Thesis

Clinical Relevance of Oncocytic Change in Papillary Thyroid Carcinoma

submitted by

Luca del Fabro

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Univ. FA Priv.-Doz. Dr.med.univ. Oleksiy Tsybrovskyy

Univ.-Prof. Dr.med.univ. Barbara Obermayer-Pietsch

Graz, February 19, 2026

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Zusammenfassung

Einleitung: Onkozytäre Transformation entsteht durch Akkumulation von Mitochondrien mit Defekten in der mitochondrialen DNA. Rezent publizierte Ergebnisse zeigen, dass der Tall-Cell-Subtyp des papillären Schilddrüsenkarzinoms (TCS-PTC) einem onkozytär transformierten Karzinom entspricht. Sowohl der onkozytären Transformation als auch TCS werden aggressives Verhalten und ungünstige Prognosen nachgesagt, obwohl neuere Studien eine differenziertere Betrachtungsweise empfehlen.

Methodik: Inkludiert wurden sämtliche PTCs, die zwischen 1995 und 2014 am Pathologischen Institut der Medizinische Universität Graz diagnostiziert wurden. Die Fälle wurden einerseits HE-morphologisch nach der aktuellen WHO-Nomenklatur reklassifiziert und andererseits mithilfe der mitochondrialen immunhistochemischen Markern Prohibitin und NDUFS4 in onkozytär versus nicht-onkozytär eingeteilt. Beide Einteilungen wurden mit klinisch-pathologischen Parametern korreliert, mit besonderem Augenmerk auf die Lymphknotenmetastasierung.

Ergebnisse: TCS und onkozytäre Tumoren manifestierten in höherem Alter ($p<.001$), waren größer ($p<.001$) und häufiger BRAF-mutiert ($p<.001$). Sie zeigten weniger Psammomkörper ($p<.001$), eine ausgeprägtere extrathyroidale Ausbreitung ($p<.001$), wuchsen infiltrativer ($p=.026$) und waren seltener bekapselt ($p<.001$). Trotzdem waren sie seltener metastasiert ($p=.034$) und wenn, waren ihre Metastasen wesentlich kleiner ($p<.001$). Im Regressionsmodell zeigte sich, dass sie verglichen mit konventionellen Subtypen zu Mikrometastasen neigen ($p=.013$) und ein geringeres Risiko für klinisch auffällige Metastasen haben ($p<.001$). Die immunhistochemische Einteilung ermöglichte eine eindeutigere Differenzierung.

Schlussfolgerung: Unsere Studie zeigt, dass TCS bzw. onkozytär transformierte PTCs mit allgemein weniger Metastasierung einhergehen als konventionelle Subtypen, womit die bisher vorherrschende Betrachtung von TCS als „aggressivem“ Subtyp in Frage gestellt wird. Außerdem konnten wir demonstrieren, dass die mitochondriale Immunhistochemie in diesem

Zusammenhang höhere Relevanz aufweist als die HE-morphologische Subtypisierung.

Abstract

Introduction: Oncocytic transformation is a result of accumulation of mitochondria harboring mtDNA mutations. Recently, tall cell subtype PTC (TCS-PTC) has been shown to be in fact oncocytic. Both TCS and oncocytic transformation have previously been associated with poor outcome while conflicting evidence suggest a more refined approach.

Methods: We selected all PTCs diagnosed at the Medical University's Institute of Pathology, Graz, Austria (N=472) between 1995 and 2014. All cases were reviewed and reclassified on H&E according to current WHO nomenclature and also categorized oncocytic versus non-oncocytic using novel mitochondrial IHC markers Prohibitin and NDUFS4. Both classifications were then correlated to clinicopathological parameters with focus on lymph node metastasis.

Results: TCS and oncocytic change were significantly associated with later onset ($p<.001$), larger tumors ($p<.001$) and BRAF V600E mutation ($p<.001$). They showed less psammoma bodies ($p<.001$), more extrathyroidal extension ($p<.001$), were more infiltrative ($p=.026$) and less encapsulated ($p<.001$). Nevertheless, metastasis was less common ($p=.034$) and overall smaller ($p<.001$). It was confirmed by regression analysis that they were more likely to come with micrometastasis ($p=.013$) and less likely to come with clinically evident metastasis ($p<.001$). IHC classification was throughout more discriminatory than H&E subtyping.

Conclusion: Our cohort demonstrates that lymph node metastasis is overall less extensive in TCS-PTC and oncocytic transformation than in conventional PTC subtypes. This is in contradiction to current belief of TCS being an "aggressive" subtype. Furthermore, we established mitochondrial IHC markers to be of significant value in diagnosing PTC and superior to H&E with regard to lymph node metastasis.

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Abbreviations

BRAF = B-type "rapidly accelerating fibrosarcoma" serine/threonine kinase[1]

cm = centimeter(s)

DTC = Differentiated thyroid carcinoma

ER = Endoplasmic reticulum

FA = Follicular adenoma

FDG = 18-Fluorodesoxyglucose

FNA = Fine needle aspiration

FTC = Follicular thyroid carcinoma

H&E = Hematoxylin & eosin staining

i.e. = "that is" [Latin: id est]

IHC = Immunohistochemistry

mm = millimeter(s)

MTC = Medullary thyroid carcinoma

MWW = Mann-Whitney-Wilcoxon (U-test for nonparametric variables)

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

OC = Oncocytic carcinoma

PET = Positron emission tomography

PTC = Papillary thyroid carcinoma

RAI = Radioactive iodine (131-I)

RAS = Rat sarcoma GTPase[2]

T3 = Triiodothyronine

T4 = Thyroxine

TGB = Thyroglobulin

TMA = Tissue Micro Array

TNM = Classification of the primary tumor's extent (T), lymph node metastasis (N) and distant metastasis (M) clinically (c) and pathologically (p)

TSH = Thyroid stimulating hormone

WHO = World Health Organization

WT = Wild type (non-mutated)

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1 Introduction

1.1 Thyroid and Thyroid Neoplasms

1.1.1 Histology of the Thyroid

The thyroid gland's key morphological element and essential to its physiology is *follicular* architecture. Thyroglobulin (TGB) as precursor molecule to triiodothyronine (T3) and thyroxine (T4) is secreted and stored within follicles, i.e. spheroid structures unique to the thyroid lined by a single layer of follicular epithelium cells. The basal membrane delineates each follicle from surrounding fibrovascular tissue. Additionally, histogenetically independent calcitonin producing cells (c-cells) are disseminated all over the thyroid's stroma.

According to their activity, follicular cells appear flat with nuclei rather prone and in seemingly immediate contact to the basal membrane with cells just as tall as nuclei are wide without significant stimulation.

On the contrary, follicles highly active in protein synthesis exhibit rather columnar epithelium (Fig. 1) whose active proliferation even appears as *pseudo papillae*. As per this secretory nature, follicular cells show great amounts of ER normally evenly dispersed throughout the cytoplasm.[3-5]

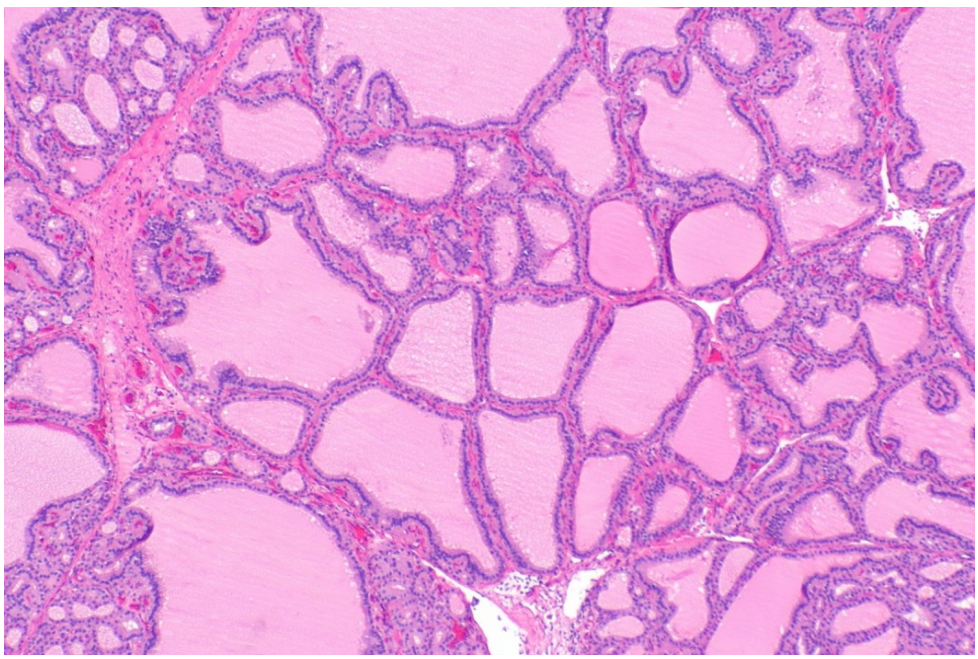

Figure 1: Macrofollicular Architecture vs. Highly Active Areas Consistent with Grave's Disease[6]

1.1.2 Neoplasms of the Thyroid

Two main types of thyroid tumors can histogenetically be differentiated; tumors derived from parenchymatous ectodermal follicular cells versus tumors derived from neuroendocrine endodermal c-cells, the former referred to as differentiated thyroid carcinoma (DTC), the latter as medullary thyroid carcinoma (MTC). By immunohistochemistry[7], both groups can easily be distinguished using antibodies against PAX-8, thyroglobulin and TPO for follicular tumors versus calcitonin showcasing c-cell origin.

Additionally, there are rare neoplasms of mixed, uncertain or entirely different histogenetic origin. A summary of the current WHO Classification of Tumors is presented in Table 1.

Table 1: Excerpt from WHO-Classification of Tumors (adapted from Baloch et al., 2022[8])

- Developmental abnormalities […]
- **Follicular cell-derived neoplasms:**
  - Benign tumors:
    - Follicular nodular disease (FND)
    - Follicular adenoma (FA)
    - Follicular adenoma with papillary architecture
    - Oncocytic adenoma
  - Low-risk neoplasms:
    - Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP)
    - Tumors of uncertain malignant potential (UMP)
    - Hyalinizing trabecular tumor
  - Malignant neoplasms:
    - FTC
    - Invasive encapsulated follicular variant (EFV) PTC
    - PTC
    - Oncocytic carcinoma
    - Differentiated high grade, poorly differentiated and anaplastic thyroid carcinoma
- Thyroid C-cell-derived neoplasms […]
- Tumors of mixed, uncertain, thymic and embryonal histogenesis […]

Benign neoplasms of the thyroid include follicular nodular disease and basically adenomatous conditions as precursor lesions to FTC. There is no real benign neoplastic precursor lesion to MTC apart from c-cell hyperplasia.

Malignant neoplasms include follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC) and oncocytic carcinoma (OC).

There is no real benign precursor of PTC equivalent to adenomas. Nikiforov et al.[9] schematically count papillary microcarcinoma, i.e. carcinoma smaller than 10 mm on gross examination, as “precursor” to “real” PTC. Microcarcinomas usually do not manifest clinically and are regularly found incidentally on routine imaging or surgical-pathological workup. When completely resected, they show excellent prognosis. However, by morphological definition and malignant potential, they do not differ from PTC in any way.

The second most common DTC, FTC, is defined by invasive nature, i.e. vascular or capsular invasion, in combination with lack of PTC-like nuclear features. Invasive nature is what separates FTC from FA. No psammoma bodies are known regarding FTC and intrathyroidal metastasis is rare.[10]

Contrary to PTC, FTC immediately shows angioinvasion resulting in distant metastasis as the main form of tumor spread.

Common etiological factors include irradiation for PTC and iodine deficiency for FTC (whose incidence is known to be higher in iodine deficient areas).

FTC is best known to harbor RAS mutations. This way, distinction between FTC and PTC has become easier, even in morphologically equivocal cases. Interestingly, NIFTP has been categorized RAS-mutated as well, showcasing rather FTC-related histogenesis than PTC-one.[8]

1.1.3 Thyroid Nodule Work-Up

Usually, thyroid nodules do not present symptomatically. Possible clinical complaints include palpable tumors, foreign body sensation, dysphagia or dysphonia as a sign of recurrent nerve impairment. Obviously, nodule size determines clinical characteristics.[11]

More commonly though, DTC emerges when suspicious nodules are found on work-up or imaging for unrelated indications or from surgery performed for other conditions such as Grave’s disease, multinodular goiter and parathyroid surgery.

A multicenter multinational retrospective analysis of 1,328 patients evaluated patients having thyroid nodule surgery. Only 34% presented with symptoms of nodular disease while the rest was asymptomatic and incidentally found to have thyroid nodules by clinicians.[12]

Due to quick and ubiquitous availability, ultrasound examination is of vital importance in initial evaluation of thyroid nodules.

Whereas serum TGB levels proved to be of low practical value, TSH should always be included in nodule workup. As caused by iodine deficiency, hypothyroidism may lead to follicular nodular disease, which in turn may lead to malignancy. Calcitonin is a valuable tool in MTC follow-up post-surgery.

The 2015 American Thyroid Association (ATA) Guidelines recommend nuclear imaging as first step in case of reduced serum TSH levels to look for "cold" nodules exhibiting less tracer uptake than surrounding tissue.[13] Common tracers include 123-I and 131-I as well as biologically similar 99m-Tc-Pertechnetate.[14]

On the contrary, increased FDG uptake on PET scan as a sign of increased metabolic activity is an obvious hallmark for malignant transformation.[15]

Key diagnostic for suspicious nodules is cytology acquired by fine-needle aspiration (FNA) and evaluated using the Bethesda System for Reporting Thyroid Cytopathology.[16,17]

1.1.4 Treatment of Thyroid Carcinoma

Thyroid carcinoma is first line treated surgically. Depending on risk stratification, lobectomy, total thyroidectomy and lymph dissection are performed. While, historically, diagnostic, i.e. prophylactic lymph dissection was common, current evidence suggests lymph dissection should only be added in case of clinical concern for metastatic disease.[18] Although not always indicated for clinical outcome, total thyroidectomy is instead prerequisite for adjuvant radioactive iodine ablation (RAI).

Successful surgical resection is confirmed through scintigraphic iodine (123-I) scan just before applying pharmacological T4 substitution. That way, elevated TSH serum levels due to post-thyroidectomy hypothyroidism stimulate thyrocytes towards increased iodine uptake. Assessment may then take place, whether patients are eligible for radioactive iodine (131-I) ablation or secondary surgical resection is recommended prior, as patients should only be exposed to as little radioactive dosage as necessary.

Pharmacological TSH suppression through T4 substitution is a key therapeutic concept, considering neoplasms of the thyroid are generally TSH-avid responding to TSH serum levels by proliferating.

Nevertheless, RAI is state of the art. RAI ablates whatever thyroid tissue remains after surgery and allows for complete thyroidectomy.

Clinical research has shown positive impact of RAI ablation post-surgery on disease specific mortality.[19]

1.2 Papillary Thyroid Carcinoma

1.2.1 Epidemiology of Papillary Thyroid Carcinoma

Overall, thyroid cancer is a rather rare form of neoplasms having made up for about 4% of all newly diagnosed malignancies in 2022 worldwide, whereas three quarters of the patients have been female over one quarter being male.[20]

Despite steadily increasing incidence rates over the past decades, rates of mortality have stayed low leading to the assumption of improvements in health care and disease prevention as the reason.[21]

Of those, PTC is by far the most common type with age-standardized incidence rates ranging from 1.29 per 100,000 men in Bulgaria to 143.3 per 100,000 women in South Korea.[22]

1.2.2 Etiology of Papillary Thyroid Carcinoma

One common etiological factor seen primarily in PTC is ionizing radiation. In the aftermath of the nuclear catastrophe in Chernobyl in 1986, incidence rates of thyroid cancer have increased in more contaminated regions compared to less contaminated places and among those, inhabitants that were of child or adolescent age at the time of the accident. In those patients, RET/PTC rearrangements were more frequent than BRAF mutations. Clinically more aggressive behavior in younger patients may be explained that way.[23]

Similar observations have been made in survivors of the atomic bombs in Hiroshima and Nagasaki, whereas BRAF-positive tumors manifested later after radiation exposure than RET-fusion ones as well as correlated to lower assumed median

radiation doses than the latter, suggesting higher influence of radiation exposure on RET/PTC rearrangement than on BRAF V600E mutations more frequently seen in patients not associated with radiation exposure.[24] This concurs with common clinical knowledge, that thyroid cancer is a late iatrogenic complication of radiation in pediatric patients, such as in lymphoma of the neck. These cases are typically RET fusion tumors gaping towards anaplastic carcinoma.

Similarly to FTC, both iodine deficiency as well as excessive iodine intake have been associated with higher risk of thyroid cancer.[25]

While it is unclear whether there is a real causal connection to Hashimoto's thyroiditis, PTC has broadly been epidemiologically linked to lymphocytic inflammation is in Hashimoto's.[26,27]

1.2.3 Histology of Papillary Thyroid Carcinoma

PTC is named after its predominantly papillary architecture. However, papillary growth in thyroid neoplasms is highly unspecific and vice versa.

More accurately, PTC is defined by distinct nuclear features. These findings are universally distinguishable on FNP and H&E sections from paraffin embedded material. They include nuclear enlargement, chromatin clearing, irregular nuclear membranes and nuclear grooving as well as pseudo-inclusions of cytoplasm (Fig. 2).[3]

Still, recent literature has shown that neither these nuclear features are truly specific to PTC nor do they represent malignancy (NIFTP).

In general, when exhibiting papillary growth, tumor cells are known to overlap as a sign of excessive proliferation.

Typically, psammoma bodies confirm diagnosis of PTC. These are concentrically layered calcifications within the stroma and/or lymph vessels (Fig. 2).

Lymphatic invasion is a typical feature of PTC. On the contrary, venous infiltration and distant metastasis as seen in FTC are very uncommon in PTC.

Multifocal carcinomas are relatively common in PTC.[28]

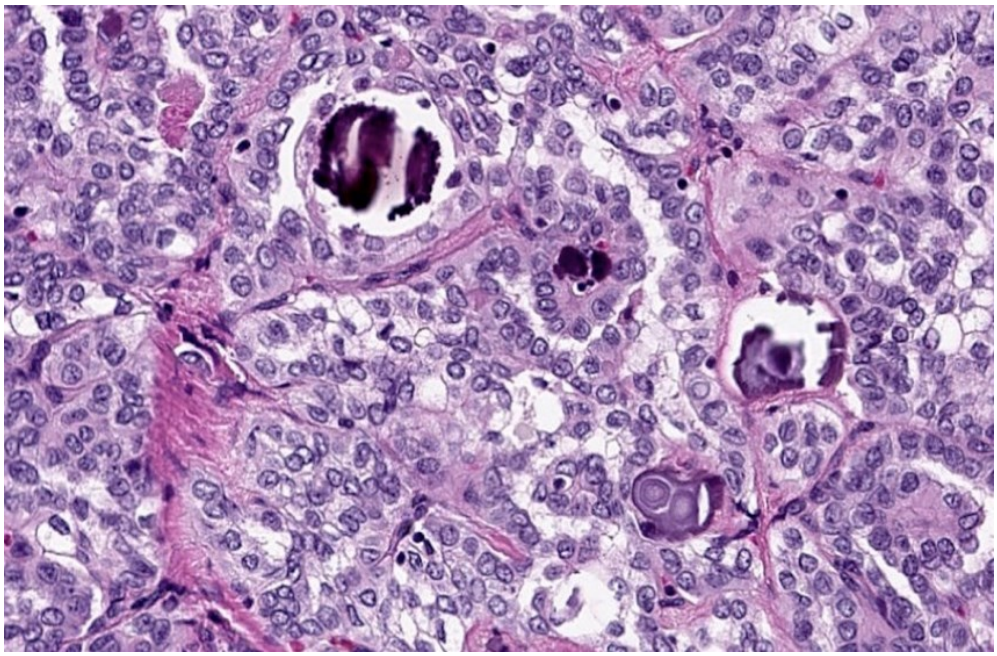

Figure 2: PTC-like Nuclear Features and Psammoma Bodies

Typical immunohistochemical markers that aid to the diagnosis of PTC are HBME-1, CK19, Galectin-3 and CD56.[29,30]

PTC comes in various morphological subtypes including but not limited to columnar call variant, clear cell variant, diffuse sclerosing variant, solid/trabecular variant, Warthin-like variant and spindle cell variant.

Importantly, the 5th WHO Classification of Tumors distinguishes between oncocytic and tall cell (TCS) PTC subtypes.[8]

1.2.4 Clinicopathologic Correlation

Compared to other malignancies, overall prognosis of PTC is remarkably favorable. A large single center study including 5,897 patients conducted by Ito et al. promises up to 20-year overall survival rates higher than 90%.[31] 20-year disease-free survival rates are reported in excess of 80%.[32]

Commonly known adverse prognostic factors include male sex, advanced disease on first surgical treatment, high pT stage and widely invasive growth.[33] More specifically, this includes tumor size, extrathyroidal extension, lymph node and distant metastasis and certain morphological subtypes.

Some studies conclude that younger patient age is consistent with poorer outcome in terms of initial disease and recurrence. In 2014, Ito et al. found larger tumor

sizes and higher rates of lymph node disease and overall recurrence rates in patients younger than 30 compared to a middle-aged and an elderly group. However, no relevance to overall survival was indicated.[34] In a study of 78 pediatric patients, younger age was associated with more aggressive morphology and more frequent metastasis.[35] Pediatric PTC's biology and genetic profile might completely differ from adult onset, though.

Psammoma bodies on H&E examination have been shown to come with overall more extensive tumor biology and adverse prognostic features such as primary tumor size, extrathyroidal extension, lymph node and distant metastasis.[36]

1.3 BRAF Status

BRAF is part of the RAS-RAF-MEK-ERK-MAP kinase pathway contributing to cell growth. Physiologically, BRAF is regulated by RAS, whereas cell growth is independent from RAS in case of mutated BRAF. BRAF activating mutation is found in a variety of malignant tumors, initially discovered in malignant melanoma.

The most common mutation is V600E. It was first described by Davies et al. in 2002[37] as one of three possible base substitutions, the original in melanoma being thymine substituted by alanine at position 1796 (T1796A) resulting in changing codon number 599 from coding for valine to coding for glutamic acid (V599E). In their ground-breaking work, they confirmed all these mutations being somatic in origin. Due to later adaptations in codon numbering, BRAF V599E has come to be known as V600E.[38,39]

Generally, BRAF mutation in PTC has been associated with worse prognosis. A recent meta-analysis ties BRAF V600E to lymph node metastasis and reduced disease-free survival without impact on disease-specific survival rates.[40] Other studies showed higher disease recurrence rates.[41,42]

On the contrary, in a single-center retrospective study published in 2024, there was no significant difference in the frequency of BRAF mutation between cases of advanced disease and cases with successful complete resection without metastasis. Additionally, BRAF status showed no influence on disease-free survival.[43]

In addition, a literature review published in 2024 assessed 47 studies for pathological hallmarks in association of BRAF status and presence of lymphocytic inflammation in PTC. BRAF positive tumors, regardless of thyroiditis, showed more frequent central lymph node disease as well as positive association to tumor size, multifocality and extrathyroidal extension. However, these pathological variables have not been analyzed for their clinical relevance. On the contrary, tumors in thyroiditis showed overall more favorable prognosis regardless of BRAF status, leading to the author's assumption of thyroiditis in some way inhibiting carcinogenesis.[44] Anyway, another explanation might be that BRAF mutation and inflammation represent two entirely different pathways to carcinogenesis.

Similarly, most analyses find an association to morphological adverse outcome factors without reviewing clinical outcome, such as Kim et al.[45]

Possible confounding factors between BRAF status and clinical outcome resulting in conflicting evidence have not been further elaborated.

1.4 Oncocytic Change

1.4.1 Oncocytic Morphology

Whereas "oxyphil cells" solely refers to the characteristic yet highly unspecific (eosinophilic) staining in H&E, the expression "oncocyte" may be quite literally translated. Large cells' dense eosinophilic appearance is a result of abundant mitochondria all over the – typically finely granular – cytoplasm.

Historically, oncocytes have been defined by excess amounts of mitochondria.[46] Immunohistochemically, these larger than normal cells harboring accumulation of mitochondria present as the entire cell homogeneously and densely stained for pan-mitochondrial markers such as prohibitin (Fig. 3).

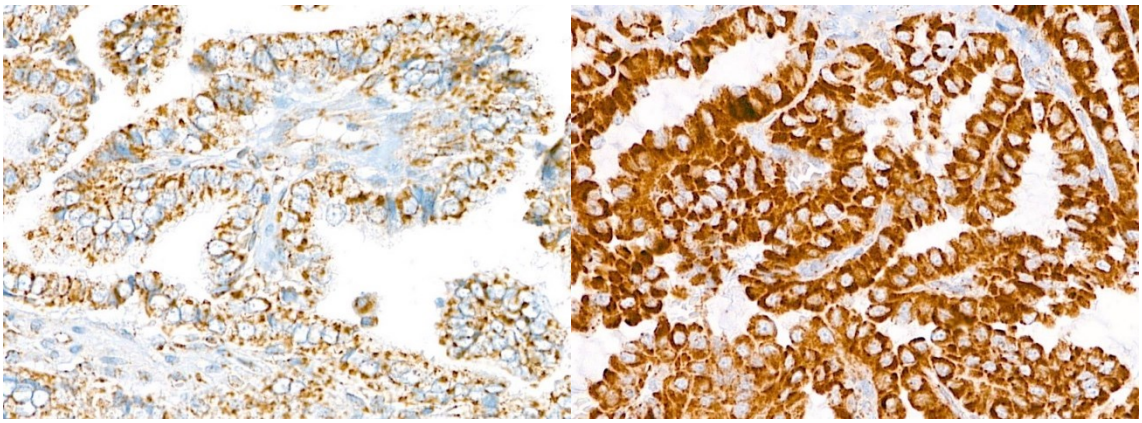

Figure 3: Mitochondrial Marker Prohibitin in Non-oncocytic (left) and Oncocytic Tumors (right)

However, a more recent study has shown that it is not solely about number of mitochondria, but distribution of other cell organelles as well. At times, subtle concentration of mitochondria towards the basolateral part of the cells with slight reduction in immunoreactivity towards the cells' luminal compartment can be observed. This sign of polarity contributes to viewing oncocytic change as a spectrum of cellular adaptations towards complete oncocytic transformation[33].

Another observation is that normal yet actively secreting thyrocytes show large amounts of ER evenly dispersed throughout the cytoplasm. In contrast, by immunohistochemistry, oncocytes have been shown to contain rather smaller amounts of ER in relation to cellular enlargement, displaced towards the cell's apical end by excess mitochondria in the basal parts of the cell[33].

Similarly, on H&E, nuclei are lifted from the basal membrane and pushed centrally, hence shaped round (Fig. 4). Most evident, these displacements were seen in polarized oncocytes as precursor to complete oncocytic transformation, yet harboring less mitochondria than fully developed oncocytes, making it harder to diagnose lesions containing such polarized oncocytes on H&E. Generally, as mitochondria make up for most of the cell content, all the other organelles lose their regular pattern of distribution[33].

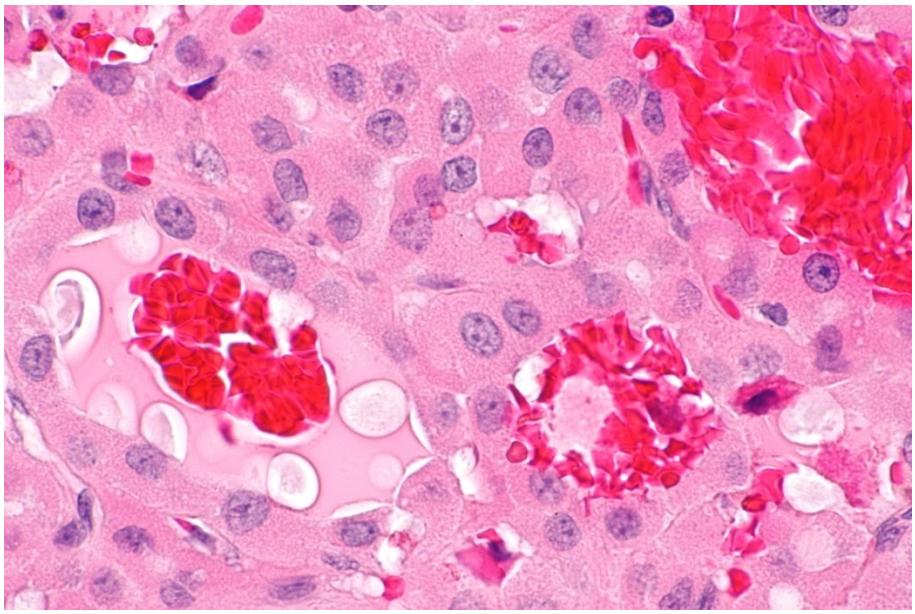

Figure 4: Evident Oncocytic Appearance on H&E[47]

On the contrary, mitochondrion-rich tumors yet morphologically distinguishable as non-oncocytic showed a varying number of mitochondria and organelles evenly dispersed throughout the cytoplasm, confirmed by electron microscopy. On immunohistochemistry, mitochondrial stain, even if dense at times, was evidently distributed in typical larger granules as opposed to fine, homogeneous stain in oncocytes. Using these findings, a simple approach to distinguishing oncocytes from non-oncocytic lesions was deduced, based on immunohistochemically aided observation of type and degree of mitochondrion accumulation and distribution of nuclei and ER[33].

Based on these observations, Tsybrovskyy and Rößmann-Tsybrovskyy[33] concluded that oncocytic change is defined not merely by mitochondrion number but by cell organelle distribution. They postulated a continuous transformation from no oncocytic features at all in normal cells, mediated by significant patterns of mitochondria and organelle distribution in what they refer to as “polarized oncocytes”, very well noticeable on IHC yet less reliably on H&E, over to “full-blown oncocytes” at the end of the spectrum, clearly visible on H&E, albeit less reliably than on IHC, too.

These findings laid the groundwork for future studies. One, oncocytes have historically been falsely characterized by mitochondrion number alone, which may have led to misclassification especially in borderline groups such as “polarized

oncocytes". Two, they illustrated what oncocytic transformation correlates to on H&E, IHC and ultrastructurally. And lastly, oncocytic change is to be separated from mitochondrion accumulation in non-oncocytic tumors. While the latter showed significantly worse outcome, the former is seemingly coexisting, but not truly causal to malignancy or poor outcome.

Current IHC markers used to stain mitochondria evolve around the mitochondrial DNA interacting prohibitin complex.[48,49]

1.4.2 Molecular Profile of Oncocytic Cancer Cells

The role of mitochondrial DNA mutations regarding cancer has at least been known since 2001. Insufficient DNA repair mechanisms account for one of multiple pathways leading to mutations, while aberrant and increased yet less efficient metabolism is one of the hallmarks of cancer. Unique to mitochondria, their genome may vary within one single cell due to their own set of DNA. Consequently, some mitochondria may harbor different mtDNA mutations than others. This concept is called heteroplasmy, when mutations arise in some but not all of one cell's mitochondria. Mutations universal to all mitochondria in one cell are referred to as homoplasmic.[50] In the case of heteroplasmy, disease or phenotype manifestation occurs only when a certain threshold is reached.[51]

In 2006, Bonora et al.[52] identified mtDNA mutations as the reason for insufficient oxidative phosphorylation – especially concerning the activity of complex I and III enzymes. Aberrant oxidative phosphorylation is therefore to be interpreted as trigger to mitochondria accumulation and high glucose dependency. This can clinically to increased FDG uptake in PET scan.[51]

In vitro, their specific oncocytic cell line happened to be the only one susceptible to lack of glucose for ATP synthesis. Offering galactose medium, the authors enforced ATP synthesis via mitochondrial oxidative phosphorylation, suggesting mitochondrial deficiency as a reason for their excessive proliferation in oncocytes. Through creating a hybrid cell line, inserting oncocyte-mtDNA into separately originated cells, they were able to rule out nuclear mutations, in fact confirming mitochondrial mutations as the cause for oncocytic transformation.

Thereafter, the same working group was able to clarify these mutations. In a series of sequencing the mitochondrial genome of several oncocytic and non-oncocytic

tumors by Gasparre et al. in 2007,[51] disruptive mutations (i.e. in the end producing inadequate proteins) have been detected. These mutations concern gene locations ND1, -2, -4 and -5, all of which code for complex I subunits of the respiratory chain and were statistically significantly associated with oncocytic phenotype.

As deficient oxidative phosphorylation and corresponding mutations have further been investigated from here on out, limitation of these studies might more likely arise from the method they used[53] to immunohistochemically classify oncocytic tumors as such, which back in the time was neither as precise nor as specific as it has been adapted since then and is in use today. Also, a solid proportion of oncocytic tumors did not show disruptive mtDNA mutations, leaving space for synergistic effects of nuclear mutations.

That said, all these mtDNA mutations are localized anywhere on the continuum of neoplasia, so far ruling out direct association to malignancy per se.

En passant, Tsybrovskyy et al. validated novel mitochondrial IHC markers regarding oncocytic change.[49] They used prohibitin as a pan-mitochondrial control and NDUFS4 as a surrogate for complex I integrity. Using these two markers, previously classified TCS test tumors were confirmed to be of oncocytic phenotype, which was then confirmed by molecular analysis of mtDNA. In TCS/oncocytes, loss of NDUFS4 was statistically significant while, if preserved, it was statistically significant for conventional non-oncocytic, non-tall-cell PTC. Furthermore, loss of NDUFS4 interpreted as complex I disintegrity was significantly associated to known mtDNA disruptions as characteristically seen in oncocytes and BRAF V600E mutation.

According to morphological studies, oncocytic change is significantly associated with lymphocytic inflammation such as in Hashimoto's disease and surprisingly even more so with Graves' disease and hyperthyroidism.[33]

Oncocytic change and similar streamlines such as general mitochondrial dysfunction, dedifferentiation and functional decline have further been linked to cellular ageing.[54]

1.4.3 Clinicopathologic Correlation

Oncocytic tumors have been found in virtually any organ. Typically, they are most associated with the thyroid gland[55], neuroendocrine structures[56] and the kidneys.[57]

Oncocytes have historically been associated with aggressive biology, extensive disease and poor clinical outcome. In current literature, consensus says there is no doubt that oncocytic carcinomas are biologically distinct entities.[58]

Tsybrovskyy and Rößmann-Tsybrovskyy significantly associated mitochondrial hyperplasia, be it mitochondrion-rich tumors or true oncocytic lesions, with later onset, concordant with age as a possible role in mitochondrial defects.[55] Lopez-Penabad et al. have received similar results. Their data also clearly shows that oncocytic carcinomas are usually larger in size on initial diagnosis.[19]

High mitochondrial number, both in oncocytic and non-oncocytic carcinomas was significantly associated with poorer disease-free survival, more so in non-oncocytic lesions harboring isolated mitochondrial increase. In general, mitochondria hyperplasia is linked to neoplasia and malignancy.

However, oncocytic change per se was not associated with earlier recurrence. Neither correlation of tumor size, tumor stage or invasion type was statistically significant.

Their extensive research allowed to postulate one reason of oncocytic tumors' so controversially discussed biological profile might be simple selection bias early on in every study, as there have never been uniform morphological criteria of how to really define oncocytic appearance and therefore find the right diagnosis. For the first time, they differentiated simple mitochondrion number from "real" oncocytic phenotype thyroid tumors. Their morphologic research using novel markers led to defining simple diagnostic algorithms as to how to correctly distinguish oncocytes from non-oncocytic lesions.[33]

As explained earlier, oncocytic change in itself is a condition occurring in all kinds of neoplasia and even nonneoplastic tissue such as hyperplastic nodules. It is important to note that the presence of oncocytes per se does not present a reasonable criterion for malignancy and/or is unlikely to itself trigger adverse outcome.[33]

One such diagnostic challenge evolving from the principles illustrated above, advocating for careful interpretation of pseudo-malignant findings in nodule workup, is oncocytic tumors usually presenting highly positive on FDG PET scan.[59]

In addition, oncocytic carcinomas may present insensitive to radioactive iodine.[60] However, Lopez-Penabad et al. noticed a decrement in iodine sensitivity on multiple longitudinal scans suggesting loss of iodine sensitivity in metastasis regardless of the primary tumors' oncocytic biology.[19] Mostly, iodine sensitivity of primary tumors is difficult to evaluate as most literature focus on 131-I uptake post-surgery.

1.4.4 Tall Cell Subtype of Papillary Thyroid Carcinoma

PTC-TCS is a subtype of PTC characterized by trabecular architecture. These consist of longer than wide, parallel papillae offering little to seemingly no stroma underneath tightly organized cancer cells, hence referred to as showing a "tram-track" pattern. "Tall cells" are defined by measuring more than three times their width in height.

It has been postulated that distinguishing TCS from conventional PTC is of immediate clinical relevance. Pathologically, TCS has been shown to come with more aggressive tumor features such as infiltrative growth, extrathyroidal extension and metastasis. In addition, also clinically, tall cell morphology has been associated with higher rates of persisting and recurrent disease.[61,62]

Despite current belief on TCS as in independent predictor for poor outcome, there is growing evidence demonstrating that poor outcome in TCS is very well linked to adverse biological features and adjusted for these features, clinical course is similar to conventional PTC.[63,64] Anyway, some studies noticed higher frequency of aggressive features in TCS.

Interestingly, recent research[49] on PTC-TCS has shown that, in fact, TCS represents oncocytic change in thyroid carcinoma. In a study comparing PTC-TCS to conventional PTC, electron microscopy showed TCS's cytoplasm to contain less ER whose distribution within the cell seemed suppressed by abundant enlarged and aberrantly shaped mitochondria.

NDUFS4 on IHC was confirmed as a valid correlate of functional complex I subchain. Preserved NDUFS4 was statistically associated with conventional PTC, whereas loss of NDUFS4 was linked to TCS. Also, prohibitin was validated as a mitochondrial marker.

Pathogenic mtDNA mutations were associated with TCS and loss of NDUFS4, whereas lack of mtDNA alterations was as significantly associated with preserved mitochondria energy metabolism and accordingly, preserved NDUFS4 staining.

BRAF V600E was significantly associated with TCS, mtDNA mutations and loss of NDUFS4. Certain tumors have led to believe BRAF mutation precedes or might even play a role in tumor cells obtaining mtDNA mutations. In some tumors, only certain clusters of tumor cells which were all deficient of complex I occurred within an entirety of tumor cells harboring V600E.

Parallels have been drawn between research on TCS and on oncocytic carcinomas which both tend to concern older patients, be larger on first diagnosis, show more extrathyroidal extension, have more frequently infested lymph nodes on initial surgery and in general, are said to come with worse prognosis.

However, further research is needed to outline whether TCS and oncocytic carcinomas provide an integral contribution to adverse biology and clinical outcome or are still confounded by for example various ways of biologic dedifferentiation such as reduced iodine uptake (RAI resistance), impaired TSH-dependency.

They concluded that tall cells as found in PTC-TCS most closely represent “polarized oncocytes” as explained above and by Tsybrovskyy and Rößmann-Tsybrovskyy in 2009. They confirmed mtDNA mutations as cause for oncocytic change.

1.5 Aim of this Study

As extensively demonstrated, implications of oncocytic change in PTC and its translation towards clinical practice is strongly disputed.

It has been postulated that oncocytic change in carcinomas result from different biology and pathogenesis compared to “classic” entities of DTC. Lately, corresponding advances have been made in the WHO Classification of Tumors and

on top recent evidence clarifies that TCS, importantly one of the more commonly diagnosed subtypes, is in fact oncocytic.

While abundant literature has linked TCS and oncocytic change to more aggressive features and worse outcome, a true cause-effect relation has never been established. There is no doubt that where adverse morphological features are present, oncocytic carcinomas come with more difficult management and possibly higher risk of recurrence. By contrast, it is questionable whether oncocytic change itself should be considered an “aggressive feature”.

Based on our initial observations, we hypothesized that TCS or oncocytic change per se is not more aggressive itself as defined by extent of initial disease and metastasis. Neither does it come with worse overall outcome or higher recurrence rates just as can be explained biologically.

Since recent works have proven historical diagnosing of oncocytic tumors solely on H&E to be imprecise, we also wanted to assess the diagnostic value of mitochondrial IHC, namely prohibitin and NDUFS4.

Hence, the aim of this study was to evaluate PTC subtyping based on H&E and oncocytic immunoreactivity and to see whether the latter is more effective. Furthermore, these classifications were used to assess clinical outcome as defined by relevant lymph node disease.

2 Methods

2.1 Study Design and Cohort Selection

We conducted a single-center population-based cohort study by retrospective data analysis.

Permission has been obtained by the ethical committee to retrospectively analyze preexisting clinical patient data and archived physical specimen. The committee's decision was documented under code "34-357 ex 21/22".

In clinical documentation, special focus was given to patients' mode of presentation, initial clinical evaluation, lab work and imaging, initial treatment and regular follow-up or recurrent treatment. Physical specimens used included histological slides and paraffin blocks.

We initially included all PTCs diagnosed at the Diagnostic & Research Institute of Pathology in Graz, Austria, between 1995 and 2014.

That way, we were able to avoid any availability or ascertainment bias as all available material was stored on our premises. By including practically any PTC cases, we tried to avoid selection bias as far as possible.

In addition, according to § 51(3) of the Austrian Physicians Act (ÄrzteG 1998), the mandatory retention period for medical information including histopathological specimens in Austria is at least ten years[65] while for liability reasons, physical evidence is mostly stored at least 30 years. This allowed for assessing at least ten years of clinical follow-up, when data collection and statistical analysis were commenced in 2024.

It must be noted that our patients have not only been treated at the Medical University of Graz but also in cooperating hospitals, clinics and primary care practices. This would render their clinical documentation slightly less available.

Only after careful consideration have some patients been initially excluded. These were primarily patients whose initial surgery was incomplete and who had completing surgery elsewhere or where first contact with our institute was already recurrent disease. Also, initially selected patients whose original diagnosis was incorrect (in fact not PTC) and, lastly, patients where PTC was part of a much

more complicated continuum towards anaplastic carcinoma or sarcomatous mixed entities were reasonably excluded.

After initial exclusion of these unsuitable tumors, a total of N=472 tumors entered the study.

2.2 Histopathological Analysis

All tumors have been reviewed by expert pathologists and had their TNM stages reclassified according to the most current 2022 5th WHO Classification of Tumours.[8,20] Consensus was reached on all cases to avoid any observer or interpretation bias.

Furthermore, tumors were specifically assessed for subtype on H&E, known adverse morphological features, tall cell and oncocytic morphology.

Tumor and metastases sizes were reconstructed by gross descriptions, macroscopical measurement on the slides and using calibrated microscopes. We decided to calculate total volumes using spherical approximation. This is because in cases with multiple lymph node metastases, their volumes can be added to reflect a total metastatic burden as a single parameter, whereas adding diameters would be mathematically incorrect. Besides, diametric measuring has been postulated to serve as an approach to approximate tumor size only but is less reliable compared to tumor volume assessment.[66,67] Ultimately, equivalent diameters have again been calculated for clinical interpretation reasons only.

2.3 Immunohistochemical and Molecular Analysis

Tissue Micro Array (TMA) is a technique allowing for efficient staining of a multitude of samples on one single slide. Samples are collected from preserved blocks and then transferred to a separate recipient block. These new blocks consisting of multiple cores are then available for usual processing.[68]

Tumor foci smaller than 3 millimeters have been excluded from TMA to increase reproducibility, as punching the right area cannot be guaranteed the smaller the target is.

First and foremost, tumors have been stained using recently validated mitochondrial markers: prohibitin and NDUFS4.

The prohibitin 1 and 2 complex is vital to mitochondrial function and staining for prohibitin serves as a pan-mitochondrial marker.[48] Moreover, complex I of the oxidative phosphorylation is stained by NDUFS4.[69] Lack of complex I as seen in loss of NDUFS4 expression has just recently been validated to be highly sensitive for true oncocytic change.[49]

Using these markers, tumors were evaluated for their oncocytic appearance as defined by mitochondrion number and organelle distribution. A cut-off of 75% oncocytes, like oncocytic carcinoma's (OC) cut-off of 75% oncocytes on H&E, was used to distinguish between immunohistochemically oncocytic and non-oncocytic tumors.

Tumors have also been stained for BRAF V600E, RAS mutation and NTRK fusion. Where necessary, molecular analysis was obtained for profiles of BRAF, KRAS, NTRK and RET oncogenes.

Where one patient's thyroid had more than one tumor focus [28], a clear distinction between true multifocality, i.e. clonally separate neoplasms, and monoclonal intrathyroidal spread had to be made. Special consideration was given to intraorgan localization, H&E subtype and most importantly genetic profiling.

2.4 Acquisition of Clinical Data

In compliance with the ethical committee's votum, we studied documentation from openMEDOCS, AuraWeb, PAS Xanthos and each of the cooperating institutions' data archives.

In the early 2000s, openMEDOCS was introduced in hospitals within the Styrian KAGes.m.b.H. as the primary documentation and survey tool in day-to-day clinical work. Usually, clinical documentation since an apparent implementation period between 2002 and 2004 can be found in openMEDOCS.

AuraWeb combines pathology data to date and clinical documentation including radiology reports from before openMEDOCS has emerged. Nevertheless, some information must have been lost along the transition around 2002 through 2004.

Where patients were treated outside of the public infrastructure defined as hospitals and institutions from KAGes and the Medical University of Graz, we could not access patient data directly as per privacy regulations. Such missing data had

been specifically marked on initial data collection and was later provided by the respective partners. These cooperating institutions include privately owned medical centers and private practices.

Collected data has been stored in a Microsoft Access 2016 database (version 16.0).

Patient and tumor data points are deemed secondary data as they have been collected out of preexisting documentation.

2.5 Statistical Analysis

Statistical analysis was performed using IBM's SPSS, version 29.

A p-value less than .05 was considered statistically significant.

Results have been rounded to one decimal, where applicable. Extreme values as of biological variability have remained included. Missing values have methodically been treated as such, e.g. in metastasis variables in non-metastatic cases.

Independent variables revolved around common H&E subtyping with special focus on tall cell and oncocytic morphology as well as oncocytic immunoreactivity. Response variables were about lymph node metastasis to express clinical relevance. Secondary outcome variables included patient characteristics and special morphological features.

Normal distribution as a requirement for several statistical tests was evaluated by nature of the variable, difference between mean and median, histogram and Q-Q plot, skew values followed by Kolmogorov-Smirnov and Shapiro-Wilk tests. An integrated decision was made.[70]

Statistical tests used included t-tests for normally distributed, Kruskal-Wallis and Mann-Whitney-Wilcoxon rank sum tests for nonparametric continuous independent variables, Pearson's Chi Square test, linear-by-linear trend tests, Fisher's exact approximation, where necessary, and ordinal or multinomial regression für ordinally, respectively categorically scaled variables.

For comparing effect sizes of Mann-Whitney-Wilcoxon-U rank sum tests, Mann-Whitney probabilistic index was calculated as follows, where U is the Mann-Whitney-U test value reported in SPSS and n_X are subgroup sizes:

$$PI = \frac{U}{n_A n_B}$$

Ordinal and multinomial regression models were compared by their parameters of overall model effect, explanatory power for variance and, lastly, modeled odds and their CI width.

2.6 Definition of Lymph Node Status

Patients, who had lymph nodes dissected, regardless whether targeted or incidentally, were classified pN0 or pN1. By definition, this renders all other patients indeterminate in terms of lymph node status (pNx).

Nevertheless, most pNx patients at no point had any metastases, in the end comparing to pN0 patients. Had they had lymph dissection, no metastasis would have ever been found, which is why in these patients an observational period in follow-up was maintained after which, if continuously disease-free, patients were classified “equivalent to pN0”.

We chose this pragmatical approach, because relevant undissected metastases on initial treatment would later manifest on follow-up.

However, it must be noted that some patients “equivalent to pN0” assumably had micrometastasis at the time of resection that never became clinically relevant, be it because of their indolent biology or because they were eliminated following adjuvant RAI. The same applies to truly pN0 patients whose micrometastases could have been missed.

For that reason, statistical analysis of lymph node status and its distribution should follow a more realistic approach. Analysis according to primarily pN0 and pN1 is not precise enough.[71] An ordinal scale distinguishing relevant metastasis was established after considering a reasonable cut-off between categories.

Now, metastasis size commonly follows a highly right-skewed distribution.[72]

Clinically, lymph node metastases are not typically found if smaller than 5 to 10 millimeters depending on the observer’s ultrasound proficiency.

In thyroid cancer, (isolated!) metastasis smaller than 2 mm is referred to as micrometastasis. For comparative reasons, we reproduced an equivalent diameter of total metastasis out of previously calculated and accumulated total volumes. This value compares to as if all the metastases were one single focus.

About 75% of our tumors had (accumulated!) total metastasis smaller than 5 mm (equivalent), at which percentile volumes started to slowly rise; about 60% had total metastasis smaller than 2 mm, respectively. We therefore chose 2 mm as cutoff for relevant metastasis in accordance with general histopathological practice as restricted by gross workup technique and specimen processing.

Again, an equivalent diameter below 2 millimeters means one focus of up to 2 millimeters diameter or several smaller foci, whose combined volume translates to less than 2 millimeters diameter. Each single focus might as well in fact only be microscopic.

This categorization was then eligible for ordinal logistic regression. Hence, the odds for more extensive metastasis as compared to no metastasis, compared between two subpopulations, were modeled along an ordinal outcome scale. This proportional odds model predicts that the independent variables' influence on ordinally ascending metastasis categories is linear, meaning the odds for reaching higher categories follow clearly monotonical trend curves and add up.

3 Results

3.1 Cohort Characteristics

Our final cohort comprised N=472 primary tumors from 421 individual patients. Basic patient and tumor characteristics are presented in Table 2.

3.1.1 Basic Tumor Characteristics

Approximately 10% of patients had more than one biologically distinct primary tumor simultaneously (true multifocality, Table 2). On the contrary, some biologically distinct entities came with more than one morphologically and genetically identical lesion (intrathyroidal spread).

Primary tumor size followed a highly right-skewed distribution (lots of smaller entities).

Table 2: Overview of Basic Patient and Tumor Characteristics

Patient Characteristics	N=421 patients
Patient sex:	
female	307 (72.9%)
male	114 (27.1%)
Female to male ratio	2.7 : 1
Age in years, median (Q1-Q3)	45 (35-55)
Multifocality	41 (9.7%)

Tumor Characteristics	N=472 tumors
Patient sex:	
female	340 (72.0%)
male	132 (28.0%)
Female to male ratio	2.6 : 1
Age in years, median (Q1-Q3)	46 (35-55)
Intrathyroidal spread	11 (2.3%)
Total tumor volume in cm^3, median (Q1-Q3)	0.9 (0.1-4.9)
Tumor equivalent diameter in cm, median (Q1-Q3)	1.2 (0.6-2.1)

An overview of morphological features can be seen in Table 3.

Table 3: Morphological Features on H&E

Morphological Features	N=472 tumors		
No, minimal, abundant psammoma bodies	305 (64.6%),	94 (19.9%),	73 (15.5%)
No, microscopic, gross extrathyroidal extension	258 (54.7%),	186 (39.4%),	28 (5.9%)
No, focal, clear infiltration	26 (5.5%),	182 (38.6%),	264 (55.9%)
No, partial, complete encapsulation	308 (65.3%),	69 (14.6%),	95 (20.1%)

Most tumors were BRAF V600E mutated and negative for RAS mutation and NTRK fusion (Table 4).

Table 4: Molecular Profile

Molecular Profile	N=472 tumors
BRAF V600E mutation	343 (72.7%)
RAS mutation	3 (0.6%)
NTRK fusion	13 (2.8%)

3.1.2 Lymph Node Status

Despite extensive due diligence, patient data about lymph dissection was unavailable in one patient, leaving 420 patients for analysis (Fig. 5).

292 had diagnostic or therapeutic lymph dissection performed, 167 of which had positive lymph nodes.

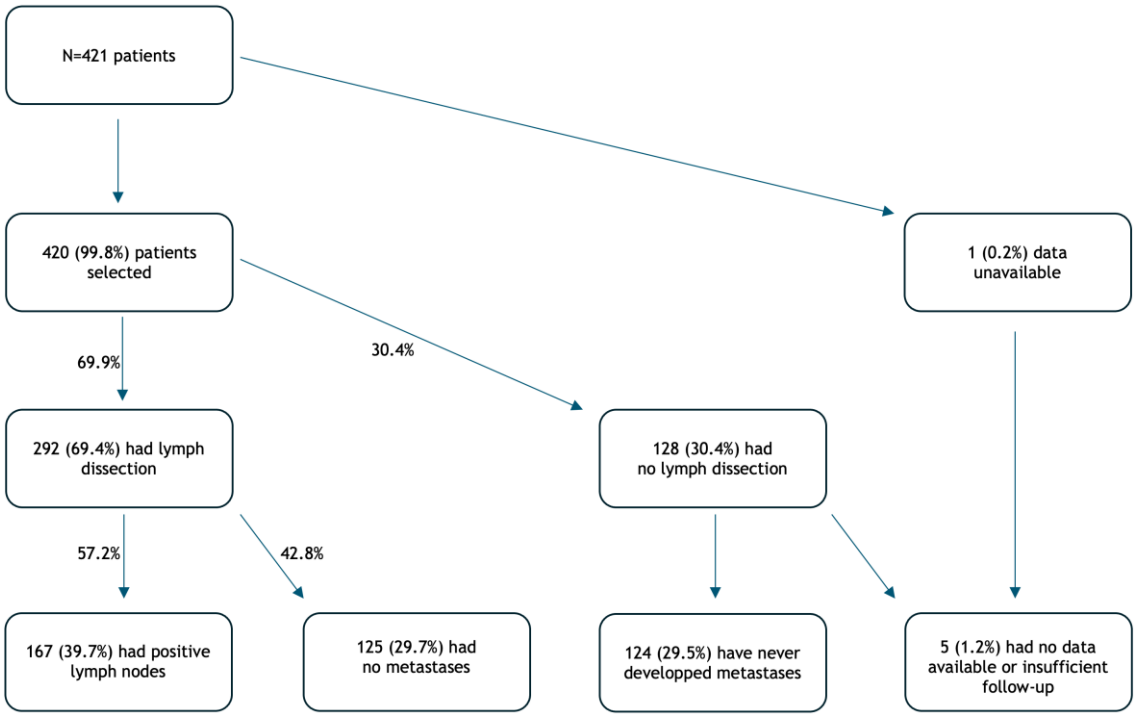

Figure 5: Lymph Dissection Flowchart

These 167 patients (39.7%) came with 169 metastatic tumors (35.8%).

Metastasis extent in these 169 cases can be seen in Table 5.

Table 5: Extent of Lymph Node Metastasis

Lymph node metastasis	N=169 tumors
Number of positive lymph nodes, median (Q1-Q3)	4 (2-8.5)
Total volume in cm^3, median (Q1-Q3)	1.2 (0.1-11.6)
Equivalent diameter in cm, median (Q1-Q3)	1.3 (0.4-2.8)

3.2 PTC Subtyping

The frequency of morphological subtypes identified on H&E are presented in Table 6.

Table 6: Morphological Subtypes on H&E

Morphological Subtypes	N=472 tumors (100%)
Classic	182 (38.6%)
Classic with pfgp *	97 (20.6%)
Follicular variant	40 (8.5%)
Tall cell subtype	97 (20.6%)
Oncocytic	24 (5.1%)
Warthin	8 (1.7%)
Diffuse sclerosing subtype	5 (1.1%)
Solid trabecular subtype	19 (4.0%)

* predominantly follicular growth pattern

Figure 6 shows frequency of oncocytic change as detected by IHC classification (with cut-off of 75% oncocytes) across the morphological subtypes. As expected, the vast majority of TCS cases were oncocytic on IHC, quite similar to oncocytic and Wartin-like subtypes. Of note, about 20.0% to 23.6% conventional (including follicular subtype) PTCs turned out to be oncocytic on IHC. By contrast, none of solid-trabecular and diffuse sclerosing subtypes were in fact oncocytic.

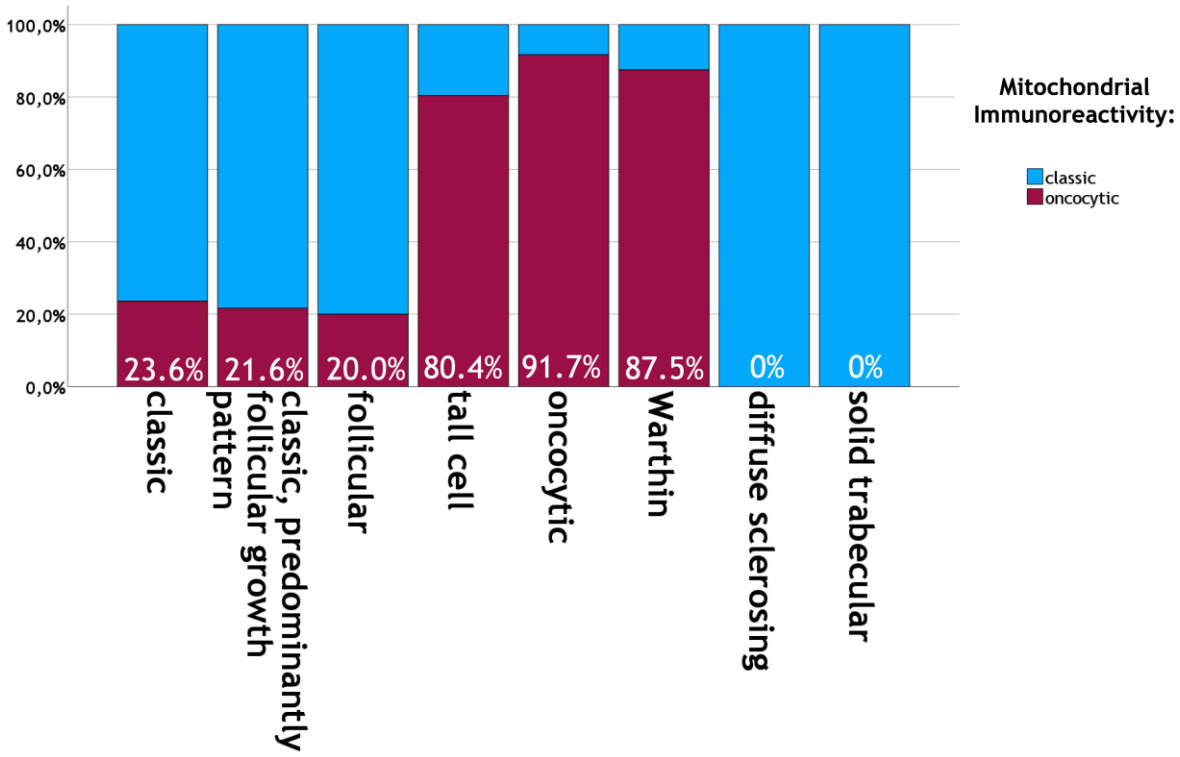

Figure 6: Oncocytic vs. Non-oncocytic Immunoreactivity in H&E Subtypes

Abundant mitochondria were observed in TCS (Fig. 9) and oncocytic PTC (Fig. 10, 11) with preserved cell polarity in TCS. Loss of NDUFS4 expression served as surrogate marker for oncocytic transformation. Preserved NDUFS4 was observed in NST cases such as classic papillary PTC (Fig. 7) and follicular variant of PTC (FVPTC, Fig. 8).

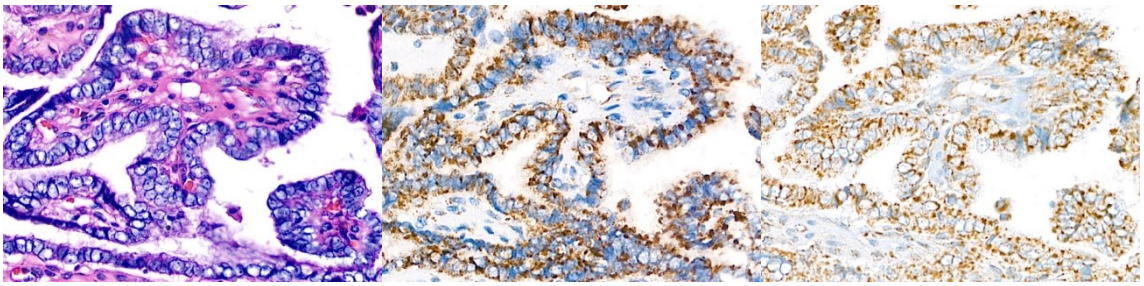

Figure 7: Classic Papillary PTC on H&E (left), showing preserved NDUFS4 expression (center) and no accumulation of mitochondria on Prohibitin stain (right), corresponding to non-oncocytic IHC profile

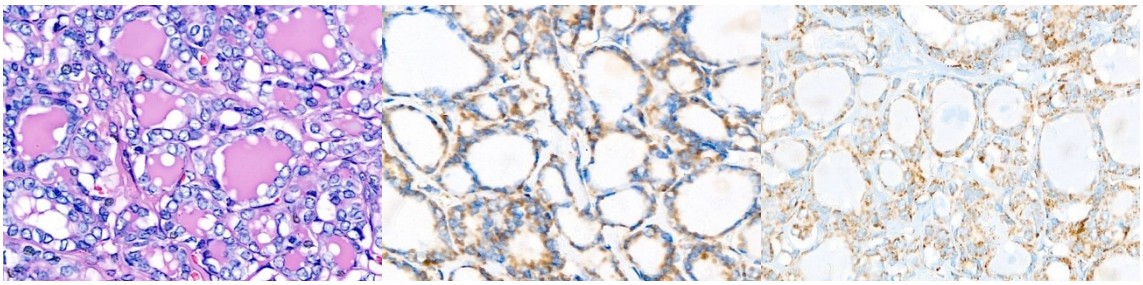

Figure 8: FVPTC on H&E (left), showing preserved NDUFS4 expression (center) and no accumulation of mitochondria on Prohibitin stain (right), corresponding to non-oncocytic IHC profile

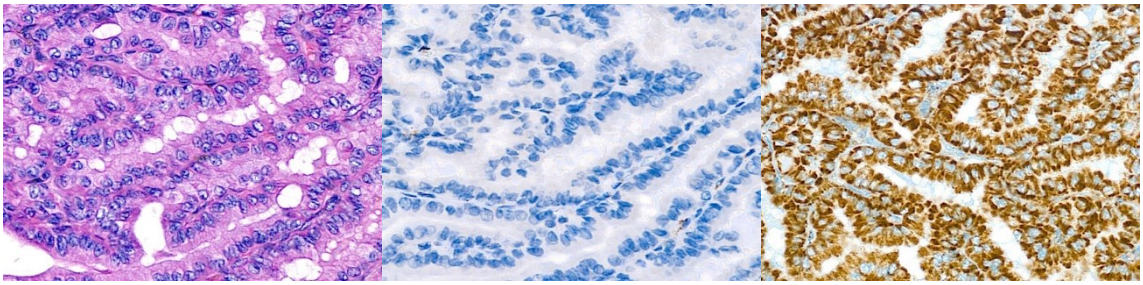

Figure 9: TCS-PTC on H&E (left), showing loss of NDUFS4 expression (center) and marked accumulation of mitochondria on Prohibitin stain (right), corresponding to oncocytic IHC profile

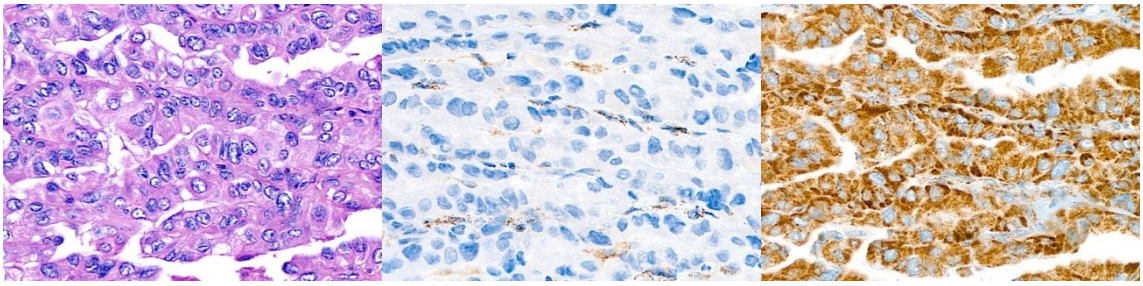

Figure 10: Oncocytic PTC on H&E (left), showing loss of NDUFS4 expression (center) and marked accumulation of mitochondria on Prohibitin stain (right), corresponding to oncocytic IHC profile

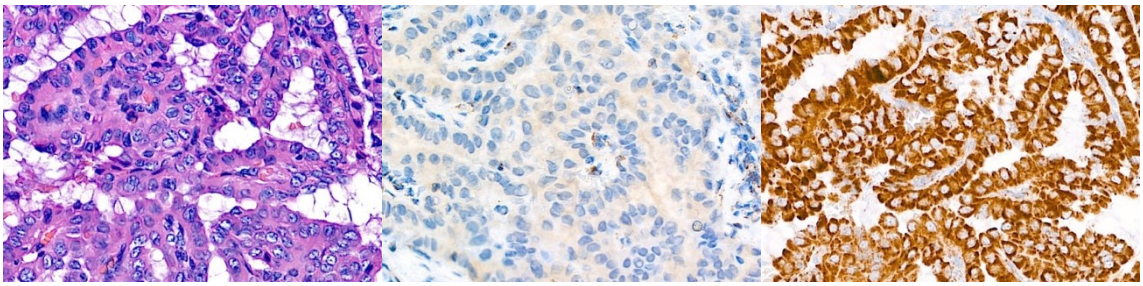

Figure 11: Classic Papillary PTC on H&E (left), showing loss of NDUFS4 expression (center) and marked accumulation of mitochondria on Prohibitin stain (right), corresponding to oncocytic IHC profile

For the sake of further analysis, we aimed to simplify the morphologic subtyping of PTC. First, we inspected the frequency of BRAV V600E mutation across the morphologic subtypes (Fig. 12).

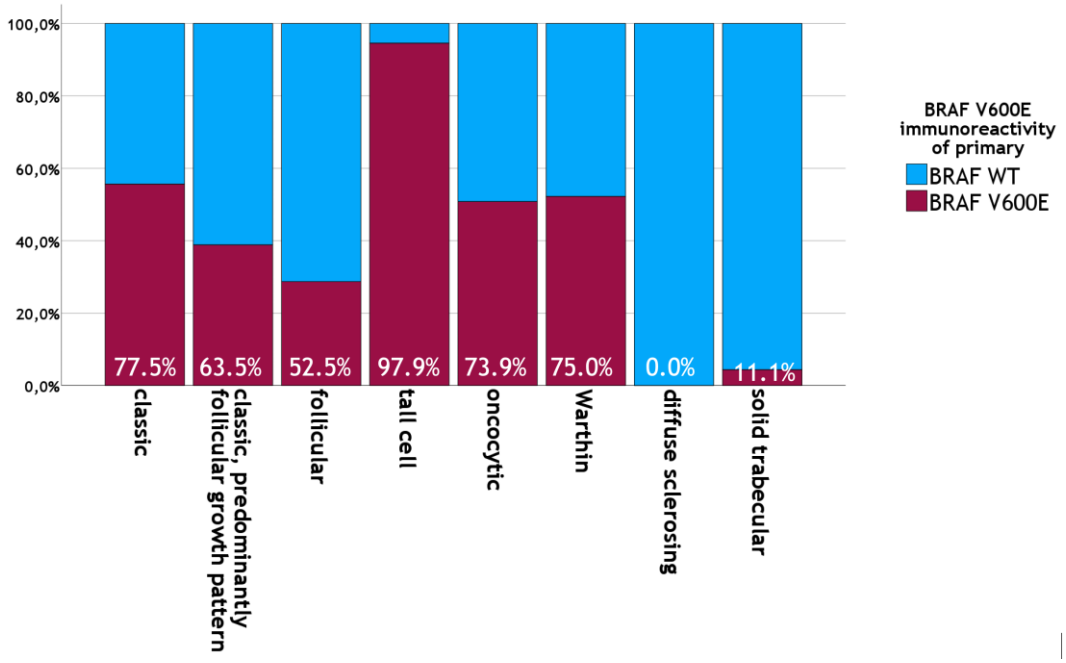

Figure 12: BRAF Status in H&E Subtypes

As can be seen, diffuse sclerosing and solid trabecular subtypes showed close to no BRAF V600E mutations. Furthermore, patients with these PTC subtypes were strikingly younger than the remaining population (Fig. 13).

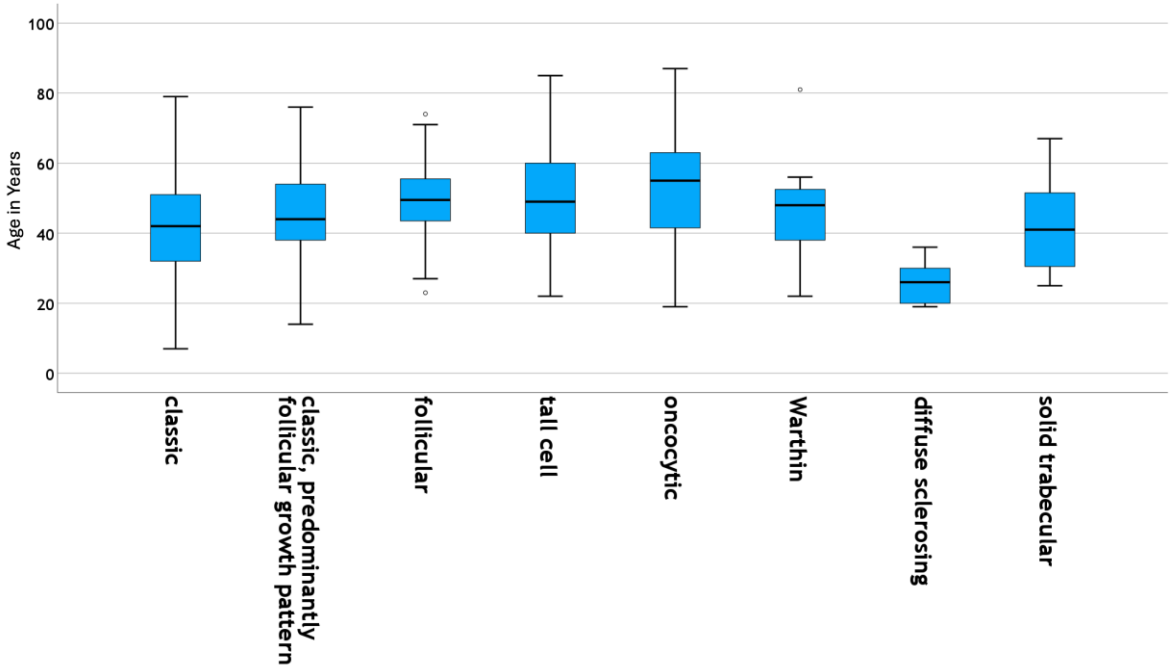

Figure 13: Age Distribution in H&E Subtypes

Due to their rarity and completely different biology depicted in the results above, diffuse sclerosing and solid trabecular subtypes (N=24) were excluded from further analysis.

Classic, classic with predominantly follicular growth pattern and follicular subtypes did not differ in presence (Fig. 14) or size of metastasis (Fig. 15). Also, no significant difference in mitochondrial immunoreactivity was observed.

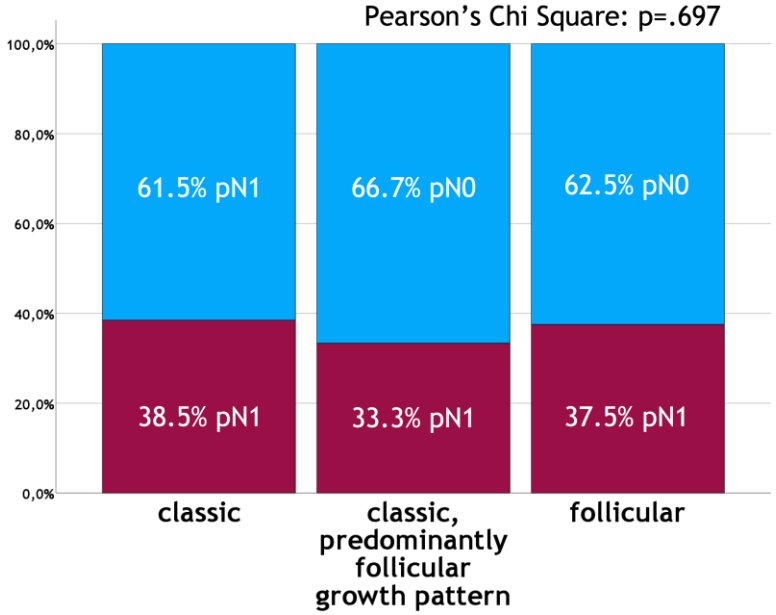

Figure 14: Lymph Node Status in Conventional Morphologies

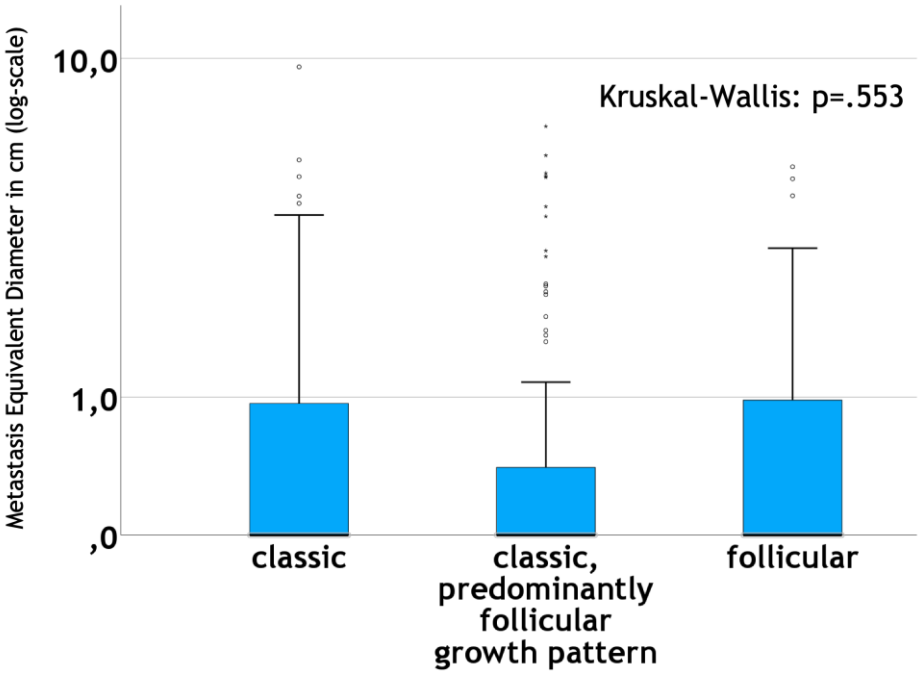

Figure 15: Metastasis Equivalent Diameter (log-scale) in Conventional Morphologies

These three subtypes were therefore combined into one "no special type" (NST) group (N=319) for further analysis.

Similarly, TCS, oncocytic and Warthin-like PTC all behaved comparably (Fig. 16 and 17) regarding metastasis and mitochondrial patterns and were subsumed under one “TCS/oncocytic” category (N=129).

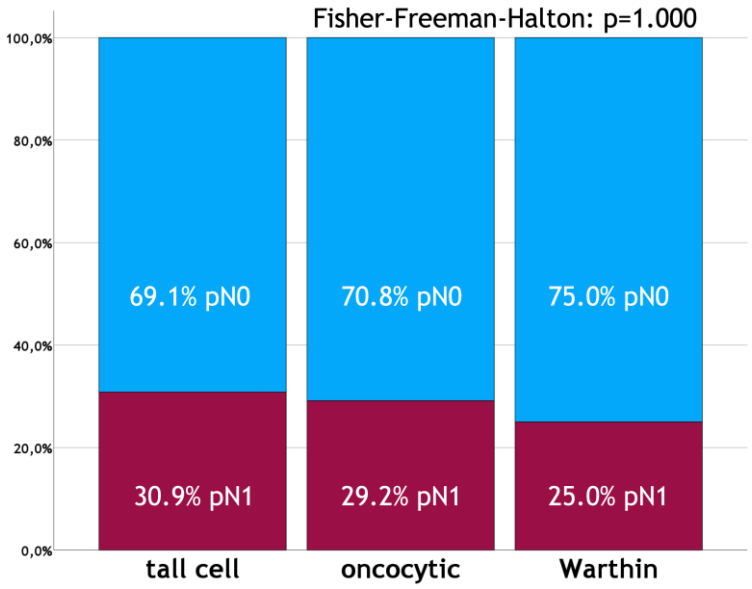

Figure 16: Lymph Node Status in TCS/oncocytic Subtypes

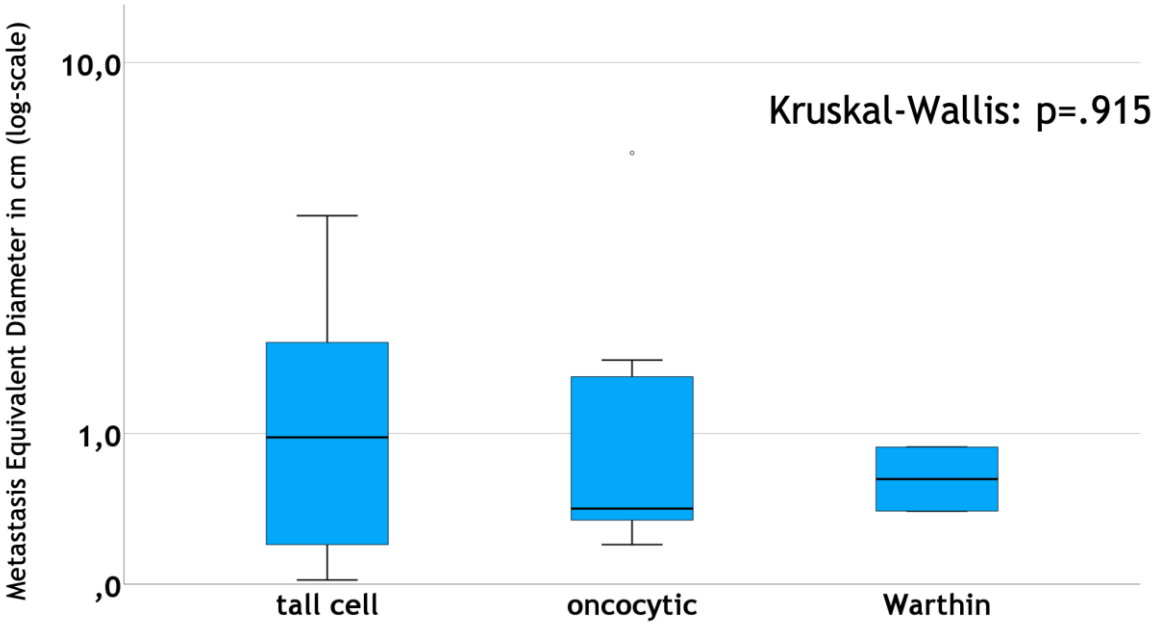

Figure 17: Metastasis Equivalent Diameter (log-scale) in TCS/oncocytic Subtypes

Ultimately, there were two morphologic PTC groups (NST and TCS/oncocytic) and two immunohistochemical PTC groups (IHC-NST and IHC-Oncocytic) left for further statistical analysis.

3.3 Clinicopathologic Correlation

319 cases were NST versus 129 TCS/oncocytic on H&E. 269 were IHC-NST versus 179 IHC-oncocytic.

TCS/oncocytic subtypes were significantly associated with later onset, larger primary tumors and BRAF V600E mutation, as were IHC-oncocytic tumors.

3.3.1 Patient Age and Tumor Volume

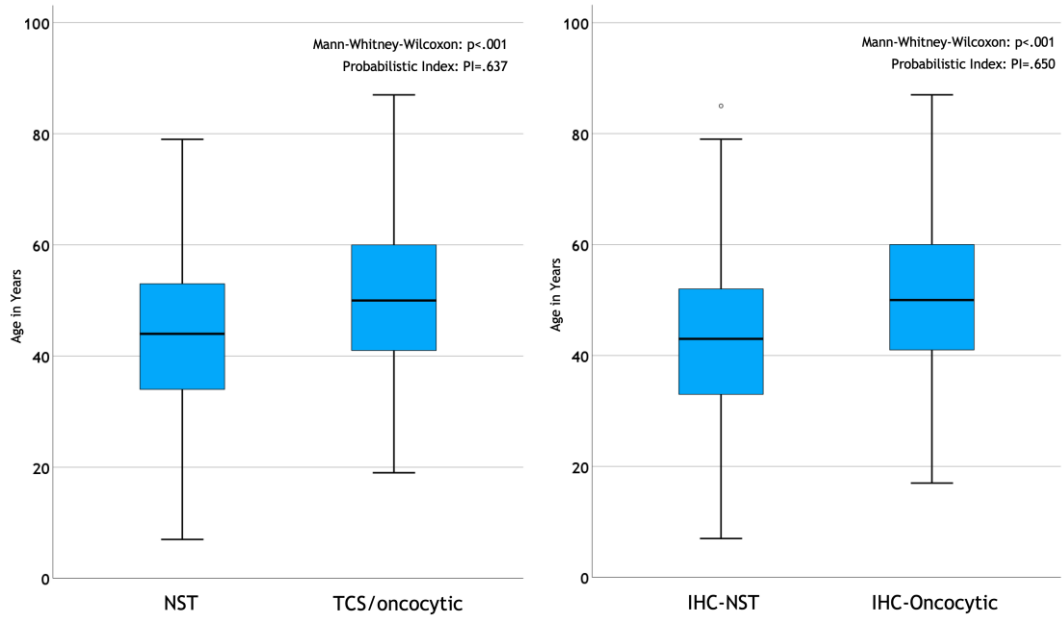

Figure 18: Age at Diagnosis in H&E Subtypes (left) vs. IHC Categories (right)

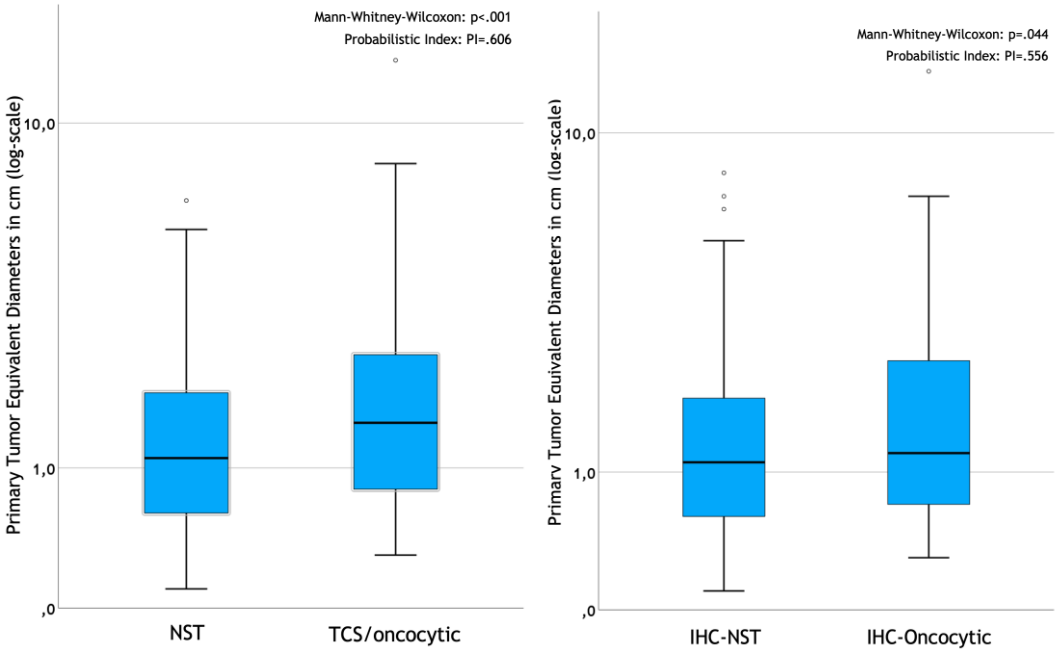

Figure 19: Primary Tumor Size in H&E Subtypes (left) vs. IHC Categories (right)

As can be seen graphically and by Mann-Whitney probabilistic index, grouping by IHC (right) was more discriminatory for patient age (Fig. 18) and less discriminatory for primary tumor volume (Fig. 19) compared to grouping by H&E subtypes (left).

3.3.2 BRAF Status

Fig. 20 presents BRAF status by H&E subgrouping as compared to IHC categorization.

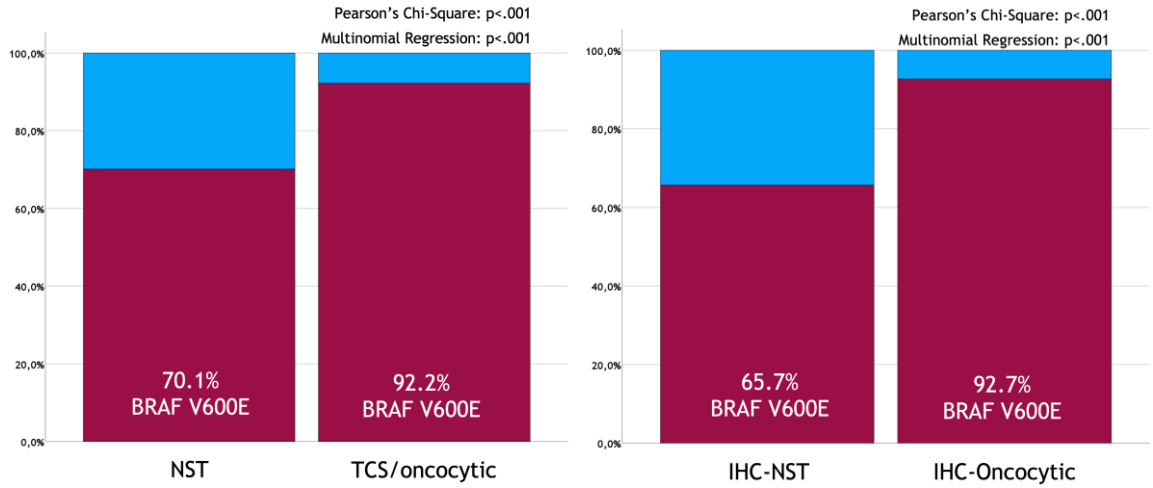

Figure 20: BRAF Status in H&E Subtypes (left) and IHC Categories (right)

In cross tabulation, IHC grouping was slightly more distinguishing between BRAF wildtype and V600E compared to H&E subtypes. This was confirmed in multinomial regression analysis (Table 7).

Table 7: Multinomial Regression Models for BRAF status by H&E vs. IHC Grouping

Comparison of Models	H&E	IHC
Likelihood ratio Chi-Square	29.192	49.078
Cox & Snell	.063	.104
Nagelkerke	.093	.152
McFadden	.057	.096
Odds e^B [95% CI]	.199, [.100; .396]	.151, [.081; .280]

3.3.3 Morphological Features

TCS/oncocytic subtypes (H&E) and IHC-oncocytic tumors showed less psammoma bodies, more extrathyroidal extension, were more infiltrative and less encapsulated.

Psammoma bodies:

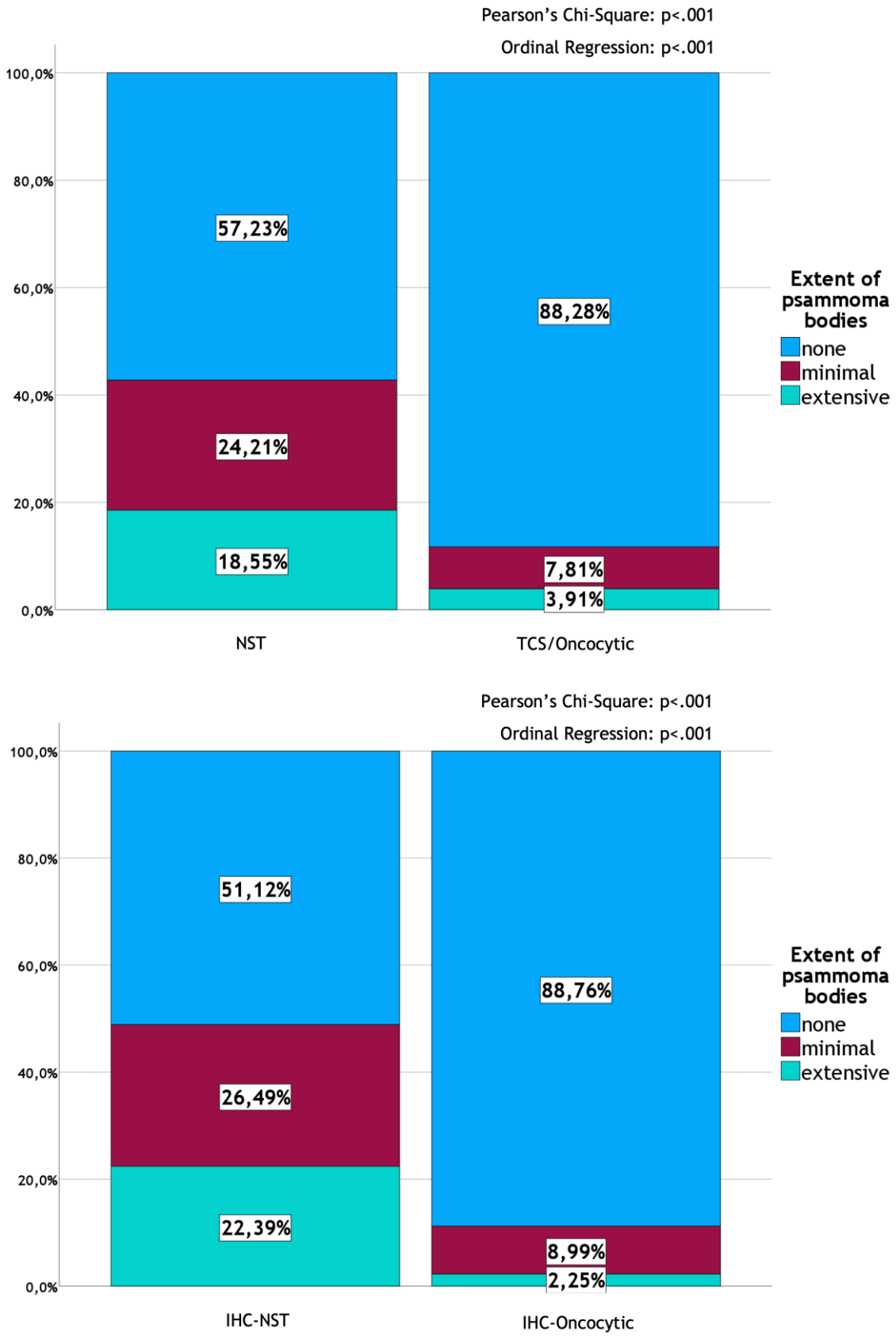

Figure 21: Extent of Psammoma Bodies in H&E Subtypes (top) vs. IHC Categories (bottom)

Similarly to BRAF, cross tabs suggest more contrasting results by IHC distinction, which was confirmed by ordinal regression (Table 8).

Table 8: Ordinal Regression Models for Extent of Psammoma Bodies by H&E vs. IHC Grouping

Comparison of Models	H&E	IHC
Likelihood ratio Chi-Square	45.965	79.587
Cox & Snell	.098	.163
Nagelkerke	.118	.197
McFadden	.059	.102
Odds e^B [95% CI]	1.744 [1.164; 2.325]	2.067 [1.544; 2.589]

Extrathyroidal extension:

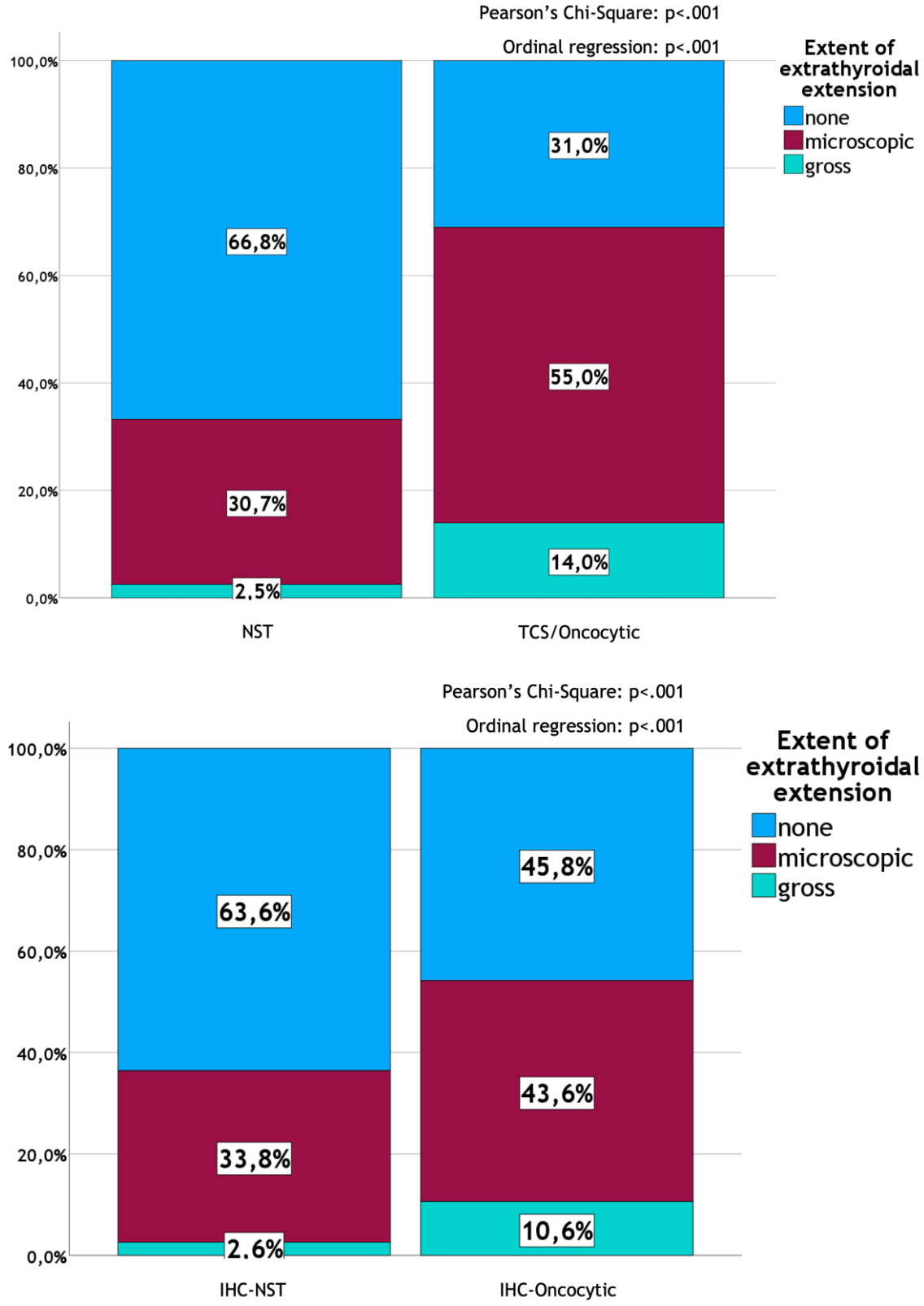

Figure 22: Extent of ETE in H&E Subtypes (top) vs. IHC Categories (bottom)

While cross tabs allow for speculation, the ordinal regression model naturally suggests IHC categorization to be weaker than H&E regarding extent of extrathyroidal extension (Table 9).

Table 9: Ordinal Regression Models for Extent of Extrathyroidal Extension by H&E vs. IHC Grouping

Comparison of Models	H&E	IHC
Likelihood ratio	54.333	17.313
Cox & Snell	.114	.038
Nagelkerke	.139	.046
McFadden	.071	.023
Odds e^B [95% CI]	-1.549 [-1,972; -1.126]	-.798 [-1.175; -.421]

Extent of invasive growth:

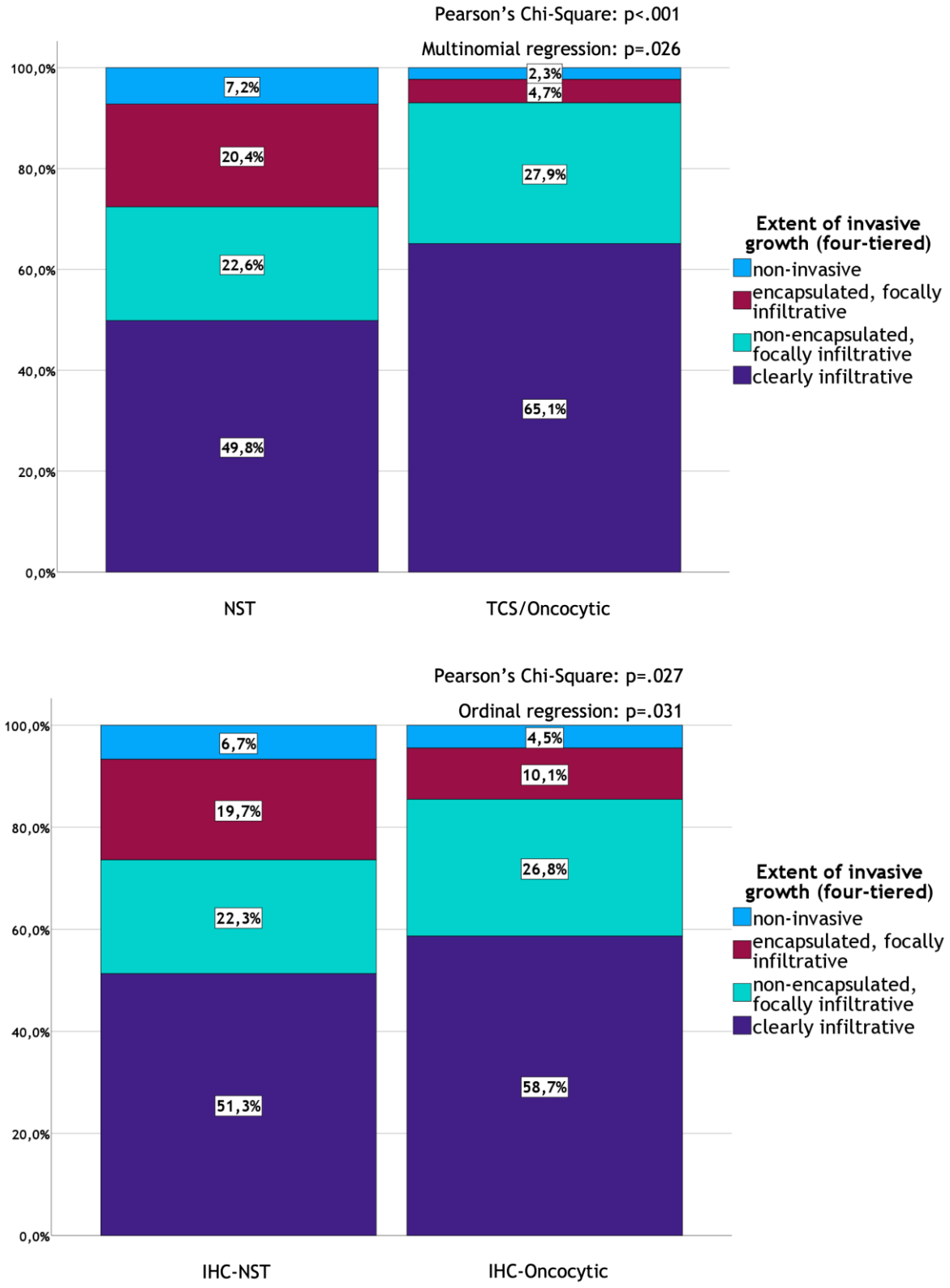

Figure 23: Extent of Invasive Growth in H&E Subtypes (top) vs. IHC Categories (bottom)

The ordinal model for infiltrative growth by H&E subtyping was invalid. Nevertheless, multinomial regression confirmed more likely infiltrative growth in

TCS/oncocytic subtypes (p=.026). By IHC, ordinal regression demonstrated higher odds for infiltrative growth in oncocytic tumors (p=.031).

Ultimately, H&E-based subtyping (NST vs. TCS/Oncocytic) allowed for better correlation with tumor size and extrathyroidal extension, whereas the IHC-based categorization (IHC-NST vs. IHC-Oncocytic) was more closely associated with tumor biology such as genotype (BRAF V600E), psammoma bodies and patient's age.

3.4 Lymph Node Metastasis

4 out of 448 tumors (excluding rare subtypes) were in the end classified pNx that had no lymph nodes removed (truly pNx) in combination with no sufficient follow-up data to confirm lack or presence of metastasis after initial treatment, leaving 444 cases, of which 155 were metastatic and considered for final analysis regarding lymph node metastasis.

TCS/oncocytic subtypes overall had smaller metastasis (Fig. 25), while no significant difference in lymph node status or number of positive lymph nodes was observed compared to NST H&E subtypes.

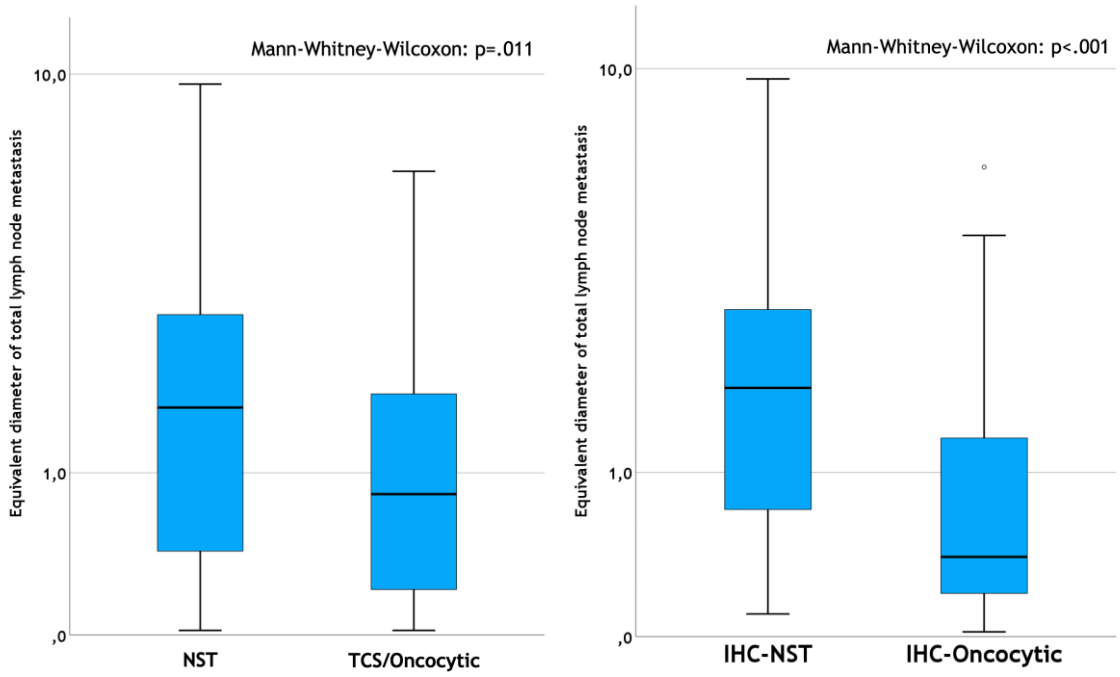

Figure 24: Metastasis Size in H&E Subtypes (left) vs. IHC Categories (right)

IHC categorization, however, was even more discriminatory and did come with further significant differences. Oncocytic IHC was associated with less frequent

metastasis (Fig. 26) and less positive lymph nodes (Fig. 27), when metastatic, compared to NST.

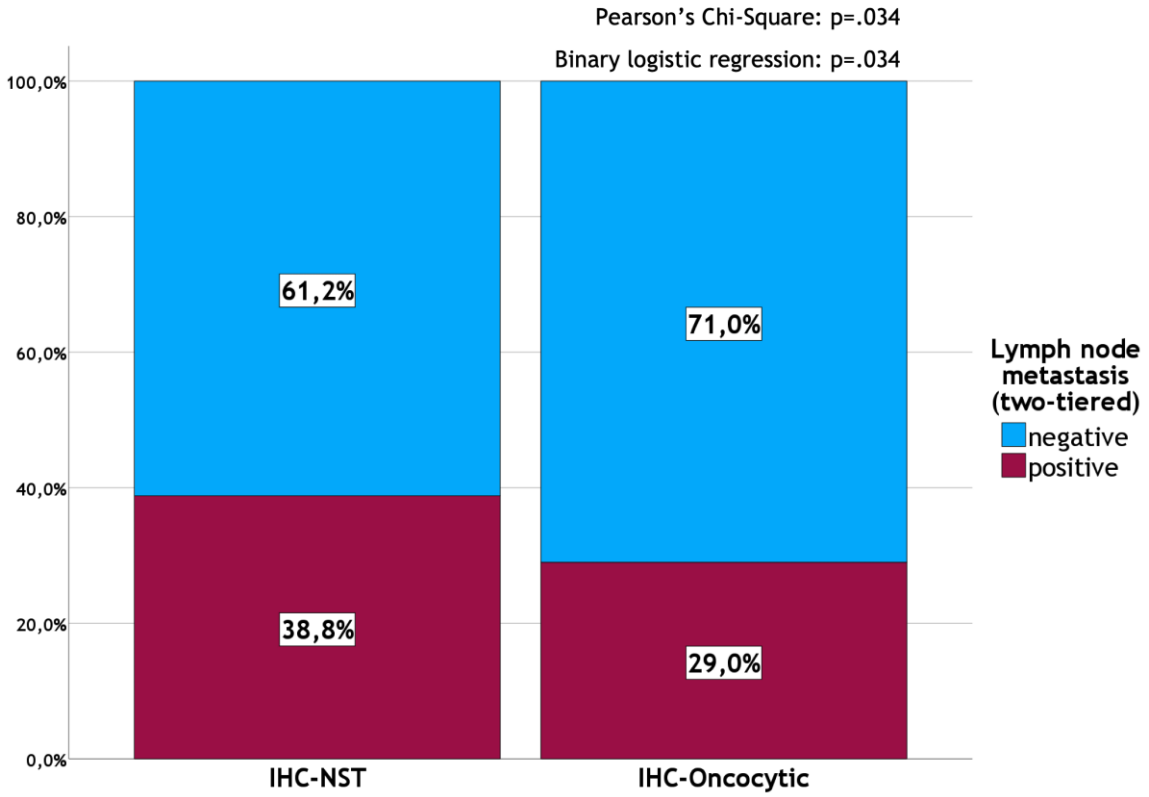

Figure 25: Lymph Node Status in IHC Categories

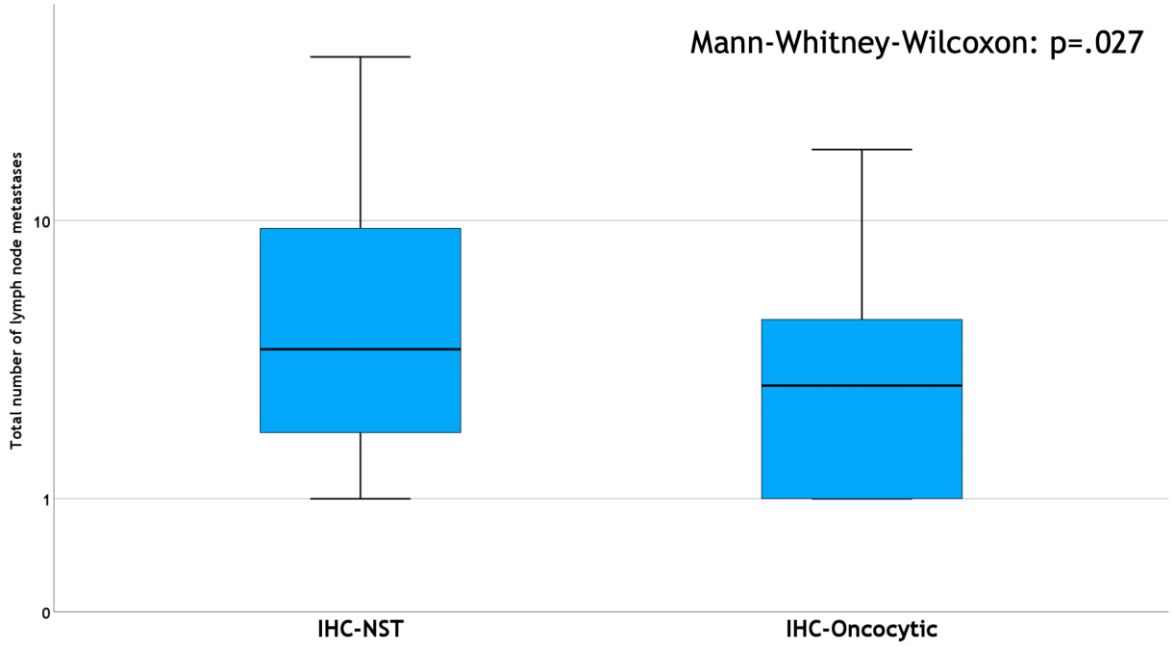

Figure 26: Number of Positive Lymph Nodes in IHC Categories

All 444 tumors were allocated into four ordinal categories regarding metastasis distinguishing between no metastasis at all, micrometastasis, clinically evident metastasis and full-scaled metastasis.

In oncocytic cases, overall metastasis was less common, shown in Table 10.

Table 10: Lymph Node Metastasis Categorization in IHC-NST vs. IHC-O

Immunoreactivity	No metastasis	<2 mm	2-10mm	>10 mm
IHC-NST	164 (61.2%)	7 (2.6%)	27 (10.1%)	70 (26.1%)
IHC-Oncocytic	125 (71.0%)	17 (9.7%)	18 (10.2%)	16 (9.1%)
Chi-Square	p<.001			

The proportional odds model was rejected for our four-tiered categorization of metastasis. This is because multinomial regression showed no statistically significant difference between oncocytic (IHC-O) and non-oncocytic tumors (IHC-NST) solely in the odds for developing clinically relevant metastasis (2-10 millimeter) as compared to no metastasis (Table 11).

Table 11: Multinomial Regression Model for Metastasis Categorization

Metastasis	IHC-NST (N=268)	IHC-O (N=176)	Regression
None	164 (56.7%)	125 (43.3%)	[reference]
<2 mm	**7 (29.2%)**	**17 (70.8%)**	**p=.013**
2-10 mm	27 (60.0%)	18 (40.0%)	p=.682
>10 mm	**70 (81.4%)**	**16 (18.6%)**	**p<.001**

Reassuringly however, the odds for developing small irrelevant metastasis referenced to no metastasis at all (multinomial regression) were higher in IHC-O than in IHC-NST and the odds for developing full-scaled metastasis (>10 mm) were significantly higher in IHC-NST than in IHC-O.

4 Discussion

Just recently, it has been established that TCS is in fact oncocytic.

At the same time, oncocytic change has been documented to come with greater disease extent, more extensive metastasis and higher recurrence rates. Independent ties have been demonstrated to more aggressive biology on the univariate level.

Conflicting evidence does not label oncocytic change as an independent predictor for more lymph node metastasis when considering common adverse morphological features such as extent of psammoma bodies or infiltrative growth.

Prior to this study, current knowledge suggests that when adjusting for aggressive morphology features, oncocytic tumors will behave just the same as their conventional counterparts on the multivariate level.

4.1 PTC Subtyping

In accordance with pertinent literature, our data makes abundantly clear that oncocytic change is an IHC diagnosis. Preexisting evidence on oncocytic tumors as defined by H&E may therefore be inaccurate.

Nonetheless, diagnosis of oncocytic PTC on H&E is to date widely acknowledged.

Furthermore, we established that certain subtypes behave similarly regarding – among other parameters – lymph node metastasis such as “conventional” (classic and FV-PTC) or “rare” morphologies (solid trabecular, diffuse sclerosing). In addition, TCS, oncocytic and Warthin-like tumors are all related as per their genetic profile and oncocytic immunoreactivity. Regarding clinical reasoning, subtyping could in fact follow a more simplified approach.

Generally, all previous evidence, be it in favor of TCS prognosis or showcasing worse outcome, relies on smaller cohorts than ours and always comes with some way of case selection.

The power of this study lies in its methodological approach in defining TCS and oncocytic change based on immunohistochemistry as recently elaborated, a relatively large cohort of IHC-oncocytic PTC and conduction as a population-based cohort study without any selection bias whatsoever.

H&E subtyping proved to be less sensitive as being oriented towards predominant appearance out of a multitude of largely redundant subentities. Dichotomized IHC classification followed a simplified, yet overwhelmingly more efficient approach.

Regardless of outcome, we observed that IHC categorization is more discriminatory and biologically more reasonable than H&E subtyping. This is because IHC more accurately represents the underlying oncocytic biology than morphologic TCS definition. While H&E mostly displayed non-significance, evident trends were established by IHC.

Clinical significance of using IHC in PTC is therefore undisputed.

4.2 Indolence in TCS and Oncocytic Change

Contrary to current most world-renowned beliefs, our data proves that TCS and oncocytic change are associated with **less** lymph node metastasis compared to non-tall-cell and non-oncocytic entities.

More specifically, our research suggests that previous evidence has been confounded and has suffered of selection bias and lack of uniform diagnostic definitions.

Firstly, our database extensively confirms common negative predictors on lymph node metastasis such as male sex, younger age, larger tumors, more extensive psammoma bodies, infiltrative growth and extrathyroidal extension. As oncocytic tumors are usually larger, more infiltrative and show more extrathyroidal extension, supposedly poor outcome in previous studies can be explained that way. But while these predictors were associated with a binary metastasis outcome, they did not come with overall larger metastasis or greater number of positive lymph nodes. This alone demonstrates that previously assumed poor outcome in TCS is flawed.

Anyway, it is without doubt that oncocytic tumors (and therefore TCS) vary biologically. That said, previously attested persistence and recurrence can also be explained by oncocytes' aberrant metabolism and overall functional/phenotypic dedifferentiation: primarily independence from TSH and iodine including RAI.

Lastly, current evidence is mostly based on H&E classification of oncocytic PTC. As we aimed to clarify, the spectrum of oncocytic change is vastly more complex than what it is widely known for and what can be observed on H&E.

Our analyses confirm previous interpretation that oncocytic transformation results in a continuous spectrum of morphologic changes. This explains why IHC categorization performed strikingly better in clinicopathological correlation as it considers intermediate morphology better than H&E evaluation which relies on more apparent morphology.

Oncocytic PTC as diagnosed on H&E is believed to be at the far end of the spectrum, consisting of what relevant literature calls “full-blown” oncocytes.

TCS, on the contrary, is an analogy to polarized oncocytes and translates to the mean group intermediating between beginning and advanced oncocytic transformation.

4.3 Favorable Outcome Regarding Lymph Node Status in TCS and Oncocytic Change

Our results even demonstrate that TCS (H&E) or oncocytic tumors (IHC) come with *even better outcome* regarding lymph node metastasis than conventional PTC. Metastasis was less common in oncocytic tumors. In metastatic cases, differences were even more pronounced seeing as oncocytic tumors had much smaller overall metastasis than non-oncocytic tumors.

Similarly, BRAF V600E, which has been shown to be discriminatory for TCS, when analyzes independently, displayed rather moderating effects such as smaller tumors, less likely metastasis and less metastasis extent.

Our novel approach on analyzing metastasis was centered around total volume and a four-tiered categorization which naturally followed a U-shaped distribution as is common in biology. It is difficult to make these situations fit into mathematical models. Anyway, we saw important results in regression modeling.

Prior to this study, it has been well established that oncocytic entities, most recognizably due to their metabolic shift, grow more slowly.

We believe, that when oncocytic tumors have already metastasized on initial contact, they did so before increasingly oncocytic shift prevents them from further proliferation.

Higher recurrence rates of TCS-PTC in current literature can therefore be explained by smaller, subclinical metastases, which are not dissected. Further research is required to clarify the necessity of lymph node dissection depending on the presence of oncocytic change in the primary tumor.

4.4 Limitations of the Study

4.4.1 Insufficient Clinical Data

In general, clinical documentation regarding presenting complaint, clinical findings and clinical reasoning was at times limited; even more so in medical specialties where certain clinical aspects were less relevant to patient care compared to other medical providers. For example, patient history and chief complaint were less thoroughly documented by surgical providers than by endocrinologists. Respectively, more care was given to imaging findings in surgical notes while medical follow-up focused on physical exam and lab work.

As a rule, the longer ago a patient was treated, the harder it was to find complete data on clinical diagnosis and treatment. This is due to a change in dogma regarding medical documentation. Although our patients were recruited when first diagnosed at our institute between 1995 and 2014, a lot of pre-operative medical documentation happened before 1995.

Furthermore, as PTC usually comes with very favorable prognoses, some patients are lost to follow-up in non-specialized and primary care as long as they are disease free.

4.4.2 Multiple Tumors in Patients

Ultimately, we diligently tried to solve the issue with patients having more than one tumor focus. In that regard, we used state of the art criteria in assigning them to didactically relevant subgroups; polyclonal multifocal versus monoclonal intrathyroid spread. In the end, though state of the art evidence such as morphology, biological features, immunohistochemistry, tumor genetics and

pathogenetic considerations was substantial to settling these cases, the decision was made following expert opinion. Further studies may clarify how to proceed with these patients or if this distinction even is relevant. Certainly, these aspects are solely academic and do not come with any relevance to patient management or further treatment.

4.4.3 Tumor Heterogeneity

In TMA, samples are manually pinched from paraffin blocks by macroscopical selection on the corresponding blocks' slides. This method allows for room for error.

As extensively demonstrated, oncotic change is a continuum and not all cell populations display the same phenotype. In addition, PTC is known to present heterogeneously in terms of H&E morphological patterns.

While tumor areas were most carefully selected and sampled, it cannot be guaranteed that the exact targeted cell populations were selected for further processing. Moreover, TMA sampling always included only these small target areas, which might not be truly representative of the remaining tissue.

However, statistical results are not expected to be any less reliable due to our large study population (law of large numbers).

4.5 Implications

This study refutes widely distributed rigid beliefs regarding clinical relevance of TCS and oncocytic change. We concur with advances that IHC evaluation of oncocytic change should be introduced to routine diagnostics as it refines current procedure.

We tried to make a point that not all metastasis is equal. Lymph node metastasis does not automatically mean aggressive disease, overall survival is still reported excellent. Depending on their biology, metastasis might as well be easily manageable and never recurrent. While more than one third lymph node metastasis on initial surgery suggests poor outcome, our research shows that patients are more often and longer disease-free than in other neoplasms rendering overall survival impeccable. However, disease-free and overall survival have not been calculated in this study and are, therefore, subject for further analysis.

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