is one of main proteins of RAS/MAPK signaling pathway (82). It is a serine-threonine kinase, activated with RAS bound to the cell membrane (214). It initiates MAPK signaling cascade, which is responsible for expression of the genes controlling cell

proliferation, differentiation and apoptosis (215). Different types of mutations on BRAF, such as point mutations, chromosomal rearrangements, in-frame insertion or deletions are detected in thyroid cancer, which leads to the activation of BRAF and uncontrolled cell proliferation (214). Furthermore, common mutations vary in different subtypes of PTC. For instance, $BRAF^{K601E}$ is a mutation often detected in FVPTC (216). The most reported mutation of BRAF is V600E in PTMC, which is the exchange of valine at residue 600 to glutamate. This point mutation occurs near the catalytic center of BRAF, as a result, the BRAF protein cannot be inactivated and MAPK signaling pathway relays the signal downstream constantly (217). Therefore, $BRAF^{V600E}$ leads to genetic instability aiding alterations in different pathways such as phosphoinositide 3-kinase-Akt serine-threonine kinase (PI3K-AKT), which facilitates tumor progression (214).

Comprehensive studies, including meta-analyses and clinical case studies have shown that $BRAF^{V600E}$ mutation is related to ETE and LNM, which are poor prognostic factors (218). A study has included 2000 patients and has emphasized the role of $BRAF^{V600E}$ in predicting the risk of PTC recurrence (219). Thus, BRAF mutations appears as an essential determinant for recurrence and poor prognosis regardless of other clinicopathological features (220). Furthermore, preoperative BRAF testing in FNAB specimens for papillary thyroid cancer plays a crucial role in improving surgical planning, decreasing false-negative diagnoses of PTC, and predicting occult central LNM (221-223). The preoperative risk evaluation, which supports the customized approach to surgical intervention and patient management in the treatment of thyroid cancer, strongly depends on the routine analysis of the BRAF mutation with FNAB (223, 224). In conclusion, $BRAF^{V600E}$ is the essential diagnostic marker for PTC and a predictor for ETE, LNM, and recurrence.

5. Prognostic Tools to Evaluate Lymph Node Metastasis

5.1. Prognostic Markers to Predict Metastasis

While few studies have investigated the role of molecular markers to predict LNM in PTMC, such studies have been gained momentum in the context of various other neoplastic diseases. Recently, the prognostic role of desmoplasia in MTC and the use of trichrome staining in thyroid disorders have been investigated.

5.1.1. Desmoplasia

Desmoplasia is the formation of fibrotic tissue driven by fibroblast growth. It is formed by the overproduction of extracellular matrix proteins and extensive proliferation of myofibroblasts (225). The tissue, which is dense and fibrous, is composed of cellular and non-cellular components. Infiltrating immune cells and fibroblasts constitute the cellular part of desmoplasia while non-cellular components include multiple extracellular matrix proteins such as collagen type I, III and IV, fibronectin and laminin besides glycoproteins. Desmoplasia has been associated with malignant tumors and studies have demonstrated the correlation between desmoplasia and the tumor growth and treatment response. Tumor cells activate fibroblasts to transform into myofibroblasts by producing signaling molecules. Myofibroblasts synthesize excessive amounts of extracellular matrix proteins and glycoproteins, thereby creating the dense fibrous tissue around the tumor. Moreover, cytokines, which are the byproducts of inflammation reactions and growth factors, produced by tumors, propagate the stromal and fibrotic changes inside or around the tumor.

Desmoplasia is linked to poor prognosis and aggressiveness of different malignant tumors. It is the main characteristic finding of pancreatic ductal carcinoma (PDAC) (226). Dense fibrous stroma surrounding the tumor facilitates the high interstitial pressure, resulting in poor vascularization. It complicates drug delivery into the tumor and results in poor response to therapy associated with poor prognosis. In addition to PDAC, desmoplasia is observed in hepatocellular carcinoma (HCC), which frequently develops following chronic liver injury and fibrotic microenvironment changes in cirrhosis (227). The fibrotic tissue in HCC aids in tumor progression and therapy resistance, complicating the management of the disease. Additionally, desmoplasia is associated with tumor growth, invasion and metastasis in colorectal, gastric, and non-small lung cancer (NSCLC) (228). The role of desmoplasia in thyroid cancer, and MTC in particular, has recently attracted attention (109). It has been established that fibrotic tissue makes MTC firmer and more palpable, facilitating the diagnosis. However, desmoplasia has also been linked to the invasion of surrounding thyroid tissue presenting a challenge for surgical resection of the tumor. In addition, desmoplasia is a significant determinant for poor prognosis for MTC (109), although research for its prognostic value in

PTC is an area for research.

5.1.2. Trichrome Staining

Masson trichrome stain is a histological stain widely used to identify connective tissue, especially collagen, in a tissue section. Using trichrome staining, collagen is dyed blue and thus differentiated from the smooth muscles which dye in red and other cells such as a erythrocytes, fibrin and cytoplasm which also dye in red. Nuclei stain purple black. The spleen has been used as a control tissue for Masson trichrome stain (229). Because trichrome staining is used to stain connective tissue, it can be used to detect the various degrees of fibrosis. In the context of PTC and thyroid nodules, fibrosis is estimated to be seen in 50-70% of the nodules (230). Ultrasonographic tolls to detect this high degree of fibrosis have been studied and various degrees of fibrosis could be detected by sheer wave elastography by analyzing the elasticity index. In one study, elasticity index was lowest in neoplastic samples (231). A positive correlation between elastography analysis and fibrosis in PTC was also noted. Hence, fibrosis and detection of fibrosis ultrasonographical and histologically by stains such as trichrome remains a vital tool in differentiating thyroid nodules (230). Furthermore, trichrome staining can be a useful tool in detecting various degrees of lymphoid tissue and stromal fibrosis which could help in differentiating between different thyroiditis groups. This has been exemplified by a study investigating the histological findings of IgG4 thyroiditis and non-IgG4 thyroiditis, where a marked stromal fibrosis of 86% in 28 tissues samples, was observed in IgG4 positive group (232).

5.2. Development of Prognostic Tools for PTMC

The prognosis of PTMC is often considered favorable as PTMC is thought to be an indolent neoplasm with low metastatic potential and high rates of long-term survival. However, some PTMC cases are known to follow an aggressive disease progression and demonstrate LNM. The factors that play a role in the development of aggressive or indolent PTMC are yet to be established. Commonly used guidelines, such as ATA, ETA, and LATS, recommend conservative approaches for the management of PTMC. Moreover, it has been suggested that the use of

invasive diagnostic and therapeutic procedures can be minimized owing to the favorable LNM node metastasis, a lobectomy is often considered sufficient. Total thyroidectomy is preferred for multifocal PTMC without lymph node involvement. While therapeutic lymph node dissections are performed when LNM is detected, the role of prophylactic lymph node dissections for PTMC have not been included in guidelines. However, the incidence of LNM for PTMC encountered at our clinical practice has been significantly higher than those cited in literature. As a result, prophylactic ipsilateral central neck lymph node dissection is routinely performed for PTMC patients in order to minimize the risk of recurrence and prevent future complications.

PTMC is not considered an aggressive thyroid tumor and therefore, several gaps in literature regarding factors that influence aggressive disease progression and LNM in PTMC remain. To address this gap and expand current knowledge on factors that impact LNM in PTMC, commonly studied BRAF mutations and clinicopathological features of tumors were investigated. In addition, the correlation between desmoplasia, which has recently gained popularity as a potential tool to assess MTC metastasis, and PTMC progression was studied. The role of trichrome staining, which shows the degree of fibrosis and has been associated with metastatic potential in tumors, as a prognostic tool was evaluated. This is one of the first report in English literature that comparatively evaluates the role of BRAF mutations, clinicopathological features of tumor, desmoplasia, and trichrome staining in predicting LNM in PTMC.

Chapter 2

RESEARCH PROPOSAL, MATERIALS, AND METHODS

6. RESEARCH PROPOSAL

PTMC, formerly known as occult papillary thyroid carcinoma, is defined as a well differentiated papillary carcinoma of the thyroid with a diameter ≤10mm. The early detection or diagnosis of PTMCs has remained challenging as disease progression is often clinically occult. Typically, PTMCs have either been identified incidentally on biopsies or thyroidectomies conducted for other indications, have presented with cervical lymph node metastases, or have been detected post-mortem. Radiographic imaging techniques have improved remarkably over the past few decades and US can currently be used to reliably identify thyroid lesions ≤10mm, enabling the detection of PTMCs. Following the detection of a suspected PTMC, FNAC or FNAB can be used to confirm the diagnosis. The incidence of PTMCs has increased significantly in developed countries owing to the availability of USG imaging and implementation of better thyroid screening practices. Although often considered indolent lesions with low metastatic potential, metastasis of PTMCs to the CLN are detected in 21.2-64.1% of cases (11-14). A consensus on risk factors for lymph node metastasis has not been reached yet. In a meta-analysis of 4573 patients, male gender, age <45 years, tumor size >5mm, multifocality, ETE, and lymphovascular invasion were correlated with a higher incidence of CLNM of PTMC (14). Similar results were obtained in another meta-analysis that included 1066 patients (15). Multifocality was associated with the highest risk of CLNM.

Besides the size of the tumor and lymphatic metastasis status, recent studies have revealed other important parameters effecting the tumor aggressiveness. This includes molecular markers such as BRAF mutations.

The aim of this study is to compare metastatic and non-metastatic PTMCs, by retrospectively analyzing our series of thyroidectomies and dissected lymph nodes in order to identify useful histopathological and molecular correlations.

7. SIGNIFICANCE

PTMC is not considered an aggressive thyroid tumor and therefore, several gaps in literature regarding factors that influence aggressive disease progression and LNM in PTMC remain. To address this gap and expand current knowledge on factors that impact LNM in PTMC, commonly studied BRAF mutations and clinicopathological features of tumors were investigated. In addition, the correlation between desmoplasia, which has recently gained popularity as a potential tool to assess MTC metastasis, and PTMC progression was studied. The role of trichrome staining, which shows the degree of fibrosis and has been associated with metastatic potential in tumors, as a prognostic tool was evaluated. This is one of the first report in English literature that comparatively evaluates the role of BRAF mutations, clinicopathological features of tumor, desmoplasia, and trichrome staining in predicting LNM in PTMC.

8. MATERIALS AND METHODS

8.1. Data Collection

Between February 2019 and December 2023, a total of 1219 patients underwent thyroidectomy operations in Koç University Hospital and American Hospital. There were 376 patients with the diagnosis of papillary thyroid carcinoma. From this group a total of 145 patients with tumor sizes 10 mm or smaller were included in the study. Those without prophylactic or therapeutic central neck dissection, and with excised lymph node number less than 3 were excluded from the study. Additionally, the patients who did not accept the use of their formalin-fixed paraffin-embedded (FFPE) tissue materials from the pathology department were also excluded from the study leaving a total of 65 papillary thyroid microcarcinoma patients.

In the digital archives of the Koç University Hospital, Department of Pathology, a retrospective database search was conducted. The patients were divided into two study groups according to the central lymph node metastasis. Patient demographics and clinicopathological features including histologic subtypes, the presence of lymphocytic thyroiditis, tumor size, multifocality, number of total removed lymph nodes, number of metastatic lymph nodes, size of metastasis, lymphovascular invasion, tumor border pattern (as encapsulated

with/without invasion, or non-encapsulated/overall infiltrative pattern), and desmoplasia, BRAF V600 mutation and Masson trichrome staining for fibrosis were evaluated.

8.2. Immunohistochemistry Analysis

Immunohistochemistry was performed for each case on a selected representative paraffin block, using a Ventana Medical System-Benchmark XT/ISH Staining module (Tucson, AZ). Formalin-fixed paraffin-embedded (FFPE) tissue materials were evaluated by immunohistochemical analyses of hematoxylin–eosin, Masson's trichrome and BRAF V600 Mutation, and compared with clinicopathological information. BRAF was scored as positive and negative.

All tumor samples were re-examined microscopically by two senior pathologists (Dr. Yersu Kapran and Dr. Orhun Çığ Taşkın) to confirm the diagnosis. The cases were classified according to the World Health Organization (WHO) classification of thyroid tumors.

We utilized hematoxylin and eosin (H&E) staining to visualize the microscopic architecture of tissues. To perform H&E staining, we first deparaffinize and rehydrate tissue slices placed on slides using a series of xylene and alcohol washes. Hematoxylin is used to stain the tissue slices after it has been rehydrated. Hematoxylin binds to nucleic acids, causing cell nuclei to appear blue. After the slides are stained with hematoxylin, the excess dye is removed by rinsing them with water and differentiating them in acid alcohol, which leaves just the nuclei stained. The blue coloration of the nuclei is enhanced by further coloring in an alkaline solution or under running water. Following rinsing, eosin is used as a counterstain on the sections, giving the extracellular matrix and cytoplasmic components a pink to red color. After that, the slides are dried using progressively graded alcohols, cleaned in xylene, and covered with a coverslip using a mounting material derived from xylene. The end product allows for the investigation of tissue morphology and disease by revealing precise cellular and tissue structures, with nuclei stained blue and cytoplasm and other tissue components in varied degrees of pink and red.

We begin with tissue preparation and deparaffinization, followed by antigen

retrieval to reveal the epitopes to perform BRAF IHC. Nonspecific binding sites and endogenous peroxidases are inhibited. After applying the main antibody against BRAF V600E, the secondary antibody conjugated to a reporter enzyme such as HRP is used to detect the reaction. The signal is viewed using a chromogenic substrate. If necessary, counterstain is applied before the slides are mounted to prepare the slides proper observation.

In our study, we aim to utilize Masson's trichrome staining IHCWorld kit to visualize and quantify collagen fibers within tissue samples, providing insights into the structural integrity and pathological changes within the extracellular matrix. The protocol begins with deparaffinizing and rehydrating the formalin-fixed, paraffin-embedded tissue sections through 100% alcohol, 95% alcohol, and 70% alcohol, followed by washing in distilled water. To enhance staining quality, sections may be re-fixed in Bouin's solution at 56°C for 1 hour, although this step is optional. The sections are then stained with Weigert's iron hematoxylin working solution for 10 minutes to highlight nuclei, followed by Biebrich scarlet-acid fuchsin solution for 15 minutes to stain cytoplasmic components. Weigert's iron hematoxylin working solution is composed of an equal volume of stock solution A and stock solution B. Stock solution A is prepared with 100 mL 95% alcohol and 1 gram of hematoxylin while stock solution B is composed of 29% ferric chloride in water, concentrated hydrochloric acid, and distilled water. Biebrich scarlet-acid fuchsin solution is prepared by mixing 90 mL Biebrich scarlet, 10 mL acid fuchsin, and 1 mL acetic acid. Following staining, it is washed in distilled water. Differentiation in the phosphomolybdic-phosphotungstic acid solution for 15 minutes ensures collagen fibers no longer appear red, after which they are stained blue with aniline blue solution for 10 minutes. After differentiation, slides are washed with distilled water. The staining process concludes with dehydration through 95% ethyl alcohol, clearing in xylene, and mounting in a resinous medium. This meticulous process allows for the distinctive visualization of collagen fibers in blue, enabling detailed analysis of tissue structure and pathology.

8.3. Scoring the desmoplasia and masson trichrome staining

To analyze the immunohistochemistry (IHC) data, including diagnostic value of certain molecular expression and prognostic relationship of the biomarkers, results of the IHC should be expressed in numerical values for statistical analysis.

Regarding our scoring the samples, a semiquantitative scoring system was used. We incorporate both the extent and intensity of the samples. Briefly, the extent was scored as the percentage of the cells that showed cytoplasmic and/or membranous staining. None= No staining, Focal=Less than 25%, Substantial=25% to 75%, Diffuse= More than 75%. The intensity was scored from 1 to 3 as 1=Weak, 2=Moderate, 3=Strong. The final immune-score is calculated by adding or multiplying each score as an alteration of the Allred score which is used in breast cancer combinative scoring systems (233).

8.4. Statistical Analysis

Statistical analysis was performed using SPSS 18 (IBM Corp. Armonk, NY) software. The Chi-square test and Fisher exact test were used to assess the relationship between categorical variables such as focality. At the same time, continuous variables were presented as mean and standard deviation (SD). Univariate analysis was performed to compare variables among the two groups using Fisher's exact test, unpaired Student's t-test, and Mann-Whitney U test when they were categorical, normally, and non-normally distributed continuous variables, respectively. A multivariate logistic regression model was conducted to assess the factors that were statistically significant in the univariate analysis on the study group. Statistical significance was defined when p value was < 0.05.

8.5. Ethical Approval

This study was approved by the institutional review board. All procedures described in this study were in accordance with national and institutional ethical standards.

Chapter 3
RESULTS

9. RESULTS

9.1. Patient Information

65 cases (48 female, 17 male) were selected based on the inclusion and exclusion criteria. The mean age of patients was 41 years, and the range was between 27 and 71 years of age. Two groups were formed based on LNM positivity (Table 1). A significant difference in age was not recorded between Group-1 (LNM Positive, n=35) and Group-2 (LNM Negative, n=30). The mean age for Group 1 and Group 2 were 41 ± 12.7 years and 45 ± 11.3 years, respectively (p=0.182). Either a lobectomy and central neck dissection (CND) or total thyroidectomy and CND were performed. Among Group-1, lobectomy and CND was performed on 11% (n=4) of patients and total thyroidectomy and CND was performed on 89%. Similarly, lobectomy and CND or total thyroidectomy and CND was performed on 16% (n=5) and 84% (n=25) of patients in Group-2, respectively. The operative strategy did not considerably impact LNM and the difference between Group-1 and Group-2 was insignificant (p=0.542). Although not statistically significant, the total number of resected lymph nodes was higher in Group-1 (9.3 ± 5.6) compared to Group-2 (7.3 ± 2.7) (p=0.076).

9.2. Pathology

A marginal difference between the mean number of tumors was observed between the groups, with Group-1 having an average of 3.1 ± 2.4 tumors compared to 2.2 ± 1.5 in Group-2, although this difference was not statistically significant (p=0.085) (Table 1). However, size of the largest tumor was significantly greater in Group-1 (7.7 ± 1.6 mm) compared to Group-2 (6.5 ± 2.3 mm) (p=0.016). When the largest tumor size was categorized into small (≤ 5 mm) and large (> 5 mm) tumor groups, 91% of patients in Group-1 had large tumors compared to 74% in Group-2

(p=0.052).

Tumor subtype analysis revealed a significant difference (p=0.003), between study groups. Aggressive subtypes such as Tall Cell (n=5) and Infiltrative Follicular (n=5) subtypes were more prevalent in Group-1 whereas subtypes with a favorable prognosis such as Warthin-like (n=3) and Encapsulated (n=3) were dominant in Group-2. While a majority of PTMC were also of classic subtype (n=22) in Group-2, Warthin-like (n=3), Encapsulated (n=3), and Oncocytic (n=2) variants were also observed. BRAF v600 mutation positivity was significantly higher in Group-1 (80%) compared to Group-2 (53%) (p=0.006). The presence of lymphocytic thyroiditis in non-tumoral thyroid tissue was significantly more common in Group-2 (86%) than in Group-1 (20%) (p<0.001).

Table 1. *Comparison of clinical and pathological features between study groups.*

	Group-1 Lymph Node Metastasis Positive N=35	Grup-2 Lymph Node Metastasis Negative N=30	P-Value
Age	41 ± 12.7	45 ± 11.3	0.182
Operation technique - Lobectomy and CND - Total thyroidectomy and CND	 4 (11%) 31 (89%)	 5 (16%) 25 (84%)	0.542
Number of tumors	3.1 ± 2.4	2.2 ± 1.5	0.085
Largest tumor size	7.7 ± 1.6	6.5 ± 2.3	**0.016**
Largest tumor size (group) - ≤ 5 mm - > 5 mm	 3 (9%) 32 (91%)	 8 (26%) 22 (74%)	0.052
Total lymph node resected	9.3 ± 5.6	7.3 ± 2.7	0.076
Tumor subtype - Classic - Tall Cell - Infiltrative follicular - Warthin-like - Encapsulated - Oncocytic	 25 5 5 0 0 0	 22 0 0 3 3 2	**0.003**
BRAF v600 mutation positivity	28 (80%)	16 (53%)	**0.006**
Lymphovascular invasion positivity	20	0	**<0.001**
Lymphocytic thyroiditis in non-tumoral thyroid	7 (20%)	26 (86%)	**<0.001**

All continuous values are mean ± standard deviation. CND: Central neck dissection.

9.3. Desmoplasia Staining

Notable variations in desmoplasia extent score ($p<0.001$) and desmoplasia intensity score ($p<0.001$) between Group-1 (2.2 ± 0.8) and Group-2 (1.4 ± 0.9) were observed (Table 2). In Group-1, no desmoplasia (0), focal desmoplasia (1), substantial desmoplasia (2), and diffuse desmoplasia (3) was observed in 3%, 11%, 49%, and 37% of patients, respectively whereas in Group-2, no desmoplasia, focal desmoplasia, substantial desmoplasia, and diffuse desmoplasia was observed in 17%, 33%, 40%, and 10% of patients, respectively ($p=0.007$). There was also a significant difference in the distribution of desmoplasia intensity scores between groups with the average score for desmoplasia being 2.3 ± 0.7 and 1.3 ± 0.8 for Group-1 and Group-2, respectively ($p<0.001$). In Group-1, moderate (+2) or high (+3) intensity desmoplasia staining was observed in 94% of cases while a majority (65%) of cases I Group-2 showed no (0) or low (+1) intensity staining ($p<0.001$). The desmoplasia total score was calculated as the sum of desmoplasia extent and intensity scores and a significant difference between groups was established ($p<0.001$). Moreover, a majority (51%) of cases in Group-1 received a score of 5 or higher, while most cases (93%) in Group-2 received a score of 4 or lower, demonstrating a correlation between desmoplasia score and LNM ($p<0.001$).

Table 2. *Comparison of Desmoplasia staining cores between study groups.*

	Group-1 Lymph Node Metastasis Positive n=35	Grup-2 Lymph Node Metastasis Negative n=30	P-Value
Desmoplasia extent score	2.2± 0.8	1.4 ± 0.9	**<0.001**
Desmoplasia extent score (categorical)			**0.007**
- no desmoplasia (0)	1(3%)	5 (17%)	
- focal desmoplasia (1)	4 (11%)	10 (33%)	
- substantial desmoplasia (2)	17 (49%)	12 (40%)	
- diffuse desmoplasia (3)	13 (37%)	3 (10%)	
Desmoplasia intensity score	2.3 ± 0.7	1.3 ± 0.8	**<0.001**
Desmoplasia intensity score (categorical)			**<0.001**
- no (0)	1 (3%)	5 (17%)	
- low (+1)	1 (3%)	14 (47%)	
- moderate (+2)	18 (51%)	9 (30%)	
- high (+3)	15 (43%)	2 (7%)	
Desmoplasia total score (extent + intensity)			**<0.001**
- 0	1 (3%)	5 (17%)	
- 2	0 (0%)	7 (23%)	
- 3	4 (11%)	9 (31%)	
- 4	13 (37%)	7 (23%)	
- 5	7 (20%)	0 (0%)	
- 6	10 (29%)	2 (6%)	
Desmoplasia total score group (extent + intensity)			**<0.001**
- ≤ 4	18 (51%)	28 (93%)	
- > 4	17 (49%)	2 (7%)	

Continuous values are given mean ± standard deviation.

Further analyses were conducted to evaluate the impact of lymphocytic thyroiditis on desmoplasia (Table 3). Cases were grouped into lymphocytic thyroiditis positive (n=39) and negative (n=26) groups. While the average desmoplasia extent score was calculated as 2.1 ± 0.8 for the lymphocytic thyroiditis positive group, the average score for the lymphocytic thyroiditis negative group was 1.4 ± 0.9, establishing a significant correlation between lymphocytic thyroiditis and desmoplasia extent score ($p<0.001$). In the lymphocytic thyroiditis positive group, a majority of cases (82%) showed substantial or diffuse desmoplasia. Moreover, 2% of cases showed no desmoplasia (0), 16% showed focal desmoplasia (1), 46% showed substantial desmoplasia (2), and 36% showed diffuse desmoplasia (3). On the other hand, in the lymphocytic thyroiditis negative group (n=26) no desmoplasia (0), focal desmoplasia (1), substantial desmoplasia (2), and diffuse desmoplasia (3) was observed in 19, 30, 43, 7% of cases, respectively. Thus, a significant correlation between desmoplasia extent and lymphocytic thyroiditis was established ($p=0.009$).

A similar correlation was observed between lymphocytic thyroiditis positivity and desmoplasia intensity scoring. The average desmoplasia intensity score was 2.1 ± 0.8 and 1.5 ± 0.9 for the lymphocytic thyroiditis positive and negative groups ($p<0.001$). In the lymphocytic thyroiditis positive group, no desmoplasia (0) was observed in 1 case (2%), low desmoplasia (+1) in 8 cases (21%), moderate desmoplasia (+2) in 16 cases (40%), and high desmoplasia (+3) in 14 cases (36%). Conversely, the lymphocytic thyroiditis negative group, no desmoplasia (0) was observed in 5 cases (19%), low desmoplasia (+1) in 7 cases (26%), moderate desmoplasia (+2) in 11 cases (43%), and high desmoplasia (+3) in 3 cases (12%). Hence, a significant difference in desmoplasia intensity between the two groups was shown ($p=0.036$). A significant difference between desmoplasia total score was also established between the lymphocytic thyroiditis positive and negative groups ($p=0.032$).

Table 3. *Comparison of desmoplasia staining scores between lymphocytic thyroiditis positive and negative thyroid tissues.*

	Lymphocytic thyroiditis positive (n=39)	Lymphocytic thyroiditis negative (n=26)	P-Value
Desmoplasia extent score	2.1 ± 0.8	1.4 ± 0.9	**<0.001**
Desmoplasia extent score (categorical)			**0.009**
- no desmoplasia (0)	1 (2%)	5 (19%)	
- focal desmoplasia (1)	6 (16%)	8 (30%)	
- substantial desmoplasia (2)	18 (46%)	11 (43%)	
- diffuse desmoplasia (3)	14 (36%)	2 (7%)	
Desmoplasia intensity score	2.1 ± 0.8	1.5 ± 0.9	**<0.001**
Desmoplasia intensity score (categorical)			**0.036**
- no (0)	1 (2%)	5 (19%)	
- low (+1)	8 (21%)	7 (26%)	
- moderate (+2)	16 (40%)	11 (43%)	
- high (+3)	14 (36%)	3 (12%)	
Desmoplasia total score (extent + intensity)			**0.032**
- 0	1 (2%)	5 (19%)	
- 2	2 (4%)	5 (19%)	
- 3	8 (21%)	5 (19%)	
- 4	12 (31%)	8 (30%)	
- 5	6 (16%)	1 (4%)	
- 6	10 (26%)	2 (7%)	

Continuous values are given mean ± standard deviation.

Desmoplasia scores were also evaluated for study subgroups of classical subtype PTMC. Two groups were formed based on LNM status among cases of classical subtype PTMC (Table 4). The average desmoplasia extent score was 2.2 ± 0.7 and 1.6 ± 0.8 for the Classical Subtype LNM Positive (n=25) and Classical Subtype LNM Negative (n=22) groups (p=0.012). Although a statistically significant difference was not observed between groups a majority of cases in the Classical Subtype LNM Positive group showed substantial (44%) or diffuse desmoplasia (40%) whereas predominantly focal (32%) or substantial (45%) desmoplasia was observed for Classical Subtype LNM Negative group (p=0.091).

An average desmoplasia intensity score 2.4 ± 0.5 and 1.4 ± 0.8 was observed for the Classical Subtype LNM Positive and Classical Subtype LNM Negative groups, respectively (p<0.001), demonstrating a statistically significant correlation between LNM and desmoplasia intensity. Moreover, the total desmoplasia score also demonstrated a similar trend with the LNM positive group showing higher total desmoplasia scores compared to the LNM negative group (p=0.005).

Table 4. *Comparison of desmoplasia staining scores between study subgroups of classical tumor type.*

	Classical Subtype Lymph Node Metastasis Positive n=25	Classical Subtype Lymph Node Metastasis Negative n=22	P-Value
Desmoplasia extent score	2.2 ± 0.7	1.6 ± 0.8	**0.012**
Desmoplasia extent score (categorical)			0.091
- no desmoplasia (0)	0 (0%)	2 (9%)	
- focal desmoplasia (1)	4 (16%)	7 (32%)	
- substantial desmoplasia (2)	11 (44%)	10 (45%)	
- diffuse desmoplasia (3)	10 (40%)	3 (14%)	
Desmoplasia intensity score	2.4 ± 0.5	1.4 ± 0.8	**<0.001**
Desmoplasia intensity score (categorical)			**<0.001**
- no (0)	0 (0%)	2 (9%)	
- low (+1)	0 (0%)	11 (50%)	
- moderate (+2)	15 (60%)	7 (32%)	
- high (+3)	10 (40%)	2 (9%)	
Desmoplasia total score (extent + intensity)			**0.005**
- 0	0 (0%)	2 (9%)	
- 2	0 (0%)	5 (23%)	
- 3	3 (12%)	7 32%)	
- 4	11 (44%)	6 (27%)	
- 5	3 (12%)	0 (0%)	
- 6	8 (32%)	2 (9%)	

All values are, mean ± standard deviation.

H&E Staining, no desmoplasia

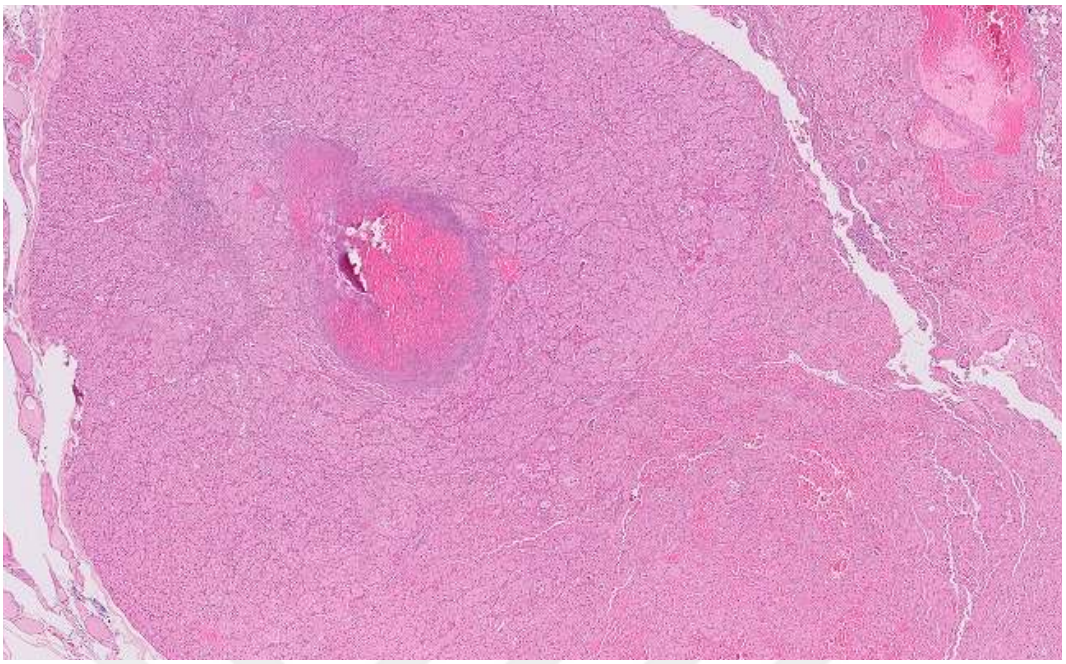

Figure 5. *Hematoxylin-eosin staining of papillary thyroid microcarcinoma specimen with no desmoplasia at 5x magnification.*

H&E Staining, focal extent desmoplasia, low (+1) intensity

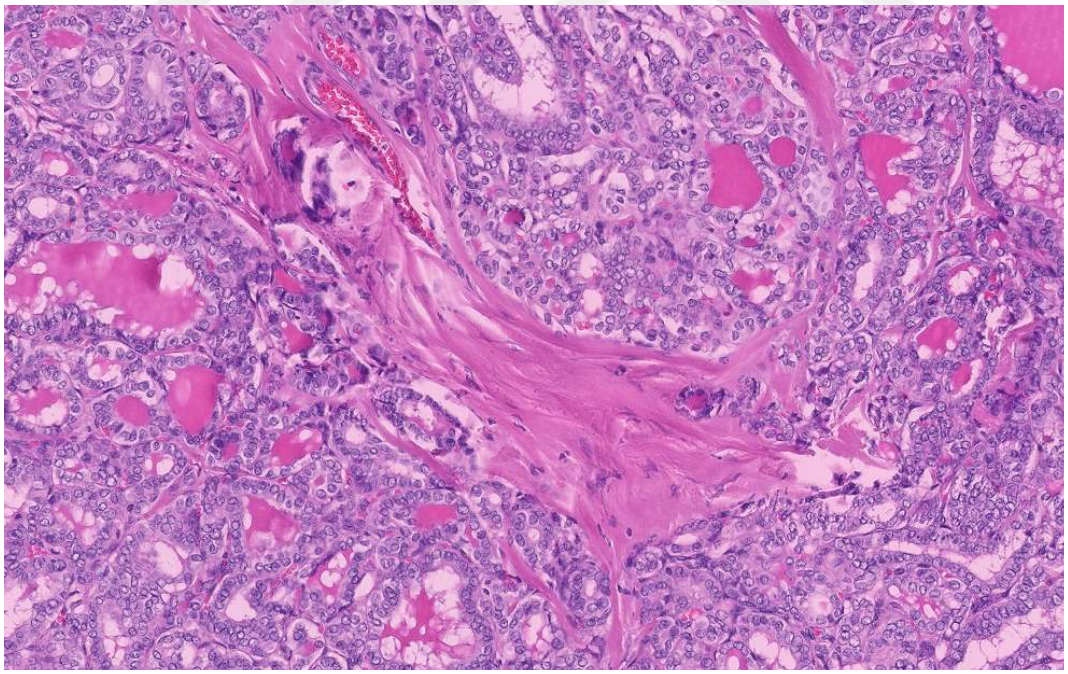

Figure 6. *Hematoxylin-eosin staining of papillary thyroid microcarcinoma specimen shows focal desmoplasia with low (+1) intensity score at 20x magnification.*

H&E Staining, substantial extent desmoplasia, low (+1) intensity

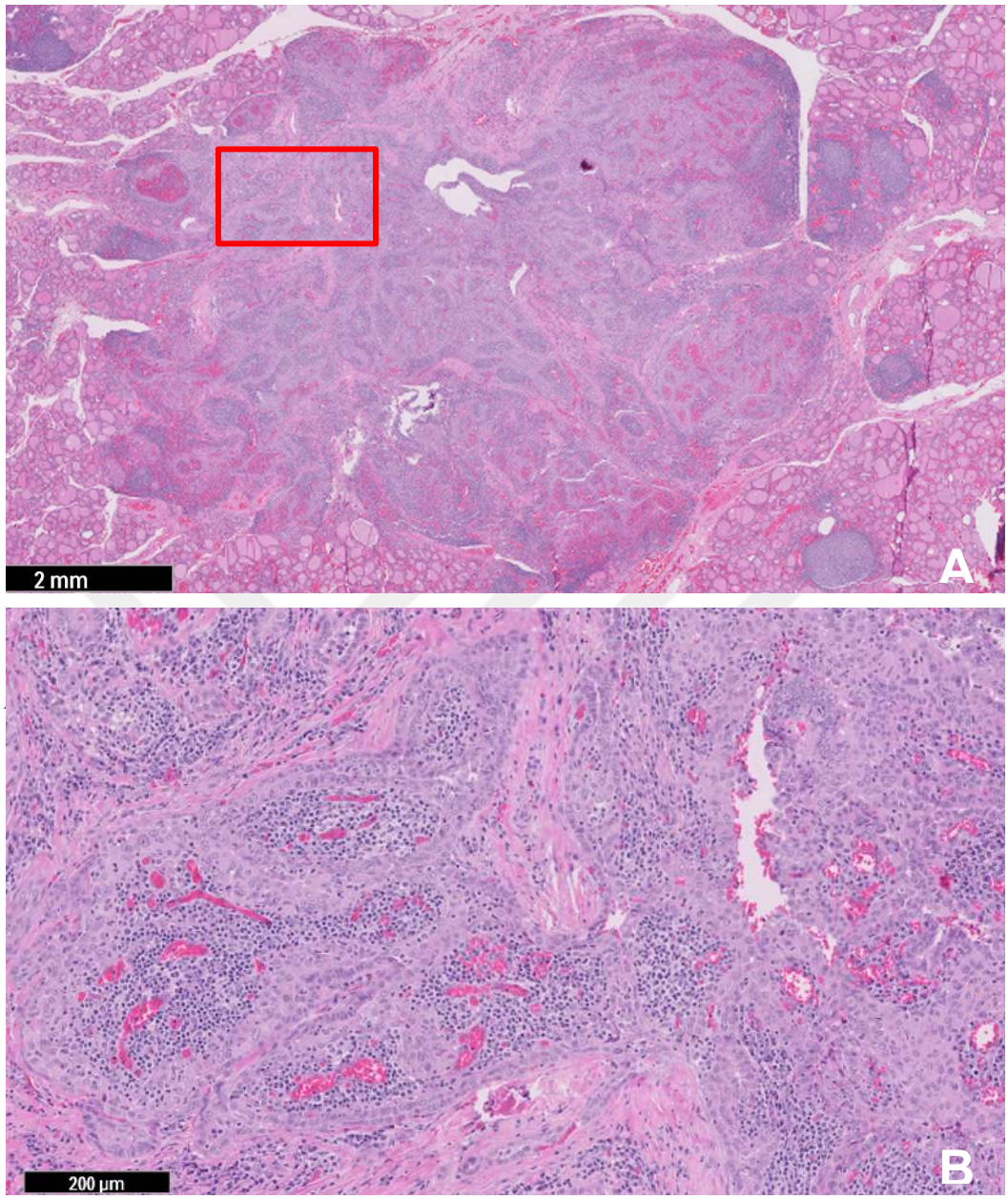

Figure 8. *Hematoxylin-eosin staining of papillary thyroid microcarcinoma specimen shows substantial desmoplasia with low (+1) intensity score at (A) 5x and (B) 20x magnification of the part indicated with the red box on (A).*

H&E Staining, substantial extent desmoplasia, moderate (+2) intensity

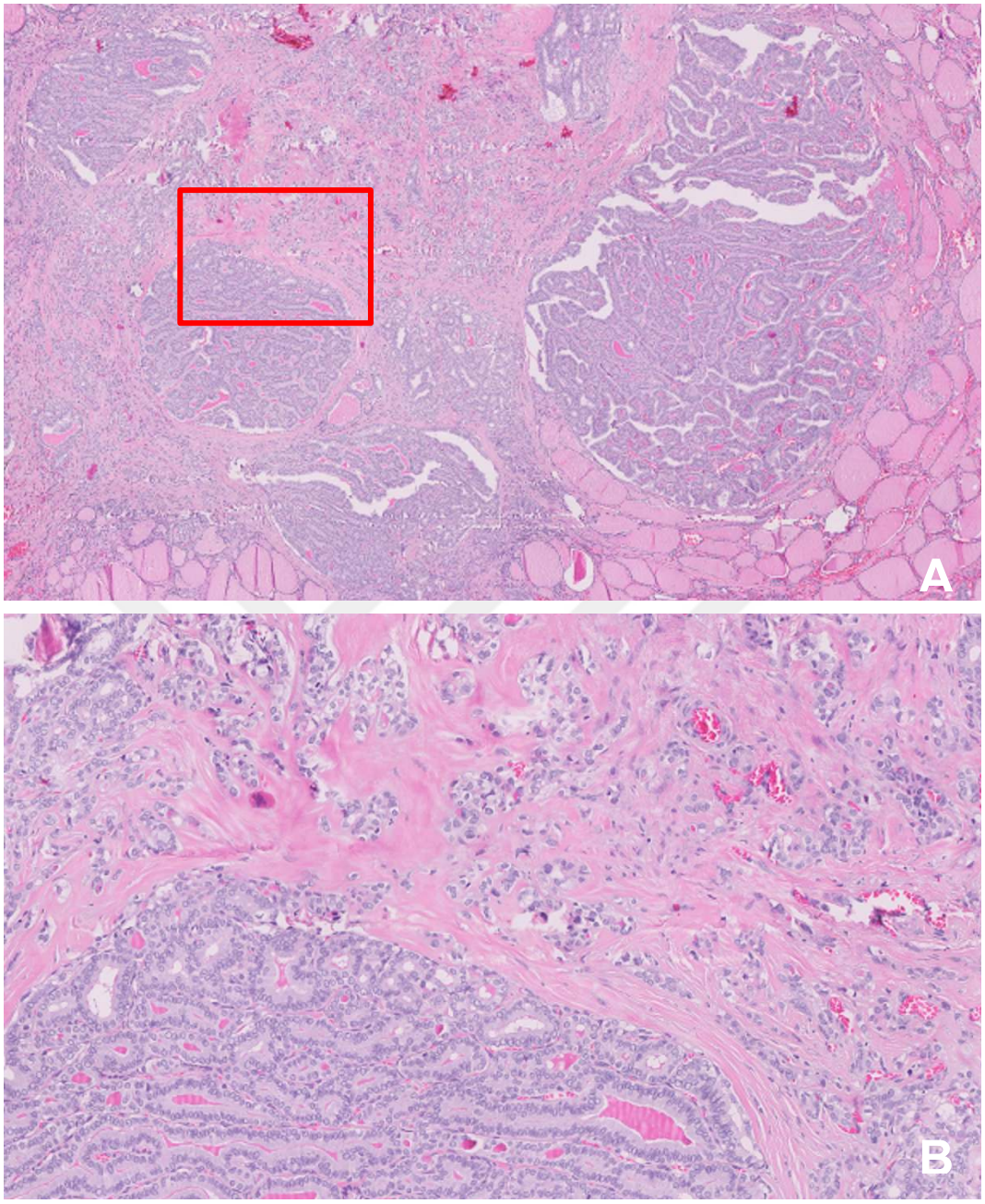

Figure 9. *Hematoxylin-eosin staining of papillary thyroid microcarcinoma specimen shows substantial desmoplasia with moderate (+2) intensity score at (A) 5x and (B) 10x magnification of the part indicated with the red box on (A).*

H&E Staining, substantial extent desmoplasia, high (+3) intensity

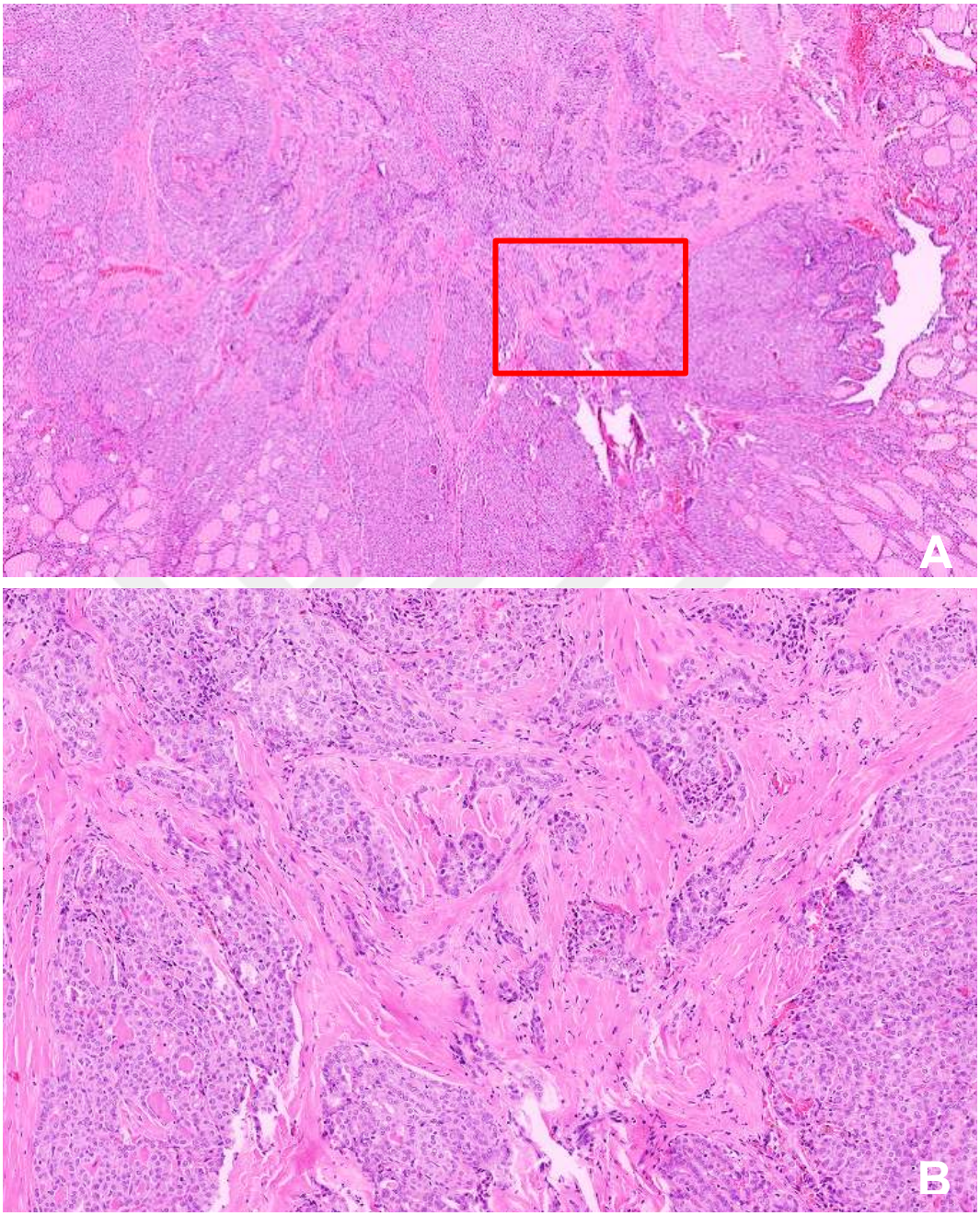

Figure 10. *Hematoxylin-eosin staining of papillary thyroid microcarcinoma specimen shows substantial desmoplasia with high (+3) intensity score at (A) 5x and (B) 10x magnification of the part indicated with the red box on (A).*

H&E Staining, diffuse extent desmoplasia, high (+3) intensity

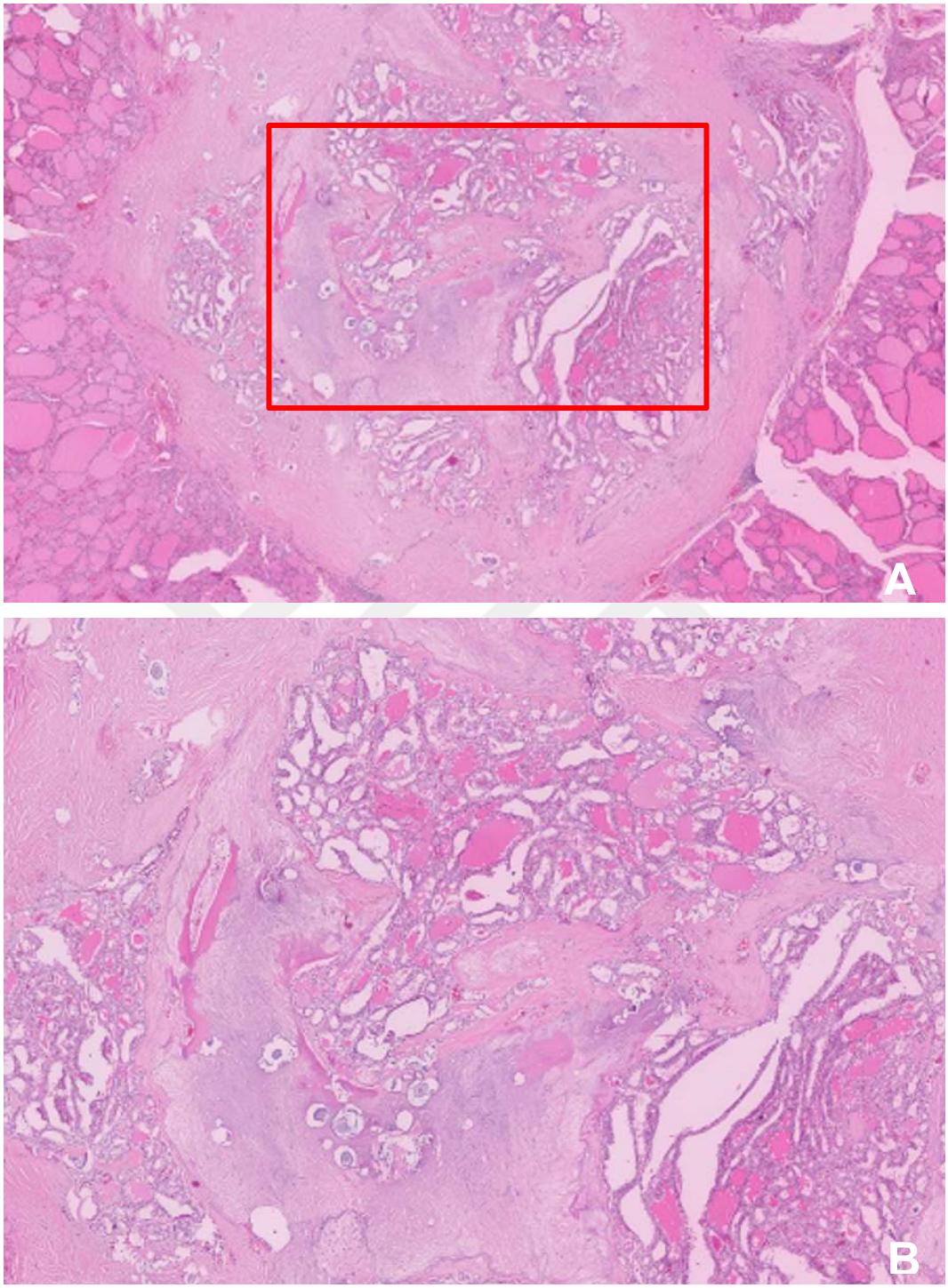

Figure 11. *Hematoxylin-eosin staining of papillary thyroid microcarcinoma specimen shows diffuse desmoplasia with high intensity score at (A) 5x and (B) 10x magnification of the part indicated with the red box on (A).*

9.4. Trichrome Staining

Trichrome staining extent and intensity were analyzed for Group-1 ang Group-2. The average was 2.43 ± 0.6 and 1.7 ± 0.6 for Group-1 and Group-2, respectively ($p<0.001$), demonstrating a strong correlation between LNM and Trichrome staining extent (Table 5). Moreover, 6% of samples from Group-1 showed focal extent staining (1), 46% substantial extent (2), and 48% diffuse extent (3) whereas Out of Group-2, 40% showed focal extent staining (1), 50% substantial extent (2), and 10% diffuse extent (3) ($p<0.001$). This categorical assessment of trichrome staining between the two groups showed a significant difference between groups, indicating a greater deposition of collagen in Group-1 ($p<0.001$).

Similarly, the intensity of trichrome staining was categorically scored. 66% of Group-1 had a score of 3, indicating the greatest intensity. The average Trichrome intensity score was 2.6 ± 0.5 and 1.4 ± 0.7 for Group-1 and Group-2, respectively ($p<0.001$). Only 10% of Group-2 had a score of 3, highlighting the pronounced disparity between the two groups and suggesting a more intense collagen network in LNM positive group ($p<0.001$). When considering the trichrome total score, 40% of Group-1 and 10% of Group-2 showed a score of 6 ($p<0.001$). In total, 74% of Group-1 fell into the category of score >4 compared to 10% of Group-2. This also confirmed the trend of greater collagen deposition and staining with LNM positivity ($p<0.001$).

Table 5. *Comparison of Trichrome staining scores between study groups.*

	Group-1 Lymph Node Metastasis Positive n=35	Grup-2 Lymph Node Metastasis Negative n=30	P-Value
Trichrome extent score	2.43 ± 0.6	1.7 ± 0.6	**<0.001**
Trichrome extent score (categorical) - focal staining (1) - substantial staining (2) - diffuse staining (3)	 2 (6%) 16 (46%) 17 (48%)	 12 (40%) 15 (50%) 3 (10%)	**<0.001**
Trichrome intensity score	2.6 ± 0.5	1.4 ± 0.7	**<0.001**
Trichrome intensity score (categorical) - low (+1) - moderate (+2) - high (+3)	 1 (3%) 11 (31%) 23 (66%)	 19 (63%) 8 (27%) 3 (10%)	**<0.001**
Trichrome total score (extent + intensity) - 2 - 3 - 4 - 5 - 6	 1 (3%) 1 (3%) 7 (20%) 12 (34%) 14 (40%)	 12 (40%) 7 (23%) 8 (27%) 0 (0%) 3 (10%)	**<0.001**
Trichrome total score group (extent + intensity) - ≤ 4 - > 4	 9 (26%) 26 (74%)	 27 (90%) 3 (10%)	**<0.001**

Continuous values are given mean ± standard deviation.

Analyzing the results of trichrome staining score in the presence and absence of lymphocytic thyroiditis yielded statistically significant findings (Table 6). The average Trichrome extent score was calculated as 2.2 ± 0.6 and 1.9 ± 0.8 for the lymphocytic thyroiditis positive and negative groups, respectively (p=0.124). Although the difference in average Trichrome extent score was not statistically significant, categorical analyses demonstrated a significant correlation between Trichrome extent and lymphocytic thyroiditis positivity (p=0.015). In the lymphocytic thyroiditis positive group, 59% and 31% received a score of 2 and 3 for trichrome extent, respectively, demonstrating substantial or diffuse staining. For lymphocytic thyroiditis negative group, 31% scored 2 and 31% scored 3.

Similarly, although the lymphocytic thyroiditis positive group had a higher average Trichrome intensity score (2.2 ± 0.8) compared to the lymphocytic thyroiditis negative group (1.9 ± 0.9), the difference was not statistically significant (p=0.199). Looking into the intensity of Trichrome staining 23% of lymphocytic thyroiditis positive group scored 1, 33% scored 2, and 44% scored 3. In the lymphocytic thyroiditis negative group 42% had a score of 1, 23% a score of 2 and 35% had a score of 3. The difference in the intensity was not statistically significant (p=0.253). Considering the Trichrome total score, a statistically significant difference was noted between the groups (p=0.025).

Table 6. *Comparison of Trichrome staining scores between lymphocytic thyroiditis positive and negative thyroid tissues.*

	Lymphocytic thyroiditis positive (n=39)	Lymphocytic thyroiditis negative (n=26)	P-Value
Trichrome extent score (continuous)	2.2 ± 0.6	1.9 ± 0.8	0.124
Trichrome extent score (categorical)			**0.015**
- focal extent (1)	4 (10%)	10 (38%)	
- substantial extent (2)	23 (59%)	8 (31%)	
- diffuse extent (3)	12 (31%)	8 (31%)	
Trichrome intensity score	2.2 ± 0.8	1.9 ± 0.9	0.199
Trichrome intensity score (categorical)			0.253
- low (+1)	9 (23%)	11 (42%)	
- moderate (+2)	13 (33%)	6 (23%)	
- high (+3)	17 (44%)	9 (35%)	
Trichrome total score (extent + intensity)			**0.025**
- 2	3 (8%)	10 (38%)	
- 3	7 (18%)	1 (4%)	
- 4	11 (28%)	4 (16%)	
- 5	7 (18%)	5 (19%)	
- 6	11 (28%)	6 (23%)	

Continuous values are given mean ± standard deviation.

The correlation between Trichrome staining scores and LNM were also evaluated for study subgroups of classical subtype PTMC. Samples were divided in two groups: Classical Subtype LNM Positive (n=25) and Classical Subtype LNM negative (n=22). A significant difference was observed between the average Trichrome extent score for the Classical Subtype LNM Positive (2.5 ± 0.5) and Classical Subtype LNM Negative (1.8 ± 0.6) groups ($p<0.001$). Among the Classical Subtype LMN Positive group, 48% had a Trichrome extent score of 2 and 52% had a score of 3 whereas in the Classical Subtype LMN Negative group 32% had a score of 1, 59% a score of 2, and 9% a score of 3 ($p<0.001$).

The average Trichrome intensity scores were significantly higher ($p<0.001$) for the Classical Subtype LNM Positive group (2.7 ± 0.4) compared to the Classical Subtype LNM Negative group (1.5 ± 0.7). Moreover, in the Classical Subtype LNM Positive group 28% had a score of 2 and 72% a score of 3, whereas in the Classical Subtype LNM Negative group, 59% had a score of 1, 32% a score of 2, 9% a score of 3. A statistically significant difference was also observed between groups for the Trichrome total score ($p<0.001$), with 80% and 9% of cases in the Classical Subtype LNM Positive and Negative groups scoring a 5 or 6, respectively. Thus, collectively findings suggests that samples of classical subtype with LMN exhibit a greater extend and intensity of collagen deposition.

Table 7. *Comparison of Trichrome staining scores between study subgroups of classical tumor type.*

	Classical Subtype Lymph Node Metastasis Positive n=25	Classical Subtype Lymph Node Metastasis Negative n=22	P-Value
Trichrome extent score	2.5 ± 0.5	1.8 ± 0.6	**<0.001**
Trichrome extent score (categorical) - focal extent (1) - substantial extent (2) - diffuse extent (3)	 0 (0%) 12 (48%) 13 (52%)	 7 (32%) 13 (59%) 2 (9%)	**<0.001**
Trichrome intensity score	2.7 ± 0.4	1.5 ± 0.7	**<0.001**
Trichrome intensity score (categorical) - low (+1) - moderate (+2) - high (+3)	 0 (0%) 7 (28%) 18 (72%)	 13 (59%) 7 (32%) 2 (9%)	**<0.001**
Trichrome total score (extent + intensity) - 2 - 3 - 4 - 5 - 6	 0 (0%) 0 (0%) 5 (20%) 9 (36%) 11 (44%)	 7 (32%) 6 (27%) 7 (32%) 0 (0%) 2 (9%)	**<0.001**

All values are, mean ± standard deviation.

Trichrome staining focal extent, low (+1) intensity

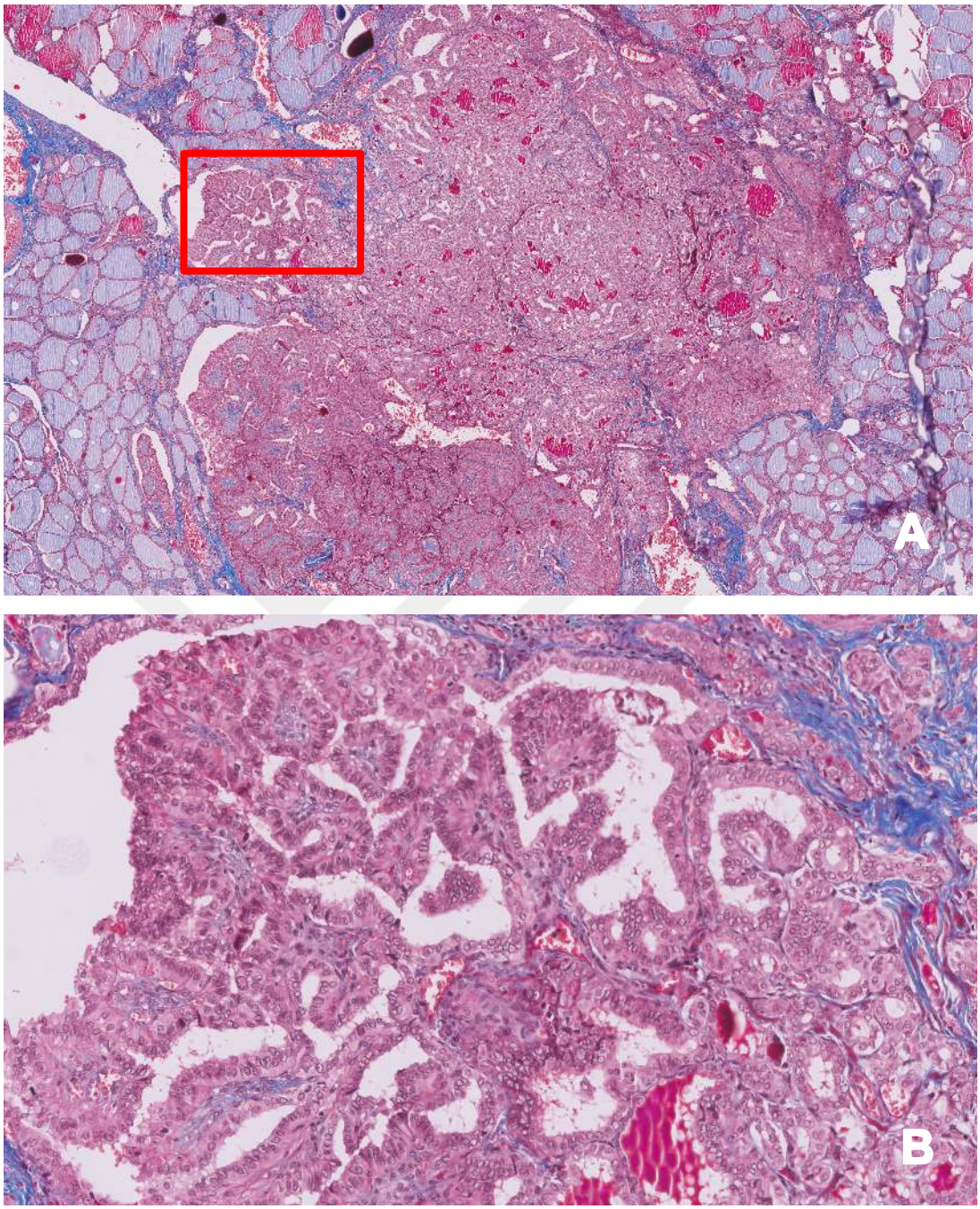

Figure 12. *Trichrome staining of papillary thyroid microcarcinoma specimen shows focal extent with low (+1) intensity score at (A) 5x magnification and (B) 20x magnification of the part indicated with the red box on A.*

Trichrome staining focal extent, moderate (+2) intensity

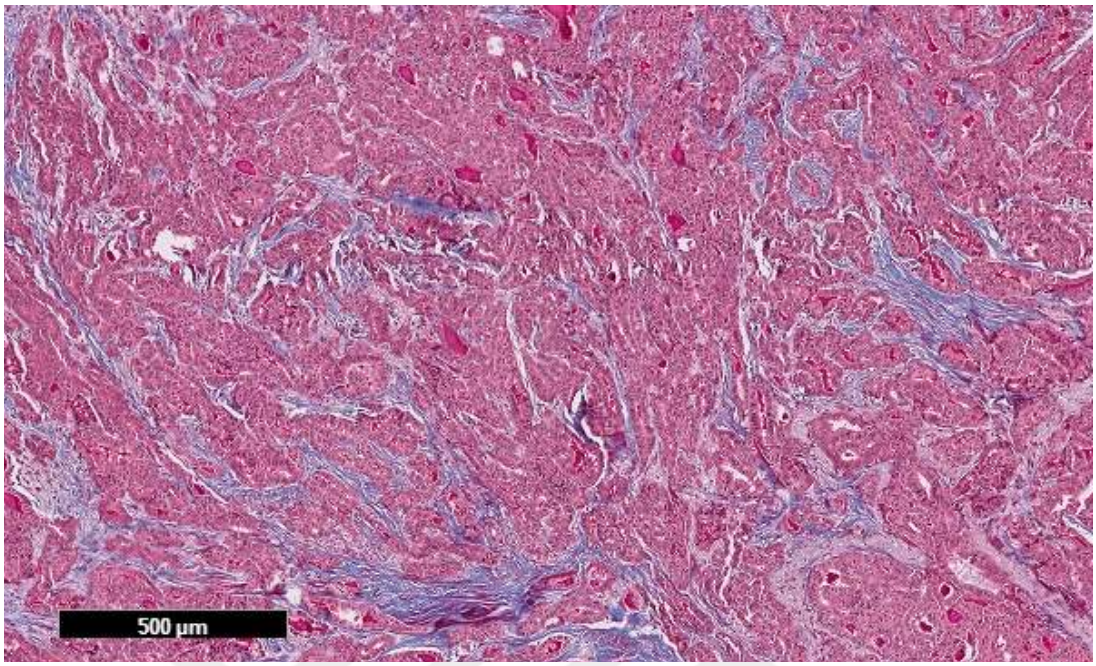

Figure 13. *Trichrome staining of papillary thyroid microcarcinoma specimen shows focal extent with moderate (+2) intensity score at 10x magnification.*

Trichrome staining substantial extent, low (+1) intensity

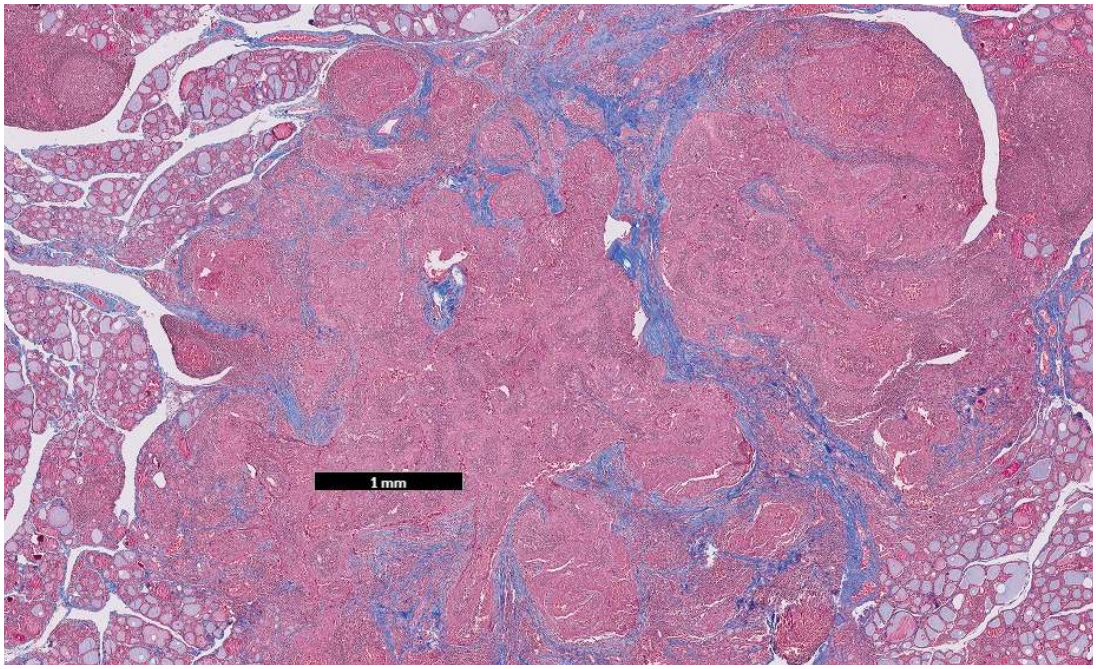

Figure 14. *Trichrome staining of papillary thyroid microcarcinoma specimen shows substantial extent with low (+1) intensity score at 5x magnification.*

Trichrome staining substantial extent, moderate (+2) intensity

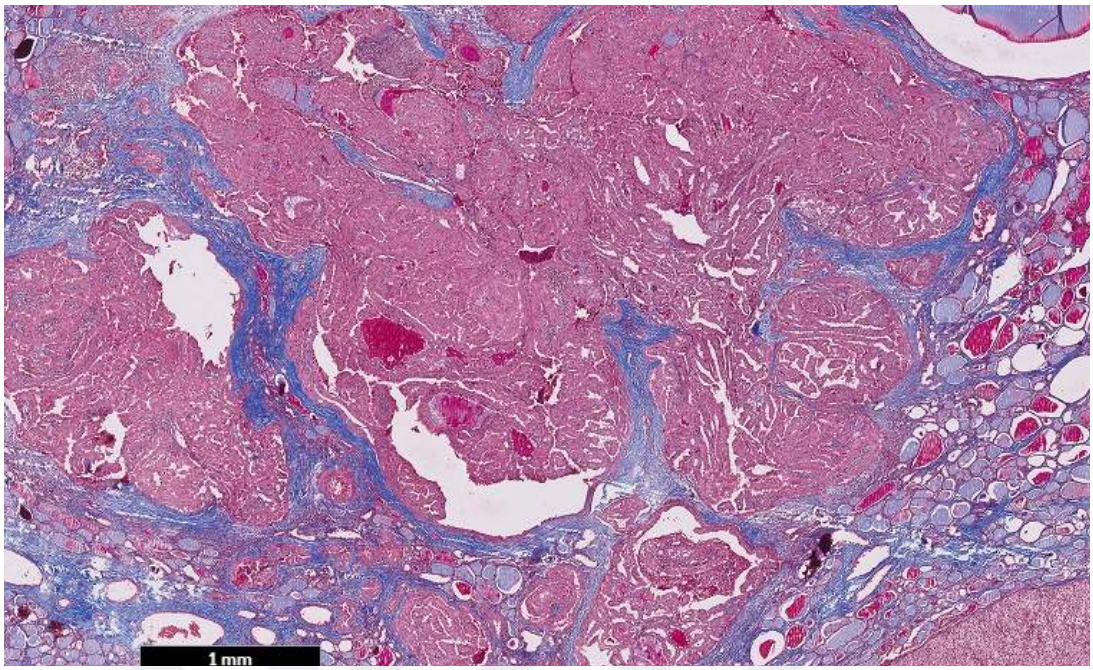

Figure 15. *Trichrome staining of papillary thyroid microcarcinoma specimen shows substantial extent with moderate (+2) intensity score at 5x magnification.*

Trichrome staining substantial extent, high (+3) intensity

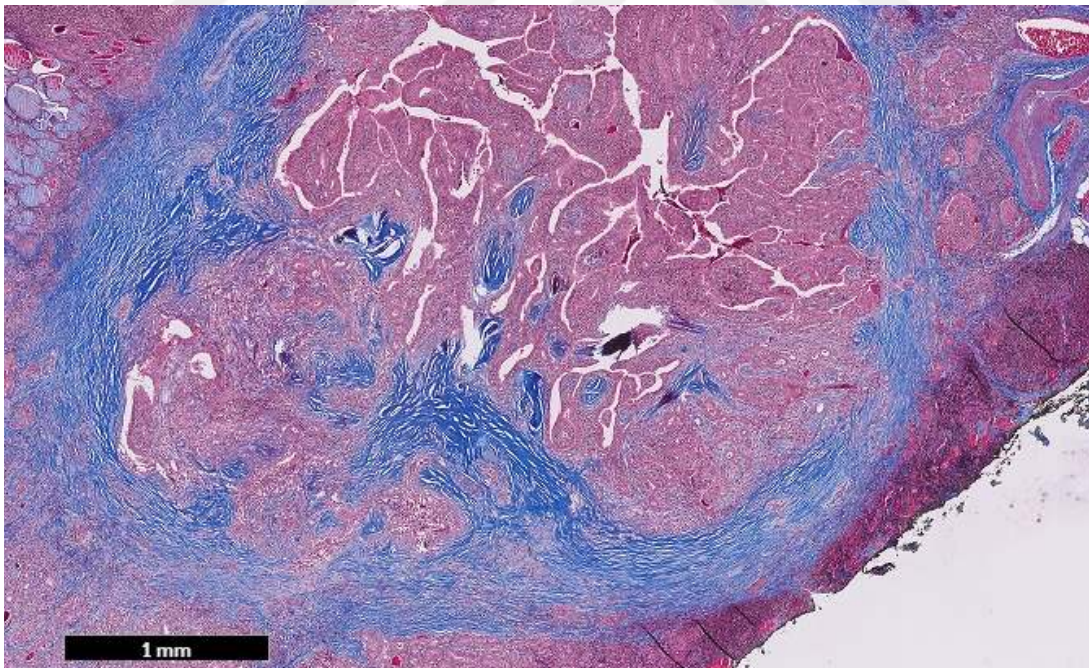

Figure 16. *Trichrome staining of papillary thyroid microcarcinoma specimen shows substantial extent with high (+3) intensity score at 5x magnification.*

Trichrome staining diffuse extent, low (+1) intensity

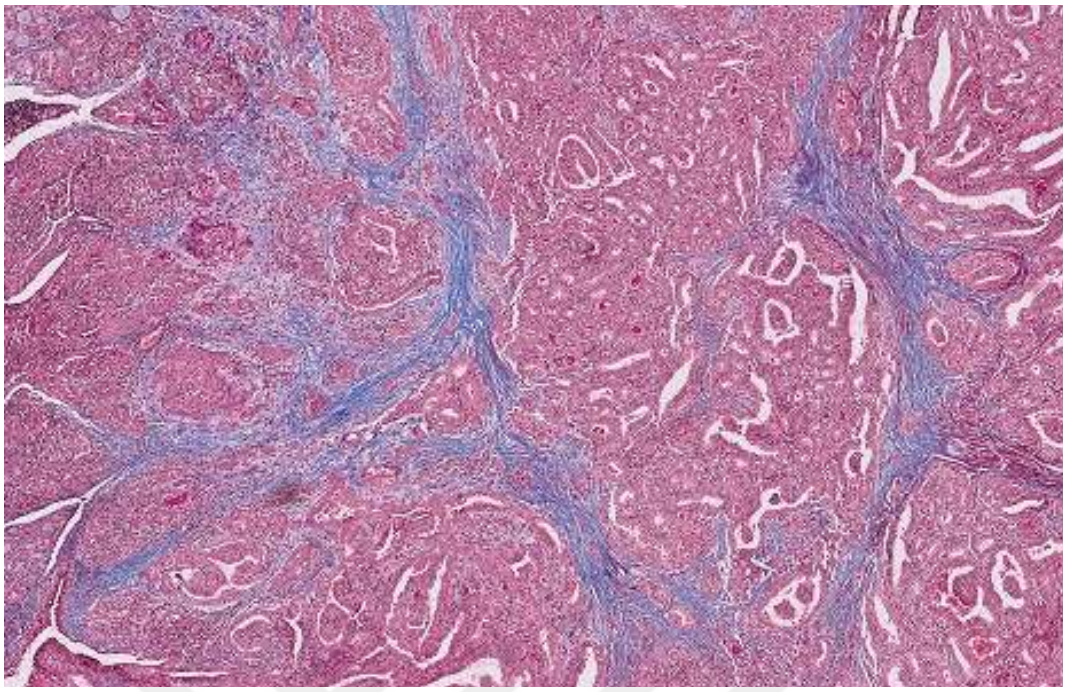

Figure 17. *Trichrome staining of papillary thyroid microcarcinoma specimen shows diffuse extent with low (+1) intensity score at 5x magnification.*

Trichrome staining diffuse extent, moderate (+2) intensity

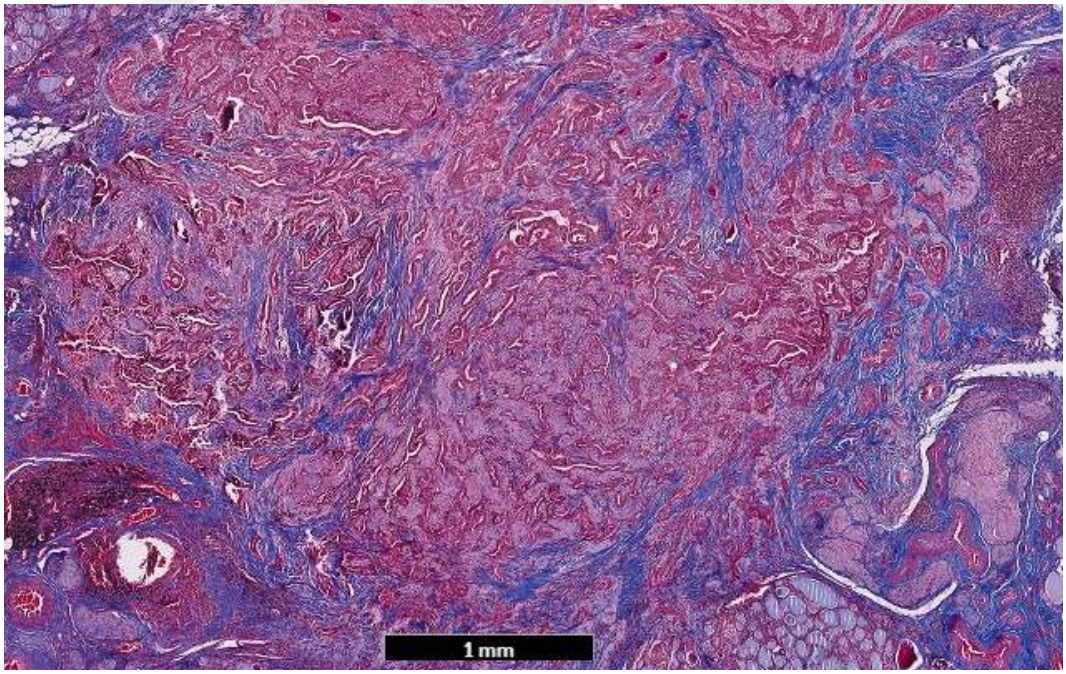

Figure 18. *Trichrome staining of papillary thyroid microcarcinoma specimen shows diffuse extent with moderate (+2) intensity score at 5x magnification.*

Trichrome staining diffuse extent, high (+3) intensity

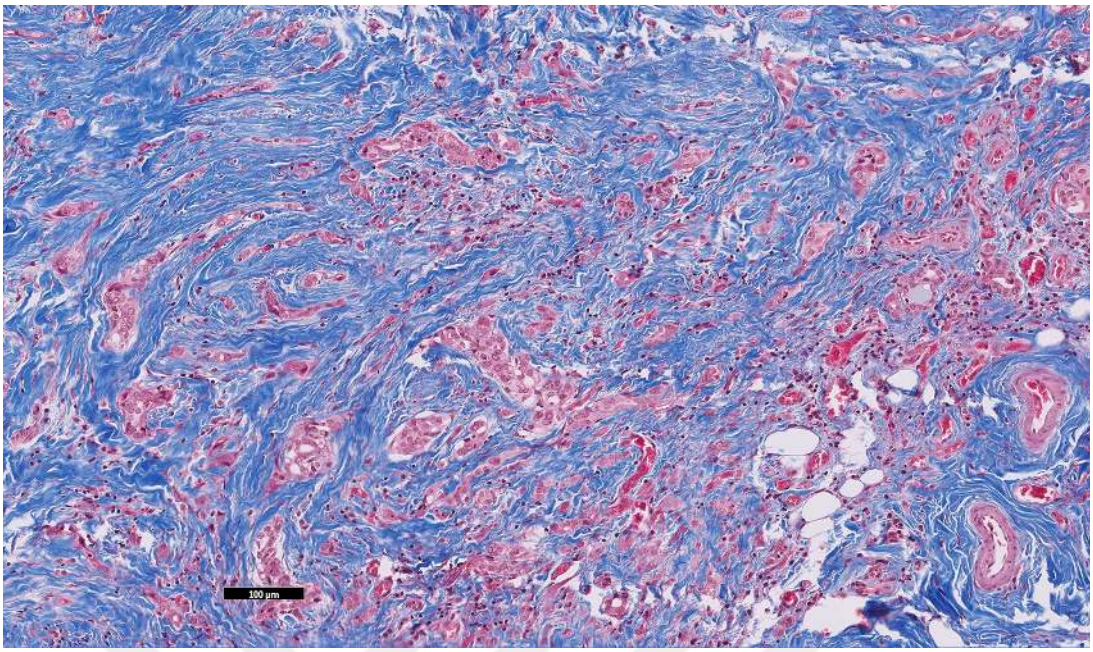

Figure 19. *Trichrome staining of papillary thyroid microcarcinoma specimen shows diffuse extent with high (+3) intensity score at 10x magnification.*

9.5. Multivariate Analyses

In order to predict the presence of positive LNM, multivariate analysis was performed using logistic regression analysis. Two models were created based on desmoplasia and trichrome extent score in separate analyses, including lymphovascular invasion, BRAF mutation positivity, tumor size, which were significant predictors of lymph node metastasis in univariate analyses.

Table 8. *Comparison of desmoplasia and trichrome scores between study subgroups of classic tumor type in patients without lymphocytic thyroiditis.*

	Classical Subtype Lymph Node Metastasis Positive n=10	Classical Subtype Lymph Node Metastasis Negative n=5	P-Value
Trichrome extent score	2.6 ± 0.5	1.2 ± 0.4	**<0.001**
Trichrome intensity score*	2.7 ± 0.5	1 ± 0	**<0.001**
Desmoplasia extent score	1.8 ± 0.6	1 ± 0.7	**0.04**
Desmoplasia intensity score	2.1 ± 0.3	0.8 ± 0.4	**<0.001**

All continuous values are, mean ± standard deviation.
**Mann-WhitneyU test is performed since the data is not suitable for normal distribution.*

Table 9. *Comparison of desmoplasia and trichrome scores between study subgroups of classic tumor type in patients with lymphocytic thyroiditis.*

	Classical Subtype Lymph Node Metastasis Positive n=15	Classical Subtype Lymph Node Metastasis Negative n=17	P-Value
Trichrome extent score	2.5 ± 0.5	1.9 ± 0.5	**<0.01**
Trichrome intensity score*	2.7 ± 0.4	1.6 ± 0.7	**<0.001**
Desmoplasia extent score	2.5 ± 0.6	1.8 ± 0.8	**0.01**
Desmoplasia intensity score	2.6 ± 0.5	1.6 ± 0.8	**<0.001**

All continuous values are, mean ± standard deviation.
** Mann-WhitneyU test is performed since the data is not suitable for normal distribution.*

In the desmoplasia extension model, it was established that substantial and diffuse desmoplasia increases the probability of LNM by 7.5 and 13.6 times independently (Nagelkerke R square score is 0.69 (p=0.047 and p = 0,02)) (Table 10). The same analysis was conducted for the trichrome model. Substantial and diffuse trichrome extent increases the risk of LNM by 8.3 and 9,8 times respectively. (Nagelkerke R square score is 0.68. (p=0.02 and p= 0,03) (Table 11).

Table 10. *Multivariate analysis of desmoplasia extent score, tumor size and BRAF mutation positivity which have correlation with lymph node metastasis.*

	B	S.E.	Wald	df	Significance	Exp (B)
Lymphovascular invasion	-22,440	8598,767	0,000	1	0,998	0,000
Tumor size	-0,252	0,215	1,366	1	0,242	0,777
BRAF Mutation positivity	-0,799	1,126	0,503	1	0,478	0,450
No desmoplasia			6,345	3	0,096	
Focal	1,718	1,645	1,090	1	0,296	5,572
Substantial	2,019	1,015	3,953	1	0,047	7,530
Diffuse	2,613	1,116	5,480	1	0,019	13,641

Table 11. *Multivariate analysis of trichrome extent score, tumor size and BRAF mutation positivity which have correlation with lymph node metastasis.*

	B	S.E.	Wald	df	Significance	Exp (B)
Lymphovascular invasion	-21,877	8533,117	0,000	1	0,998	0,000
Tumor size	-0,184	0,199	0,851	1	0,356	0,832
BRAF Mutation positivity	-1,074	0,962	1,246	1	0,264	0,342
Focal			6,680	2	0,035	
Substantial	2,121	0,906	5,477	1	0,019	8,342
Diffuse	2,288	1,075	4,532	1	0,033	9,851

Chapter 4

DISCUSSION AND CONCLUSION

10. DISCUSSION

In trying to minimize unnecessary procedures while maintaining the highest degree of patient safety, PTMC treatment preferences have varied among clinicians, ranging from active surveillance to prophylactic central neck dissections. It has been argued that minimizing invasive diagnostic and therapeutic procedures in the management of PTMC should be prioritized due to the indolent and often subclinical nature of the lesions (180, 234). Such strategies would also play a role in reducing treatment costs for patients. However, despite remaining quiescent for most patients, PTMC has been frequently associated with a multifocal presentation, which has been associated with a more aggressive course of disease progression. Furthermore, it has been demonstrated that 20-46% of PTMC cases are multifocal (179). Similarly, although PTMC is characterized by low aggressiveness and low potential for distant metastasis, high recurrence rates (40% at 30 years for patients over 55, and 10% for younger patients) have been recorded in literature (235). In addition to age and multifocality, other prognostic factors including radiation exposure have been associated with an increased risk for PTMC progression and metastasis (188). Therefore, the diagnostic and therapeutic algorithm for PTMC is still disputed. Moreover, although recent ATA guidelines do not recommend performing FNAB for thyroid nodules <10mm, according tp a survey of 661 members of The Endocrine Society, 454 American Association of Clinical Endocrinologists members, and 365 ATA members, 67% of clinicians concluded that they would perform FNAB for thyroid nodules <10mm if US demonstrated features suggestive of malignancy (i.e. irregular margins, hypoechoic nodules, taller than wide shape, microcalcifications, microlobulations, rim calcifications, or soft tissue components) (193, 236). Likewise, although conservative management strategies such as active surveillance for low-risk PTMC have been suggested, these have not been widely adopted. In fact, a recent retrospective study (n=4923) has

demonstrated that at least one high-risk clinicopathological feature (aggressive PTMC subtype, multifocality, LVI, perineural invasion, ETE) was observed in 45.7% of patients that could be categorized as low-risk based on ATA guidelines (237). All included patients had undergone lobectomies and completion thyroidectomy was performed on 3.5% of PTMC initially described as low risk. The clinical decision to perform a completion thyroidectomy was made based on the identification of LVI, aggressive variants, or ≥ 5 metastatic lymph nodes. As such, it was demonstrated that in clinical practice risk stratification might fall short in assessing PTMC progression and that more reliable tools to predict PTMC prognosis is required.

Hence, while some have argued that the indolent nature of PTMC does not warrant radical surgical approaches and that PTMC are commonly overtreated, others have reasoned that the risk for LNM (undetected using conventional US at the time of PTMC diagnosis) is high (up to 50.7%), necessitating therapeutic surgical interventions to prevent recurrence and future complications (238). Several studies have aimed to elucidate mechanisms leading to recurrence. Among other variables, the role of PTMC characteristics (i.e. histopathological and clinical presentation) and surgical strategies in recurrence have attracted significant attention over the past few decades. For instance, among 2070 patients with PTMC, tumor recurrence was experienced for 3.5% (235). Recurrence was reported in 3.3% and 14% of patients with PTMC $\leq$5mm and 6-10mm, respectively. A meta-analysis of 3,523 patients categorized PTMCs into two groups (239). The recurrence rate for the nonincidental and incidental groups were 0.1% and 7.9%, respectively. 80% of recurrent PTMC were associated with lymph node involvement and distant metastases were observed for 13% of recurrences. In another meta-analysis of 15 studies recurrence rates of 2.83% and 2.84% were recorded for patients who underwent total and non-total thyroidectomy, respectively (240). Similarly, in a study of 22,283 cases of PTMC without lymph node involvement or distant metastases, it was demonstrated that surgical approach (lobectomy vs. total thyroidectomy) did not significantly impact OS or disease specific survival (241). Age, sex, and multifocality were identified as prognostic determinants impacting OS, with older age, male sex, and multifocal disease associated with poorer prognosis. Contrary to these findings, a meta-analysis of 11 cohort studies (13,801 patients) revealed that while differences in mortality rates were not observed

between patients who received a total thyroidectomy compared to a lobectomy, recurrence rates were lower in the total thyroidectomy group, suggesting that surgical approach can be instrumental in preventing recurrence (242).

Over the past few years, predictive algorithms and nomograms to identify risk factors for LNM or aggressive progression in PTMC have been employed. For instance, data from 7,344 PTMC patients categorized as low-risk (clinically N0 and lack of gross ETE) were used to generate a nomogram to predict increased risk for LNM in low-risk patients (243). Such models have reduced reliance on cut-off values and enabled the classification of features like age and tumor size as continuous variables, thereby potentially allowing a more nuanced analysis of risk factors. Although promising, one of the major challenges associated with the development of such models is the integration of all relevant risk factors. As such, some models have focused on demographic characteristics (such as age, sex, comorbidities such as Hashimoto's thyroiditis) while others have looked into tumor characteristics (tumor location on thyroid gland, liquefaction, and microcalcifications (243, 244). Recently, in addition to the use of predictive algorithms and nomograms, machine learning (ML) based tools have been developed to empower pre-operative clinical decision making for PTMC (245). A gradient-boosting decision tree (GBDT) model was developed by integrating an assessment of radiomics features of PTMC on US images and independent risk factors (sex, age, body mass index (BMI), and serology including TSH, Tg, and anti-thyroglobulin antibodies). The GBDT model was used to predict CLNM in PTMC. Results demonstrated that the model outperformed the clinical assessment made based on stand-alone US findings, despite a small sample size (274 patients) and omission of criteria that could influence CLNM (i.e. BRAF mutational status or analysis of invasion characteristics). Similarly, other ML models were trained using radiomics features extracted from CT images and risk factors (sex, age, tumor size, and nodal status) to non-invasively predict CLNM in 452 PTMC patients (246). The ML-based models exceeded radiologists' prediction of CLNM, suggesting that such tools can be employed to facilitate clinical decision making. In addition to traditional regression analysis, several other ML-based tools have been developed using various algorithms including extreme gradient boosting, random forest, adaptive boosting, gaussian naive bayes, and multi-layer perceptron to assess risk factors for LNM in PTMC on a population-based study, with extreme gradient

boosting appearing superior over other algorithms (247). Although not yet suitable for daily clinical practice yet, studies have demonstrated the role of ML-based strategies in developing tools to support clinical decision-making in PTMC management, particularly in predicting prognosis and metastatic potential of PTMC.

At our clinical practice, we have observed that the incidence of micrometastases of PTMC is significantly higher than that suggested in literature (184). It has been well established that LNM increases the risk for recurrence (248). However, a consensus on risk factors for LNM has not been reached yet and no molecular or clinicopathological marker has been reliably used to predict LNM in PTMC patients. Similarly, despite the development of promising ML-based tools, current understanding of PTMC LNM remains lacking. Therefore, the aim of this study was to identify molecular markers and clinicopathological features that can be employed in evaluating the prognosis of PTMC, particularly to predict LNM.

10.1. The Role of Clinicopathological Features of PTMC in Prognostic Evaluation

The role of clinicopathological features have been thoroughly investigated in literature to identify factors potentially leading to CLNM, LLNM, poor prognosis, and aggressive progression. In a meta-analysis of 4,573 patients, male gender, age <45 years, tumor size >5mm, multifocality, ETE, and LVI were correlated with a higher incidence of CLNM of PTMC (187). Similar results were obtained in another meta-analysis that included 1066 patients (249). Multifocality was associated with the highest risk of CLNM. In addition to multifocality, male gender, age ≤45 years, tumor size >6mm, and ETE were correlated with an increased risk of CLNM. Multifocality, ETE, and CLNM were identified as risk factors for LLNM. Additionally, it was demonstrated that LLNM is more commonly observed in patients over the age of 55 years (250). In another meta-analysis of 8,345, male gender, age <45 years, tumor size >5mm, multifocality, and ETE were correlated with a higher incidence of CLNM (186).

Aggressive Subtypes

Subtypes of PTC hold a clinical significance due to their aggressive nature. WHO

classification from 2022 has highlighted that compared to classic PTC type, the tall cell, columnar cell, and hobnail subtypes of PTC present significantly more aggressive clinicopathological features (251). A large study of 11,570 patients has classified diffuse cell, solid, tall cell, columnar cell, and hobnail subtypes as aggressive based on their exhibition of aggressive characteristics (i.e. ETE or metastasis to lymph nodes) (252). Similarly, the tall cell variant has been identified as an aggressive variant based on ETE, LVI, and LNM behaviors (189). While aggressive behavior has been associated with the tall cell and diffuse sclerosing variants, no differences in overall survival was recorded on a propensity score matched study that included 83,198 patients (253). Microfollicular variant PTMCs have been associated with a 26% increased risk of multifocality and twice the likelihood of LNM compared to the classical variant based on multivariate analyses of 30,926 cases (254).

In our study apart from the classical subtype of PTC, there was not enough data on the other subtypes, which presents as a potential limitation to our study. Nevertheless, the subtypes that were found in the metastatic group are Tall Cell (n=5) and Infiltrative follicular (n=5) and the subtypes found in the non-metastatic group were Warthin-like (n=3), Encapsulated (n=3) and Oncocytic (n=2). Infiltrative follicular type and Tall Cell were found in the group with LMN. This is parallel to the literature given the evidence for their aggressive nature (72, 74). Although Whartin and Encapsulated subtype have a good prognosis with a low rate of LNM, the concomitant presence of desmoplasia and the extent of trichrome staining was significant. Hence, desmoplasia and trichrome staining might not be utilized as valuable prognostic factors in those two subtypes.

Larger Tumor Size

The correlation between the size of PTMC and the tumor's ability to metastasize has been studied extensively. A clinicopathological study of 126 patients has demonstrated that large PTMC size (>5mm) is associated with a higher risk of LNM (190). Similar findings have been demonstrated in other single-center studies as well as several meta-analyses suggesting that larger tumors are more likely to present with LNM (186, 187, 249, 255). In addition to a higher risk of LNM, larger tumor sizes (>5mm) have also been linked to a higher incidence of bilateral

presentation, ETE, and CLNM (250). However, some studies have also established that the size of PTMC does not significantly impact prognosis as larger size is not correlated strongly to recurrence (256).

In this study, when the tumor size was analyzed as a continuous variable a significant difference was not observed. However, parallel to the literature, the findings of this research showed that 91% of the tumors with LMN had a size >5 mm (p=0.052).

Lymphovascular Invasion

Some PTMCs have been shown to demonstrate aggressive progression including higher metastatic potential and higher rate of recurrence. These aggressive PTMCs have largely been attributed to the presence of advanced features at initial presentation (257). Advanced features include CLNM, LLNM, mETE, ETE, LVI, and distant metastasis. Among 30,180 PTMC patients, 18.7% presented with at least one of these features and among these aggressive features only mETE and LVI were not associated to a lower survival rate. Notwithstanding, both mETE and LVI were linked to nodal metastasis and LNM (both CLNM and LLNM). ETE was also associated with distant metastasis, thereby suggesting that mETE and LVI can play a significant role in the pathogenesis of LNM. Moreover, in several meta-analyses, invasive characteristics such as ETE, capsule invasion, and LVI have been identified as risk factors in PTMC metastasis (186, 187, 249). In a retrospective study evaluating 814 PTMC patients, it was reported that even patients with minimal ETE were at a greater risk of LNM compared to those without ETE (143). Similar to ETE, LVI and capsule invasion have also been considered poor prognostic features and have been associated with a higher risk of developing LNM (190, 258, 259). However, other studies have suggested that minimal or microscopic ETE is associated with multifocality and larger tumor sizes, but does not have a significant impact on clinical progression and recurrence unless gross ETE is also observed (260). Similarly, some studies have found no association between ETE or LVI and LNM (261).

Alongside LVI, extrathyroidal and capsular invasion can be observed in cancerous tissue. Since PTMC is <10mm, in literature, extrathyroidal invasion of PTMC is noted to be rare. However, in some subtypes of PTMC cancer LVI and

capsular invasion are common, and it is well established that they are correlated to LMN. Our results have confirmed these findings and suggest that LVI was significant for LMN ($p<0.001$).

Multifocality

Multifocality has also been recognized as a prognostic factor in PTMC with higher rates of LNM observed for multifocal PTMC compared to unifocal PTMC (186, 187, 249). However, some studies have found no difference between the metastatic potential of multifocal versus unifocal tumors and have not identified multifocality as a significant risk factor in evaluating the prognosis of PTMC (190, 262). Notably, in a retrospective study that included 975 females (between the ages of 18-55) the role of multifocality on CLNM was investigated and results demonstrated that while two foci did not significantly increase the incidence of CLNM compared to unifocal PTMC, the risk for CLNM was significantly higher for patients with multifocal disease (>2 foci) (263). A different trend was observed in males (between the ages of 18-55): CLNM risk appeared to increase significantly when unifocal and multifocal (2 foci) PTMC were compared. The allocation of the tumor to the isthmus and the presence of multiple foci is significant in thyroid cancer.

The findings of this study showed no correlation between LMN and multifocality ($p=0.085$). However, a potential explanation for this observation lies in the possibility of each focus could harbor a different subtype of thyroid carcinoma. While a biopsy done on the dominant nodule might present a classical type of thyroid cancer, a biopsy done on smaller foci might show a more aggressive subtype such as TCV. Since the analyses in this study were conducted on the dominant nodule, this presents as a limitation to the results. Extensive molecular investigation on all present foci could lead to a better understanding of the nature of tumor subtypes.

10.2. The Role of BRAF Mutations in Prognostic Evaluation

Several studies of varying cohort sizes have assessed the recurrence rate of PTMC and potential risk factors that predispose PTMC patients to experience recurrence have been evaluated. However, currently no molecular marker can definitively

predict recurrence potential. $BRAF^{V600E}$ point mutations have been associated with higher recurrence rates in PTMC as well as higher stages and metastasis in PTC (264-266). Similarly, despite the lack of agreement among various studies, associations between $BRAF^{V600E}$ mutations and older age, male gender, tumor size, and ETE have been described (81). A study that included 2245 patients demonstrated that $BRAF^{V600E}$ mutations are associated with a recurrence risk of 20% in PTMC (266). Similarly, in a retrospective which included 2,717 patients (60 of whom experienced recurrence) BRAF mutational status was identified as a risk factor for recurrence (267). However, assessment of BRAF status had only been conducted on 311 patients, suggesting that selective bias could have influenced results. Another retrospective study (n=400) identified that patients with the $BRAF^{V600E}$ mutation had a higher risk of CLNM (268). Notably, among patients with the $BRAF^{V600E}$ mutation, clinically N0 PTMC patients who were treated with a prophylactic central neck dissection had a worse 3-year disease-free survival rate than those who received a therapeutic central neck dissection for clinically N1 PTMC. It has also been established that BRAF mutations result in impaired NIS-dependent ^{131}I uptake, which could help explain findings demonstrating higher risk of recurrence in BRAF mutated PTC (269). Such findings have suggested the use of $BRAF^{V600E}$ not only as a prognostic marker predicting tumor progression, LNM, and recurrence, but also as a tool to guide clinical practice. On the other hand, some recent studies have demonstrated that while $BRAF^{V600E}$ is linked to features associated with aggressive progression (ETE, multifocality, and larger tumor sizes (>5mm)), RAI therapy response in intermediate- and high-risk PTMC patients was not influenced by mutational status, suggesting that the presence of $BRAF^{V600E}$ might not change therapeutic strategies (122).

In addition to the correlation between BRAF mutational status and recurrence of PTMC, the correlation between aggressive features and $BRAF^{V600E}$ has also gained widespread attention. In a study that included 121 PTMC or PTC (<15mm) patients, aggressive features (ETE, LNM, high risk variants including tall cell, columnar, hobnail, DSV, and solid) were observed in 54.2% and 19.4% of PTMC patients with and without $BRAF^{V600E}$ mutations, respectively (270). Similarly, 19.4% of PTC patients without BRAF mutations exhibited aggressive features compared to 82.1% in those with $BRAF^{V600E}$. Another study that included 100 PTMC patients, $BRAF^{V600E}$ mutations were associated with aggressive

features, however, no correlation between mutational status and lymph node, distant metastasis, mortality, or tumor recurrence was observed (271). Contrary to these, others have found no association between $BRAF^{V600E}$ mutations and PTC aggressiveness (272). Moreover, when 393 tumor genetic alterations were analyzed to assess the role of mutations in classifying PTMC as high- or low-risk, it was demonstrated that $BRAF^{V600E}$ mutations did not correlate strongly with high-risk PTMC (255). Thus, it has been suggested that $BRAF^{V600E}$ mutations could not sufficiently predict PTMC aggressiveness. Single-center studies have also supported these findings, displaying no correlation between PTMC focality or size and BRAF mutations (273). Similarly, when 504 patients with PTMC were evaluated for TERT and $BRAF^{V600E}$, 3.2% had TERT ± $BRAF^{V600E}$ and no correlation was found between mutational status and aggressive characteristics or LNM (274). Likewise, in another study that included 89 PTMC patients, $BRAF^{V600E}$ was not linked to tumor staging, CLNM, aggressive features such as capsular invasion, or multifocality (275).

Our results have demonstrated that $BRAF^{V600E}$ mutations are observed in 68% of all patients. 80% of metastatic PTMC patients and 53% of non-metastatic PTMC, were shown to have the $BRAF^{V600E}$ mutation($p=0.006$). Multivariate analysis did not show a role of BRAF in predicting LNM. Remarkably, among patients with LNM (n=35), 7 patients did not harbor the $BRAF^{V600E}$ mutation. Although the use of BRAF mutational status to predict LNM and assess eligibility for active surveillance has been suggested in several studies, our results show that these strategies can be misleading. Because not all patients who present with LNM have the BRAF mutation and multivariate analysis failed to show a correlation between BRAF mutational status and LMN, BRAF mutational status cannot be reliably used to predict LNM and inform clinical treatment planning. Moreover, the use of BRAF mutational status to decide eligibility for active surveillance in the management of PTMC can lead to the progression of disease and therefore to poor prognosis in patients with wild type BRAF and aggressive PTMC.

10.3. The Role of Desmoplasia of PTMC in Prognostic Evaluation

The association between desmoplasia and metastasis, particularly LNM, in thyroid cancer has been investigated in several studies. The role of desmoplasia has been particularly researched in MTC. The desmoplastic stromal reaction is linked with

LNM (276, 277). A study analyzing 196 MTC cases has shown that desmoplasia is a reliable and reproducible morphological parameter indicating LNM (109). Compared with other morphological features such as vascular invasion and lymphocytic infiltration, desmoplasia has high specificity in predicting LNM (278). None of the tumors that have shown lymph node involvement was evaluated as non-desmoplastic while LNM was not detected in any of the tumors evaluated as non-desmoplastic. Moreover, the study has shown that peritumoral invasion was not observed in most tumors lacking desmoplasia, which is associated with well-defined, regular tumor borders. The lack of desmoplasia has also been described as benign behavior of the tumor, predicting the lack of LNM. Another study has investigated the correlation between nodal involvement and molecular desmoplastic stromal reaction markers such as fibroblast activation protein α (FAPα) and tenascin-C (Tn-C) in MTC (279). These molecules produced by activated fibroblasts or myofibroblasts play active roles in the formation of desmoplasia. The findings of this study have shown that there is a significant correlation between the grade of desmoplasia and LNM. Prominent desmoplasia in the histological level was observed in cases with LNM. Moreover, LNM was not found in non-desmoplastic tumors and expression of Tn-C and FAPα in tumors had increased significantly in cases with nodal involvement regardless of tumor size. This study also determined that there is a significant correlation between FAPα expression and LNM in PTC as well as MTC, suggesting that desmoplastic markers can be used to predict LNM in different types of thyroid cancer.

Guidelines about MTC therapy state that the primary treatment for MTC is total thyroidectomy with CLNM. However, clinicians argue that a surgical approach without considering tumor features would lead to overtreatment and increased complications of extended surgery. One study has indicated that surgical intervention should be adjusted based on specific features of MTC tumors including desmoplasia (276). Surgical intervention deviates from the classical approach to total thyroidectomy without central lymph node dissection if preoperative criteria suggest sporadic, unifocal MTC with biochemical cure and frozen sections excluding desmoplasia and capsular infiltration. Otherwise, it is indicated that tumors showing desmoplastic reactions should be resected with lymph node dissection due to the high risk of LNM. Similarly, another study has stated that nodal invasion, the number of node metastases, and the biochemical cure rates are

all substantially correlated with expanding desmoplasia and diminishing tumor capsule integrity (280). Therefore, node dissection may not be necessary for patients with desmoplasia-negative encapsulated unifocal sporadic MTC. These studies suggest that more personalized treatment modalities should be designed based on the histopathological characteristics of tumors. It has been recommended that surgical intervention for PTC and PTMC can be modulated based on the presence or degree of desmoplasia, enabling a more tailored approach to surgical treatment regarding histopathological features.

Desmoplasia is a marker for aggressive tumor behavior, which has been linked to mutations like BRAF, often considered a predictor of poor prognosis in PTC. Several studies have demonstrated that BRAF-mutated PTC tumors display distinctive morphological features such as desmoplasia (281). A study has proposed that desmoplasia and well-defined morphological features can predict BRAF mutational status with %80 accuracy and substantial interobserver reproducibility can be achieved to select the most appropriate tumors, especially multifocal PTCs, for molecular testing. Moreover, a study including 50 PTC patients, has indicated that desmoplasia is considerably more common in BRAF mutant samples than in wild-type samples (72% vs. 29%), suggesting a potential correlation to more aggressive tumor features (282). This demonstrates that desmoplasia in relation to BRAF mutations may contribute to the aggressiveness and spread of PTC.

The impact of desmoplasia on the prognosis of PTMC is yet to be investigated. Recently, several studies investigating determinants of LNM, particularly CLNM, have demonstrated that desmoplasia in PTMC is a significant predictor for metastasis and aggressive tendency of tumors. A study including 336 PTMC patients has shown that besides the number of metastatic lymph nodes and size of metastatic foci, the presence of desmoplasia is also significantly associated with decreased recurrence-free survival (283). Another study has advocated that desmoplastic reaction, especially with tumor calcification, is significantly associated with LNM (284). Of 28 PTMC with LNM, desmoplastic changes in tumors were observed in 27 patients. In addition, calcification or psammoma bodies, which are morphological findings related to LNM, were observed in tumors without desmoplasia. Invasive parameters such as peritumoral and vascular invasion have also been linked to desmoplasia in PTMC. Similarly, a study discussing the risk stratification of PTMC has underscored that desmoplasia,

defined as an aggressive morphological feature, is strongly associated with larger and subcapsular PTMCs (277). Therefore, it was concluded that PTMC with desmoplasia require more aggressive management and attentive follow-up.

Several studies in current literature have found that desmoplasia is correlated with aggressive behavior of tumor and LNM, so tumors that have desmoplastic changes require aggressive management constituting extended surgical intervention and an attentive follow-up period (109, 226). However, in certain cases, such as inflammation or changes resulting from FNAB, fibrotic changes might be incorrectly identified as desmoplasia. This misinterpretation can lead to unnecessary aggressive treatment in patients with PTMC. Moreover, since Hashimoto's thyroiditis, a condition that leads to significant inflammation of the thyroid, is common in Turkey, it is crucial to assess desmoplasia in PTMC with great attention (285). This careful evaluation is necessary to avoid incorrect diagnoses of PTMC with desmoplastic reactions. A study focusing on the pathology of Hashimoto's thyroiditis related PTC has investigated intratumoral fibrosis and desmoplasia (286). The findings have indicated that a considerable proportion of these carcinomas have diverse levels of fibrosis, ranging from dense fibrous septa that separate tumor nodules to nearly complete fibrotic destruction of the tumor. These tumors usually exhibit a fibromatosis-like desmoplastic response, with thick hyalinized collagen and bland-appearing spindle cells. Abundant fibrosis can obscure the cytological and morphological features of papillary carcinoma make diagnosis a greater challenge. Moreover, another study found results that could help differentiate what the main cause of fibrosis is in patients with PTC and Hashimoto's diseases. Results show that when lymphocytic infiltration was dominantly seen amongst the fibrotic tissue, this fibrosis could be induced to the inflammatory nature of Hashimoto's disease rather than the progress of the cancer. Notwithstanding, it is possible that the concomitant presence of both chronic lymphocytic thyroiditis and cancerous tissue to have synergistic contributions into the development of fibrosis (287). Additionally, another study identified fibrosis as a common chronic alteration following FNAB (288, 289). 66% of the cases that had undergone FNAB have exhibited fibrosis. This was significantly higher compared to the non-FNAB group, where only 15.3% of the cases showed fibrosis. Statistical analyses demonstrated that there was a substantial ($p<0.001$) difference in the fibrosis rates between the FNAB and non-FNAB groups. The influence of FNAB

and Hashimoto's thyroiditis on the occurrence of fibrosis is observed irrespective of the aggressiveness of PTMC. Consequently, this factor must be considered during the assessment of desmoplasia to estimate the potential for metastasis in PTMC.

Our results have also supported recent findings in literature regarding the association between desmoplasia and LNM. Among PTMC patients with LNM, 49% demonstrated substantial desmoplasia and 37% showed diffuse desmoplasia compared to 40% substantial desmoplasia and 10% diffuse desmoplasia in the non-metastatic group ($p=0.007$). Furthermore, the average desmoplasia extent score ($p<0.001$) and desmoplasia intensity score ($p<0.001$) was significantly higher for the LNM positive group, although desmoplasia alone would not enable an accurate prediction of LNM. In 98% of Hashimoto's thyroiditis patients diagnosed with PTMC, desmoplasia was observed compared to 80% in patients without Hashimato's disease, suggesting that chronic lymphocytic thyroiditis might have caused the apparent desmoplastic changes leading to desmoplasia positivity in the absence of LNM. Moreover, desmoplasia extent ($p<0.001$) and intensity ($p<0.001$) scores were significantly higher for the lymphocytic thyroiditis positive group. These findings are also supported by several studies in the literature which have demonstrated high incidence of prominent stromal desmoplastic changes in Hashimoto's thyroiditis associated PTC (286, 290). Another factor that can impact desmoplasia is FNAB. Considering that PTMC sizes do not exceed 10 mm by definition, acquiring FNAB samples can lead to an increase in fibrotic tissue within the lesion, thereby resulting in a higher degree of desmoplasia. The impact of FNAB on desmoplasia has also been established in literature: FNAB induced needle trauma can result in increased desmoplastic changes, potentially leading to higher observed desmoplasia (291). However, the role of FNAB on desmoplasia has not been evaluated in this study as FNAB was performed on all patients included in this study.

10.4. The Role of Trichrome Staining of PTMC in Prognostic Evaluation

Trichrome staining is excellent in differentiating collagen deposits from the surrounding tissues and thus it plays a vital role in detecting fibrosis. Presence of fibrosis in cancer microenvironment has been suggested to play a critical role in cancer progression and metastasis. Particularly, alterations observed in the

extracellular matrix (ECM) such as stiffness, are thought to have a prognostic value. It has been shown that increased collagen density results in ECM stiffening (292). Additionally, the tumor microenvironment is abundant with cancer-associated fibroblasts which can promote tumor progression and increase the migration potential (293).

In a study investigating the ECM of 26 BRCA positive breast cancer tissue, stiff ECM was shown to play a role in the nerve invasion potential of the cancerous tissue (294). ECM stiffness was verified by Trichrome staining. It was shown to upregulate pro-metastatic genes, particularly neurotrophic genes such as the significant increase in NGF mRNA expression in BRCA positive breast cancer tissues. In the same study, cancerous cells were also noted to migrate faster in a stiff matrix (294). Moreover, the stiffness of the matrix was associated with higher nerve invasion by cancer tissue which results in a poorer prognosis of breast cancer (294). Similar results were demonstrated in an analysis of the interaction of human prostatic carcinoma and the ECM. Cancer cell migration was promoted and stimulated through network of fibronectin which formed the physical connection between cancer cells and cancer-associated fibroblasts, all of which occur in the matrix (295). The potential impact of collagen density on cancer cell mobility and metastasis was also shown in a study analyzing gastric cancer metastasis into the peritoneum. In total, 36 gastric cancer samples were divided into four groups according to the 8th edition of the AJCC staging system. The samples with the worst prognosis, stage IV, were associated with the highest collagen deposits out of the all groups (296). Moreover, Masson Trichrome staining showed that collagen deposits were increased in metastatic nodules which had genes (LGALS1) that upregulated collagen-I, collagen-III, and fibronectin-1 secreting genes (COL1A1, COL3A1, FN1). Increased collagen expression in the human peritoneal mesothelial cells promoted gastric cell adhesion and metastasis into the peritoneum (296). Hence, it was suggested that peritoneal fibrosis created a favorable environment for metastasis and cancer cell migration, which is consistent with the effects of ECM stiffening as mentioned above.

Thus, these findings seem to point out that collagen deposits identified by Trichrome staining could play a predictive role in cancer metastasis. However, another viewpoint exist.s A study aimed at determining the relationship between the content of collagen in early-stage invasive breast cancer and the long-term

incidence of metastasis to the bone. Collagen content was differentiated from the surrounding tissue using Trichrome stain and 60 samples of breast carcinoma with various degrees of metastasis, were included in the study (297). Results showed that as breast carcinoma became more invasive (i.e. LNM and bone metastasis), collagen area decreased. Breast carcinoma without LNM had a collagen area of 32.39% while breast carcinoma with LNM had an area of 25.37% and breast cancer with bone metastasis had an area of 22.71% (297). Given the existence of two contrasting conclusions on the impact of collagen deposits on cancer metastasis, more research needs to be conducted as establishing early predictors of metastasis could aid in the detection of cancer with a poor prognosis.

The role collagen deposition and the extent of fibrosis as a potential prognostic value was explored. One study investigated collagen deposition within brain metastases. It is thought that collagen deposition within brain metastasis might be associated with a poor prognosis especially after brain surgery, leading to the development of leptomeningeal failure (LMF). Samples from 55 patients were stained with Trichrome stain and the degree of Trichrome staining was shown to have correlation with the amount of collagen in the brain metastasis samples. Greater Trichrome staining was associated with a higher risk of LMF (298). This could potentially serve a prognostic tool and guide for higher surveillance. Patients with an area with a high Trichrome score could be followed up more closely and carefully rather than a patient with a low Trichrome score. Hence, routine implementation of Trichrome staining could help guide treatment plans. Additionally, although this study focused on the prognostic value of fibrosis, the fact that the samples belonged to a metastasized brain carcinoma further support findings suggesting the correlation between metastasized carcinomas and presence of fibrosis. A study investigating prognostic factors to evaluate poor and favorable bladder cancer progression explored the correlation of bladder cancer and collagen deposits. 66 bladder cancer pathological samples were analyzed and grouped into two groups: 34 cases of low-grade urothelial carcinoma and 32 cases of high-grade urothelial carcinoma (299). After measurement with sheer wave elasticity and histological analysis with Masson Trichrome stain, the high-grade group was shown to exhibit a higher degree of elasticity indicating a higher degree of tissue stiffness. Moreover, higher collagen deposits were seen in the high-grade bladder cancer group with an area of 11.45% while low-grade carcinoma group had an area of

7.64%. It was shown that the higher the pathological grade of the cancer, the higher the collagen deposits (299). Similar to ECM stiffness analyses, collagen deposits seem to play an important role in determining the prognosis of the cancerous tissue and shows its potential utility as a prognostic tool.

In addition to these findings, it is thought that dense connective tissue might serve as a protection the cancer tissue and provide a favorable environment for cancer growth. In one study, the effects of intratumoral fibrosis were evaluated in the context of peritoneal metastasis. Compared to liver and lung metastasis of colorectal cancer, peritoneal metastasis had significantly more fibrotic components shown by Trichrome staining (300). However, the density of tumor infiltrating lymphocytes $CD3^+$ and $CD8^+$ was lower in high fibrotic areas such as the peritoneal environment of the metastasized cancer than in low fibrotic areas. There is evidence that decreased tumor infiltrating lymphocytes in gastric cancer is correlated with decreased overall survival (301). Therefore, deactivation of lymphocytic infiltration due to greater intratumoral fibrocystic changes is also thought to lead to a worse prognosis (300). Collagen deposits in various cancerous tissue detected by Masson Trichrome staining seem to have a real potential in detecting cancerous tissue that tend to have a worse prognosis or a tendency for metastasis. Hence, the development of tools or routine assessments that can detect and quantify collagen deposits can potentially have an impact in calculating the potential outcome and designing the most adequate treatment strategy. However, establishing the relationship between cancerous cells and collagen deposits is an area that still calls for more research.

As demonstrated in breast carcinoma, brain metastases, bladder cancer, and peritoneal metastases, Trichrome staining can play an instrumental role in displaying metastatic potential. To evaluate the role of trichrome staining in predicting metastasis, subgroup analyses were performed following the exclusion of chronic lymphocytic thyroiditis patients. These patients were excluded since IgG4 variant of chronic lymphocytic thyroiditis has been associated with dense stromal fibrosis, lymphoplasmacytic infiltration, and lymphoid follicle formation, and thereby increased trichrome staining (232, 302). Subgroup analyses revealed a strong correlation between extent ($p<0.001$) and intensity ($p<0.001$) of Trichrome staining and incidence of LNM. 40% of LMN positive group scored the highest score (6) while only 10% scored this in the LMN negative group. Conventionally,

the pathological assessment of thyroid nodules begins with H&E staining and Trichrome staining is not routinely performed following FNAB (303). Considering these findings, we believe that routine implementation of routine trichrome staining to evaluate PTMC can enable the prediction of LNM and inform clinical decision making. Moreover, for clinical N0 PTMC, prophylactic neck dissections are not routinely performed. However, in clinical N1 PTMC, thyroidectomy and therapeutic central neck dissection is performed followed by adjuvant RAI. PTMC patients can be assessed through desmoplasia and trichrome staining characteristics and strategies such as prophylactic RAI administration in the absence of pathological lymph nodes can be considered to prevent recurrence and future lymph node involvement.

10.5. Limitations and Bias

Although we noted a significant presence of desmoplasia and Trichrome staining in the metastatic group, it is critical to acknowledge some potential bias factors. Desmoplasia could have been induced by a scar after FNAB and/or the notable presence of chronic lymphocytic thyroiditis within our study population, which reflects the high incidence of Hashimoto's thyroiditis in the Turkish population. Hence, this could present potential bias factors. Moreover, one of the primary limitations of this study is the multifactorial nature of lymph node metastasis (LNM). Thus, our investigation into whether desmoplasia in thyroid tissue constitutes a significant risk factor was limited by the inherent complexity of LNM pathophysiology. In comparing groups with and without metastases in terms of desmoplasia, we encountered uneven distributions of critical risk factors, including tumor size and BRAF mutations, which were more prevalent in the metastatic group. To mitigate these biases and better isolate the impact of desmoplasia, we employed multivariate analysis. However, the retrospective nature of our study introduces constraints that may not allow for the complete elimination of bias. This inherent limitation of retrospective analyses means that, despite our efforts to control for confounding factors, the potential for residual bias cannot be entirely excluded. Additionally, desmoplasia, a phenomenon also observed in chronic lymphocytic thyroiditis, was prevalent across our study groups. This is particularly relevant given the high frequency of desmoplasia within the Turkish population, which our sample pool reflects. Consequently, the unequal representation of these

independent factors, coupled with the limited sample size, introduces a primary bias in our research findings, complicating the assessment of desmoplasia's role as an isolated risk factor for LNM.

11. CONCLUSION

In this study the prognostic role of BRAF mutations, clinicopathological features, desmoplasia, and Trichrome staining in predicting LNM of PTMC was evaluated. Findings have demonstrated strong associations between desmoplasia score (extent and intensity) and LNM. Similarly, strong correlations between Trichrome staining score (extent and intensity) and LNM was established. However, the presence of chronic lymphocytic thyroiditis or FNAB-induced scarring (fibrosis) could partially account for desmoplasia. For multifocal PTMC, all assessments were based on the largest tumor. Mix tumor types and agressive features of smaller tumors might explain findings of LNM despite the lack of apparent aggressive features in the largest tumor. Due to financial limitations, several molecular factors (i.e. matrix metalloproteinases) that have been suggested to play a role in determining PTMC prognosis were not included in this study. Future studies will aim to evaluate the role of other novel molecular markers as LNM of PTMC is most likely directed by multifactorial mechanisms.

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