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HISTOPATHOLOGICAL, CYTOLOGICAL AND IMMUNOHISTOCHEMICAL CORRELATIONS IN THYROID NEOPLASMS

ABSTRACT OF DOCTORAL THESIS

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List of Abbreviations Used in the Text

ATA - American Thyroid Association

CEA - carcinoembryonic antigen

FTC - follicular thyroid carcinoma

CK7 - cytokeratin 7

PTC - papillary thyroid carcinoma

CMT - medullary thyroid carcinoma

EMA - epithelial membrane antigen

MEN - multiple endocrine neoplasia

PET - CT - positron emission tomography-computer tomography

protooncogene

RET - protoncogene receptor tyrosine kinase

PCNA - nuclear cell proliferation antigen

PNST - peripheral nerve sheath tumour

TSH - thyroid stimulating hormone

TTF1- thyroid transcription factor 1

Current State of Knowledge in Thyroid Tumours

The thyroid gland is a small, butterfly-shaped organ in the front of the throat that is of overwhelming importance in the functioning of every cell, tissue or organ. Thyroid carcinoma is a rare malignancy, accounting for about 1% of all human tumours found in the general population in developed countries, yet it is the most common malignancy of the endocrine system. Of the endocrine neoplasms, thyroid gland tumours occur most frequently, occurring in 92% of cases. Studies have shown an increase in incidence of 8.7-9 cases/100000 inhabitants per year, showing a marked increase of 310% over the last five decades. The increase in the incidence of these neoplasms is most likely explained by the application of modern diagnostic methods that can accurately detect even very small, infracentimetric tumours. Mortality from thyroid cancer has also fallen from 44% to 0.3% thanks to current methods of diagnosis and treatment. The vast majority of patients with thyroid carcinomas of various types have a very good prognosis after treatment, with survival rates of over 98%.

Cases reported in the medical literature report a 50% prevalence of nodules detected during autopsies in subjects who were not known to have thyroid pathology and a prevalence ranging from 1.0% to 35.6% of thyroid ocular carcinomas detected in different series of systematic autopsies.

Thyroid pathology, although it accounts for only 1% of all malignancies, is an increasingly common condition worldwide. This is why, year after year, this topic is discussed and analysed in presentations at various sessions of meetings of the medical scientific world of endocrinologists. The incidence of thyroid cancer has tripled in the last 30 years, with a marked increase in small tumours, while mortality has remained stable according to data provided by the American Association of Endocrinopathology and the American College of Endocrinopathologists.

The study of the characteristics and advanced understanding of the mechanisms underlying the biological features of thyroid carcinomas have led to the implementation of guidelines for the application of therapeutic regimens and protocols. However, there are still a number of practical questions that remain unresolved or controversial, for example the extension of surgery to potentially aggressive cases. The increased incidence of thyroid cancer is thought to be the likely result of two coexisting processes: on the one hand increased detection due to thyroid-specific carcinogens and on the other hand the

identification of large reservoirs of subclinical papillary lesions that do not influence the patient's health.

The thyroid gland can be thoroughly investigated by imaging methods such as ultrasound, scintigraphy, CT, PET-CT, MRI, but these cannot determine the malignancy of the tumours, but they play a very important role in determining the regional and distant assessment of tumour extension.

Pathological management of thyroid investigations begins with cytological examination. This is the preoperative stage widely used as a preoperative screening method capable of establishing a definite diagnosis of benignity or malignancy.

Although fine-needle aspiration biopsy followed by cytological examination are considered to be highly sensitive and sensitive procedures, the diagnosis of certainty is subsequently established only by histopathological examination.

The limitations of this technique are the difficulty in differentiating malignant cells in lesions containing a large number of apparently benign cells (e.g. in the case of cytodiagnosis of follicular neoplasm, which can easily be confused with follicular adenoma). Capsular or vascular invasion is also a controversial issue in establishing the definitive diagnosis of malignancy or benignity, thus these smears require additional complex cytochemical investigations and sometimes adjuvant computerized morphometry methods.

In general, thyroid tumours have a favourable outcome, with a five-year survival of more than 98%, but there are also aggressive variants with varied clinical behaviour, unfavourable outcome and prognosis.

Histological diagnosis of these aggressive forms can be a real challenge for the pathologist, as many share common features. All these cases require complex histopathological diagnosis, tailored to each individual case to achieve maximum efficiency.

In these cases, computerized nuclear morphometry can be an effective, low-cost assessment tool in evaluating the establishment of histological features. Using nuclear morphometry, we can assess a number of important parameters in terms of size and shape of nuclei, with an important role in facilitating the diagnosis of various neoplasms. A search of the literature dedicated to the application of computational morphometry for the histological study of thyroid lesions reveals the existence of a relatively small number of papers compared to publications focusing on thyroid cytological microscopic morphology. This can be explained by the complexity of the computational investigation approach, which requires specialized software technology based on artificial intelligence.

Working Hypothesis

Based on research studies in the literature, s - outlined the working hypothesis which states that the most common neoplasm of the endocrine system, is found in the thyroid gland, with a significant increase in recent decades, largely explained by the application of modern diagnostic methods. Although thyroid tumours generally have a favourable outcome, there are also aggressive variants with a poor prognosis.

The PhD thesis aimed to study thyroid tumors with emphasis on histopathological correlations, identification and definition of clinico-epidemiological, immunohistochemical and cytological parameters, as well as the analysis of data obtained by measuring nuclear parameters by means of microscopic morphometry investigation, in order to expand the knowledge on thyroid carcinomas.

The thesis comprises three studies on incidentally detected thyroid tumours and selected cases with potentially aggressive thyroid tumours.

In the first study, thyroid glands were evaluated in a series of 526 autopsies performed between January 2017 and February 2020 in the Forensic Medicine Department of the County Clinical Emergency Hospital of Braila, with the aim of identifying the prevalence of thyroid carcinomas among the various nodular thyroid lesions incidentally detected during necropsies. The distribution of thyroid tumours in the studied group, the correlations with respect to the patients' environment (urban/rural), the distribution according to age, sex, location of tumours in the thyroid lobes, the influence of environmental factors, possible associations with autoimmune thyroiditis, metabolic diseases, obesity and cardiovascular diseases were studied.

In study 2, the research group was enlarged as follows: to the 51 tumors detected incidentally during autopsies, 17 additional cases were added, selected from the histopathological registers between 2016 and 2021, with thyroid tumors with histological forms that had aggressive potential, from patients operated on in the surgical wards and from patients in the oncology ward of the Emergency Clinical County Hospital, Braila.

A total of 68 cases with thyroid tumours were included in Study 2. Of these 60 were papillary carcinomas, 4 follicular carcinomas, two poorly differentiated thyroid carcinomas, one case of medullary thyroid carcinoma and one case of squamous thyroid carcinoma.

Papillary carcinomas were reanalysed to determine histological subtype, of which the vast majority were microcarcinomas, but there were also five carcinomas considered to have high aggressive potential: 2 cases of papillary carcinoma, high-cell variant, one case of

papillary carcinoma with diffuse sclerosis, one case of papillary carcinoma, hobnail variant, and one case of papillary carcinoma, diffuse follicular variant.

Cases from patients in surgical wards and those in oncology wards also received fine-needle aspiration cytopuncture prior to specific therapeutic and surgical procedures. In these patients it was possible to make correlations between cytological smears and those obtained after processing histopathological and immunohistochemical specimens. The distribution of histopathological forms of thyroid tumours in the studied group, correlations in terms of age, sex, types of surgery applied, their inclusion in national oncological programmes, and in advanced cases the monitoring of the application of advanced palliative care treatments in specialised centres were followed.

For the third study, 36 cases with different benign (10 follicular adenomas) and malignant thyroid lesions (10 follicular carcinomas, 10 follicular variant papillary carcinomas, 4 cases of papillary carcinoma, variant with diffuse sclerosis and 2 undifferentiated cases), selected from the Departments of Pathological Anatomy and Forensic Medicine of the Emergency Clinical County Hospital of Braila, were analyzed by nuclear morphometry over a period of 5 years (2017-2018).

The aim of this study was to develop and apply a complementary nuclear morphometry method that allows the differentiation of benign from malignant tumours, using a system that can be easily reproduced in any laboratory, as a tool to help in cases with difficult to diagnose thyroid lesions.

The use of morphometric analysis in thyroid cytology is not a routine practice, it is only used to a limited extent in scientific research studies**.**

Study Objectives

The general objectives of the doctoral thesis were to find the incidence of thyroid tumours in a series of autopsies in the first study, and in the following two studies to identify clinico-biological, histopathological, cytological and immunohistochemical correlations of some types of potentially aggressive thyroid tumours, as well as to develop and apply a nuclear morphometry method in order to differentiate benign thyroid tumours from malignant thyroid tumours.

To this end, in order to achieve the proposed objectives, the doctoral study began with a thorough review of the literature by searching scientific publications from the last five

years and conducting a meta-analysis to identify all articles with potential relevance to the proposed purpose and that met the following criteria:

- included thyroid tumours;
- The reviewed scholarly articles were mostlywritten in English (a few in Spanish) and were accessed through the virtual library of U. M. F. "Carol Davila" Bucharest.

The selection criteria for the study groups were then established:

- Thyroid tissue specimens came from collections during autopsies or thyroidectomy operations;
- the examined specimens had described clinico-pathological characteristics for each case;
- The subjects included in the study were from the general population, urban or rural environment, with no age restriction, being of both sexes;

The following complex histopathological procedures and analyses were performed in the doctoral study:

- Selected thyroidectomy specimens were histologically processed, microscopically analyzed and immunohistochemically tested using a panel of 7 antibodies: CK7, TTF1, thyroglobulin, EMA, VIM, PCNA, P53.
- cases with potentially aggressive thyroid tumours also received fine needle aspiration cytology and subsequent evaluation of the cytological smears obtained
- informed consent has been obtained from patients or legal guardians ;
- the data in the autopsy files were examined;
- digital photographs were taken prior to sampling, with the histological specimen in situ, digital photographs during macroscopic orientation, before and after sectioning of specimens harvested/removed from autopsy rooms;
- Microscopic photographs were taken with the Mshot camera integrated into the trinocular system of the microscope.
- cases with uncertain thyroid lesions, such as follicular adenomas, papillary carcinomas in the follicular variant, follicular carcinomas as well as poorly

differentiated carcinomas were evaluated by microscopic morphometry techniques

General Research Methodology

The case reports presented in this paper include thyroid tumours operated and diagnosed in the surgical wards of the Emergency County Hospital of Braila, as well as thyroidectomy specimens collected during serial autopsies performed in the forensic medicine department.

In the present study, a series of thyroid tumours collected during 526 autopsies between January 2017 and February 2020 and a number of 36 cases with thyroid tumours, selected from the registers of the Pathological Anatomy Service, over a five-year period (2017-2021), operated in the Surgical Departments or under the care of the Oncology Department of the Emergency County Hospital of Braila, were evaluated.

The aim was to study the distribution of histopathological forms of thyroid tumours in the study group, the correlation of morphometry in the differential diagnosis of benign and malignant thyroid lesions and especially in the forms with high aggressive potential, to evaluate the clinical impact and to identify prognostic and aggressive factors of these tumours.

The study was approved by the Ethics Council of the Emergency County Hospital of Braila and was conducted in accordance with the principles of the Universal Declaration of Helsinki, as well as the legislation in force in our country (Law 104/2003 on the handling of human cadavers).

The accompanying annexes of biological products (in which patients/legal guardians express their consent to the use of biological material, including for scientific purposes) were checked by the institutional ethics committee, respecting the ethical standards of the "Declaration of Helsinki" of 1975, revised in 2000, and conducted in accordance with national legislation.

Study 1 : Incidence and Histopathological and Immunohistological Correlations in Thyroid Carcinomas

It was based on the general assumption that detection of thyroid carcinoma has a higher overall incidence than other types of tumours.

In the first study, serial thyroid gland samples were taken during 526 autopsies over a three-year period from January 2017 to February 2020, from which the study group was formed.

Total thyroidectomy specimens were examined macroscopically, thoroughly in order to detect any pathological changes.

A complex database was developed that included the following variables: age, gender, environmental factors, smoking and clinical factors considered to be related to risk for thyroid cancer.

It was also noted the presence of comorbidities present, represented by chronic diseases, especially cardiovascular disease, metabolic diseases, diabetes mellitus, as well as the presence of overweight.

The thyroid gland was harvested during autopsy by carefully dissecting and separating it plane by plane from the soft tissues surrounding the thyroid lobe.

Thyroid tumours were found in 51 cases out of 526 autopsies, these thyroid tumours were mostly papillary microcarcinomas (47 cases), which followed an indolent course with no clinical outcome, three cases were undifferentiated carcinomas and one case was classified as squamous thyroid carcinoma.

The risk of malignancy correlated with age: the highest incidence of malignant nodules was found in patients in the 60-70 age decade; malignant nodules had an approximately equal distribution in the 2 sexes.

The distribution of malignant nodule localization was higher in the right lobe, being 54.1% in the right thyroid lobe and 43.1% in the left thyroid lobe, with 9% having multifocal localization, spread in both thyroid lobes.

The incidence of thyroid pathology associated with cardiovascular disease had the following distribution:

- 67.3% had chronic cardiovascular disease - myocardiofibrosis type;
- 5.3% had chronic cardiovascular disease associated with acute myocardial ischemia;
- 7.8% had acute myocardial ischemia.

Neoplasia was found in 51 cases, being three times more common in men than in women.

The vast majority of tumors were papillary microcarcinomas (47 cases), undifferentiated carcinomas 3 cases, one case squamous. The youngest patient was 40 years old and the oldest 94 years old. The association with other thyroid pathology seen in neoplastic cases was as follows:

The urban/rural distribution found in patients with neoplasia was approximately equal. Regarding the association relationship between obesity and thyroid pathology showed: 33.3% of cases with presence of excess adipose tissue, obesity being of all grades, 52% of cases had normal adipose tissue represented, and 19.5% of cases had poorly represented adipose tissue.

Of the 51 thyroid tumours 47 were micropapillary carcinomas, three undifferentiated and one squamous.

Papillary carcinomas typically showed papillary projections consisting of fibrovascular rods, lined with cuboidal cells, eosinophilic cytoplasm, nuclei with irregular margins and pale, hollow irregular appearance, with classic nuclear features: vesicular, crowded, overlapping, optically clear, ,,Orphan Annie eyes" type nuclei with irregular outlines.

A typical but not considered pathognomonic feature that was encountered was the presence of psammomatous bodies, a feature that was evident in five cases of papillary thyroid microcarcinomas.

Carcinomas smaller than 1 cm were classified as papillary microcarcinomas.

Five tumour cases were selected for immunohistochemical staining: one squamous cell carcinoma, three undifferentiated carcinomas and one papillary carcinoma.

The immunohistochemical tests expressed the following results, and the data obtained have been summarized in the table below:

Table 1. Expression of immunohistochemical markers

Crt. no.	Markeri IHC	Undifferentiated carcinoma (case 1)	Squamous cell carcinoma	Undifferentiated carcinoma (case 2)	Papillary carcinoma	Undifferentiated carcinoma (case 3)
1.	Cytokeratin 7	diffuse positive	zonal positive	zonal positive	diffuse positive	zonal positive
2.	EMA	diffuse positive	diffuse positive	diffuse positive	diffuse positive	diffuse positive
3.	TTF 1	negative	negative	negative	negative	negative
4.	Ki67	5-6% positive nuclear	25% nuclear positive	inconclusive	3-5% positive nuclear	10% positive nuclear
5.	p53	negative	negative	negative	negative	negative
6.	Vimentin	negative	negative	inconclusive	negative	negative
7.	Thyroglobulin	positive	positive	positive	positive	positive

Cytokeratin 7 was diffusely positive at the tumor cell membrane in papillary carcinomas and had focal expressed expression in squamous cell tumors and undifferentiated tumors. Cytokeratin CK7 was zonally positive in tumor cells from squamous cell carcinoma islets having positive, conserved control in follicular epithelial cells. Cytokeratin CK7 was positive in the nuclei of tumour cells and in subcapsular callospheres in two cases of undifferentiated thyroid carcinomas, and in the third case of undifferentiated carcinoma, which showed areas of extensive necrosis with expression artefacts, the CK7 marker was expressed zonally in tumour cells.

Cytokeratin CK7 expressed diffuse positivity in papillary cystic tumor cells and in dystrophic sclera in papillary carcinoma.

EMA - epithelial membrane antigen was diffusely positive in all thyroid carcinomas.

The TTF1 marker was negative in most cases with papillary tumors, expressing a positive conserved control in follicular cell epithelia, and had negative expression at the tumor level in the first case of undifferentiated carcinoma.

The TTF1 marker was almost diffusely positive in the other two cases of undifferentiated tumours at the nuclear level and showed a positive reaction zonally, the intensity being weaker in areas of necrosis with various brownish tones.

TTF1 marker expressed a diffuse positive response in a case of papillary carcinoma at the tumor cell level.

Thyroglobulin was positive in all cases, with 7-8% positive expression in the nuclei of tumour cells in undifferentiated carcinomas and 3-5% in the nuclei of tumour cells in papillary carcinomas and 20-25% in the nuclei of cells in squamous carcinomas.

The Ki67 proliferation index was expressed in tumor cell nuclei in 25% of well-differentiated basal and parabasal squamous cells; no expression was observed in areas of keratin and parakeratin formation.

Tumor antigen of p53 protein was negative in all neoplastic cases, including undifferentiated and squamous differentiated cases, nuclear antibodies were affected by autolysis and necrosis, and antigen-antibody reaction had lost expression.

The Ki67 proliferation index expressed positive, brownish nuclei on a brown background in 25% of well-differentiated basal and parabasal squamous tumor cells in the upper layers, not visualized nduing because keratin and parakeratin do not express fact or proliferation. The Ki67P proliferation index expressed positivity subcapsularly, in tumour cells in 5-6% in papillary carcinomas, respectively 75-80% in tumour cells from two of the undifferentiated carcinomas and was inconclusive in the third case of undifferentiated thyroid carcinoma, where it expressed positivity of maximum 3% in thyroid tissue, nuclear antibodies being affected by autolysis and necrosis so that in the antigen-antibody reaction the nucleus lost its expression. Ki67 tumour proliferation index expressed a positivity of 3-5% in the nuclei of tumour cells in squamous carcinoma.

The Vimentin marker was inconclusive in undifferentiated carcinomas and was negative in papillary tumour cells, expressing only in stroma and blood vessels, which were used as a control test.

Study 2: Cytomorphological Study in Thyroid Tumours

In study 2 the study group comprising 51 tumours discovered incidentally during autopsies was enlarged by introducing 17 additional cases selected from histopathological registers between 2016-2021 with thyroid tumours from patients operated on in the surgical wards and from patients on the oncology ward records who presented with tumours of potentially aggressive shapes.

A total of 68 cases with thyroid tumours were included in the study. Of these 60 were papillary carcinomas, 4 follicular carcinomas, two poorly differentiated, one medullary and one squamous.

Papillary carcinomas were carefully re-examined in order to determine the histological subtype. Microcarcinomas were identified in the vast majority of cases (55 cases), but also 5 cases with potentially aggressive papillary carcinoma variants including: high cell variant (2 cases) and one case each for diffuse sclerosing, hobnail variant, diffuse follicular variant.

The cytomorphological features found in the studied cases that showed aggressive potential have been summarized in the table below:

Table 2 Morpho-pathological features in potentially aggressive tumours

Cytomorphological features	Architecture	Background	Nuclei	Nuclear chromatin	Mitoze	Cytoplasm	Psammoma bodies
High-cell carcinoma	cells 3x taller than wide	absent	elongated shape with specific features of papillary carcinoma	microgranular	regular	eosinophilic with oncotic features	rare
Hobnail variant	micropapillary	absent	apically located, oval/ cuboidal shape	clear	rare	eosinophilic	rare
Variant with diffuse sclerosis	three-dimensionally grouped cohesive cells	lymphocyte present	specific features of papillary carcinoma	clear	rare	dense with large vacuoles	plentiful
Diffuse follicular variant	follicular structures	absent	pleomorphs with papillary carcinoma features	fine-grained	rare	a little	rare
Undifferentiated carcinoma	solid, trabecular, insular pattern	tumour diathesis	pleomorphs/ round, oval shape	microgranular ,,salt and pepper"	present	mild, oncotic type	absent
Follicular carcinoma	well-defined follicles, solid/trabecular pattern	follicular cells, lymphocytes	round/oval cores	granular	present	eosinophilic, granular	absent
Medullary carcinoma	carcinoid, tubular, follicular, glandular pattern	amyloid deposits	average size in round/polygonal cells	microgranular	usual	amphophyll	absent
Squamous cell carcinoma	malignant cells, isolated or pseudo-syncytial aggregates	tumour diathesis	pleomorphic, hyperchromatic	macrogranular	usual	dense, keratinized mature	absent

Patients diagnosed preoperatively by fine-needle aspiration in cases with aggressive variants underwent operative surgical treatment with extensive neck dissection, received imaging scans to rule out the possible presence of metastases, and were more rapidly included in national oncology programs. Moreover, these patients required close

surveillance and monitoring, and in advanced cases of disease they received complex palliative care. According to the literature, aggressive variants are responsible in most cases for the occurrence of recurrences and increased morbidity. Recognition of aggressive variants requires more aggressive surgical treatment from the outset with optimization and minimization of post-treatment sequelae and avoidance of reoperations.

On some cytological smears it was not possible to specifically diagnose an aggressive histological subtype of thyroid carcinoma. However, even in these cases, the morphological features visualized were detailed, signaling the possibility of an aggressive variant of thyroid carcinoma;

Study 3 - Nuclear Morphometry Study in Thyroid Tumours

The third study aimed to study the correlation of nuclear morphometry data in the differential diagnosis of benign and malignant thyroid lesions, especially in the potentially aggressive forms.

The study material was composed of 36 cases with different selected thyroid lesions diagnosed in the Pathology Department of the County Hospital of Braila over a period of five years (2017-2021). Of these, the diagnostic classification allowed the grouping into benign (follicular adenomas - 10 cases) and malignant lesions (follicular carcinomas - 10 cases, papillary carcinomas - follicular variant - 10 cases, papillary carcinomas - variant with diffuse sclerosis - 4 cases, undifferentiated carcinomas - 2 cases).

The control group consisted of normal thyrocytes in thyroid tissue adjacent to thyroid lesion tissue.

Special cases with oncotic, Hurthle-like cells showing nuclear abnormalities were excluded from the study in order to avoid major errors in the measurements and later in the statistical analyses.

The data collection process from the measurements was performed using a manual collection method with interactive selection of representative cells and morphometric features of interest.

The morphometric analysis was performed without knowing the final diagnosis, thus eliminating any bias. Digitization of the slides was performed at 400x magnification for higher image resolution. It allowed appropriate micrometer-scale morphometry measurements to be made. Capturing a larger microscopic field of cells would have required the use of a lower power magnification (200x), but this would have limited the resolution of

the image. Calibration of the measurements was performed using a micrometer slide with a calibrated scale of 0.1mm Mshot.

An average of 5-10 microscopic fields were analysed on each slide of the selected cases at 400x magnification. An average of 50 nuclei per microscopic field were measured. Only nuclei with well visualized outlines were included. Stromal cell nuclei, areas with fragmented nuclei or overlapping nuclei were excluded. Measurements were performed on 10 nuclear morphometric parameters. To obtain these nuclear parameters, the drawing tools of the Digimizer software were used, followed by the processing of the obtained data.

Statistical analyses

The data collected were statistically analysed using Minitab v19 software.

Two-way mixed ANOVA followed by Bonferroni post hoc test was used to assess the differences between normal cell and tumor cell groups in selected cases with follicular adenomas, follicular variant papillary carcinomas, follicular carcinomas, and undifferentiated carcinoids.

Nuclei of papillary thyroid carcinoid cells showed significantly higher values than nuclei in the normal thyroidocyte group, as well as nuclei in the thyroid adenoma group.

Table 3 Comparison of nuclear parameters

	Normal cell group - Follicular adenoma cell group		**Normal cell group - Follicular carcinoma cell group**		**Normal cell group - Follicular variant papillary carcinoma cell group**		**Normal cell group - undifferentiated carcinoma cell group**	
Variable	**Mean ± SD Normal N=2754**	**Mean ± SD Follicular adenoma N=2754**	**Mean ± SD Normal N=2488**	**Mean ± SD Follicular carcinoma N=2488**	**Mean ± SD Normal N=2384**	**Mean ± SD Papillary carcinoma follicular variant N=2384**	**Mean ± SD Normal N=472**	**Mean ± SD Undifferentiated carcinoma N=472**
Area	12.908 [e] ±**3**.279	24.841 [c] ±**12**.061	13.035[e] ±**3**.688	33.524 [b] ±**8**.143	13.250 [e] ±**3**.342	52.081 [a] ±**11**.147	12.963[e] ±**3**.153	20.018 [d] ±**9**.474
Perimeter	13.440 [e] ±**1**.693	18.097 [c] ± 3.981	13.560 [e] ±**1**.873	21.68 [b] ±**2**.616	13.562 [e] ±**1**.695	26.665 [a] ±**2**.92	13.425 [e] ±**1**.635	17.177 [d] ±**4**.338
Roundness	0.887 [b] ±**0**.062	0.911 [a] ± 0.054	0.877 [b] ±**0**.073	0.885 [b] ±**0**.066	0.894 [b] ±**0**.060	0.914 [a] ±**0**.085	0.893 [b] ±**0**.059	0.815 [c] ±**0**.131
Avg. Intensity	0.420 [d] ±**0**.048	0.560 [c] ±**0**.081	0.421 [d] ±**0**.050	0.633 [b] ±**0**.058	0.423 [d] ±**0**.048	0.691 [a] ±**0**.072	0.421 [d] ±**0**.048	0.387 [e] ±**0**.062
Intensity, Red - Avg	0.375 [d] ±**0**.050	0.650 [c] ±**0**.096	0.375 [d] ±**0**.053	0.746 [b] ±**0**.054	0.378 [d] ±**0**.051	0.816 [a] ±**0**.072	0.376 [d] ±**0**.050	0.373 [d] ±**0**.069
Intensity, Green - Avg.	0.366 [b] ±**0**.058	0.336[c] ±**0**.081	0.366 [b] ±**0**.060	0.394 [a] ±**0**.071	0.369 [b] ±**0**.058	0.872 [a] ±**0**.059	0.367 [b] ±**0**.059	0.349 [bc] ±**0**.064
Intensity Blue - Avg.	0.520 [d] ±**0**.044	0.694 [c] ±**0**.090	0.521 [d] ±**0**.044	0.757 [b] ±**0**.058	0.522 [d] ±**0**.043	0.872 [a] ±**0**.059	0.521 [d] ±**0**.043	0.438 [e] ±**0**.062
Hue	242.17 [d] ±**7**.98	293.150[b] ±**5**.070	242.54 [d] ±**7**.810	298.50 [a] ±**2**.74	242.71 [d] ±**8**.09	293.83 [b] ±**2**.45	242.39 [d] ±**8**.39	258.09 [c] ±**20**.12

The above table presents a synoptic presentation of the morphometric characteristics of the cases analysed.

Two-way mixed ANOVA test was applied to compare the mean values of morphometric parameters of normal cell group and follicular adenoma (FA) group, normal cell group and follicular carcinoma (FC) group, normal cell group and follicular papillary carcinoma group (PFAC) and normal cell group and undifferentiated carcinoma group.

Table 3 shows the results of Bonferroni post hoc analysis of nuclear morphometry parameters. Statistically significant differences between groups are indicated by different letters ($p < 0.05$). Subscript letters have been used for ease of comparison, and to better visualize significant differences between the groups analyzed.

The nuclear morphometric data obtained were noted with the letters ,,a", ,,b", ,,c", ,,d" in descending order of the values obtained. The highest values obtained noted with the letter-subscript ,,a" are observed in papillary carcinomas, follicular variant, followed by follicular carcinoma, noted in the table with the letter-subscript ,,b", followed by cases with follicular adenomas, noted with the letter-subscript ,,c" and undifferentiated carcinomas noted with the letter-subscript ,,d".

In cases of undifferentiated carcinomas the morphological parameter ,,roundness" showed significantly increased waves with a standard deviation of 0.131. This parameter defines the marked irregularity of the nuclear membrane found in undifferentiated tumour cells. In the Digimizer software, the morphological parameter of ,,roundness" defines a value between 1 and 0, with values closest to 1 being the most regular, a perfectly round circle having a value equal to 1.

Also in the Digimizer program, the average nuclear brightness intensity was calculated, considering a completely white area with a brightness intensity equal to 1, while a completely black area has an intensity value equal to 0.

In Digimizer software, the measured parameter, "Hue" is a measure of the colour of an image. Hue is determined by the wavelength frequency of the light measured by the software for each core being measured. The "Hue" parameter is expressed as a number between 0 and 360 degrees, where 0 represents red, 120 represents green and 240 represents blue.

Hue values were higher in benign and malignant tumour cells compared to normal cells, with tumour nuclei being closer to the blue wavelength value in the visible spectrum.

Paired t-test results for nuclear morphological parameters compared the normal cell group with the benign cell group, and the normal cell group with the malignant cell group.

Statistically significant differences between the groups were identified based on the means obtained, with a $p<0.05$.

The follicular adenoma neoplasia group had significantly higher mean values of nuclear parameters than the group with thyrocytes considered normal.

The follicular carcinoma group had higher nuclear parameters than normal cells and the follicular adenoma group.

The follicular variant follicular carcinoma group showed the highest values of nuclear parameters defining nuclear size.

Papillary carcinomas with aggressive forms showed significantly higher values of nuclear size parameters than the group with normal thyrocytes as well as thyroid adenomas.

In undifferentiated carcinomas the morphological nuclear parameters characterizing size were also slightly increased compared to normal. In these cases of undifferentiated carcinomas, the Roundness parameter is significantly increased, showing the increased degree of irregularity of the nuclear membrane.

Among the most reliable parameters analysed were: nuclear area, nuclear perimeter and the degree of irregularity and roundness of the nuclear membrane.

The present study focused predominantly on cases with follicular architecture, as follicular tumours most commonly cause differential diagnostic problems in cases of thyroid pathology.

The present study showed significant changes especially on the area, perimeter and roundness parameters, while the red, green, blue and hue parameters did not yield conclusive data.

The highest values for morphometric parameters such as area and perimeter were found in papillary thyroid carcinoma, follicular variant.

In the case of nuclei from undifferentiated thyroid carcinomas, the highest values were found for the parameters of the degree of irregularity and roundness of the nuclear membrane.

Computer-aided image analysis can range from basic, simple software requiring human intervention to complex applications involving artificial intelligence. The current state of development of software resources allows the integration and processing of histopathological images with automated measurements. It acts as an expert system, allowing automated interrogation of a complex database, facilitating the work of morphopathology.

A review of the literature reveals that authors rarely provide concrete details and data on how the software applications with which the automated morphometric measurements were made were implemented.

In our study the cases in the malignant lesion group (follicular carcinomas, papillary carcinomas - follicular variant, papillary carcinomas - diffuse sclerosing variant, and undifferentiated carcinomas) had significantly increased values of nuclear morphometric parameters compared to the benign lesion group represented by follicular adenomas. The highest values were found in follicular variant papillary carcinoma. Undifferentiated carcinomas also had a higher value of the parameter measuring the degree of irregularity of the nuclear membrane.

Personal Contributions

In Study I, written in Chapter 6, we presented the incidence of thyroid carcinoma found following 526 autopsies, from which thyroid glands obtained for histopathological analysis were harvested. A total of 51 cases were identified that were positive for latent papillary thyroid carcinoma.

The prevalence of papillary thyroid carcinoma was 9.69% and was within the range reported in the literature studies of 1.0% to 35.6%. Comparing these prevalence values in autopsy series studies is difficult to integrate because different examination methods were used, such as: the ranges used for sectioning (from 4μm to 4mm), diagnostic criteria and specimen selection. In particular some examination methods may have a more accurate method in observing carcinoma prevalence and may lead to a marked increase in the detection of papillary thyroid carcinomas.

Thyroid nodules were found in a proportion of about 40% in the thyroid gland, but most of them were benign, a proportion of about 10% of the solitary nodules found were malignant. Nodules detected in polyendocrine glands were mostly of colloid appearance and low risk of malignancy. In the cases detected with thyroid tumours, the most common thyroid tumours were papillary carcinomas, of which the most common histological subtype was papillary microcarcinoma. It was observed that papillary microcarcinoma is an incidental finding, extremely frequent, with a sclero-fibrotic appearance, thus suggesting that in the vast majority of cases the development is indolent, latent and long-lasting.

The general adult population appears to be uniformly exposed to factors involved in the occurrence and development of papillary thyroid carcinoma. In our study the urban/rural

distribution found in patients with thyroid neoplasia was approximately equal, with the literature reporting an increased incidence of thyroid disease found in residents of large cities. The risk of malignancy correlated with age, with most malignant nodules present after 60 years of age, and benign nodules more commonly detected between 30-50 years of age.

In our study the association of thyroid cancer in patients with pre-existing thyroid disease or other diseases such as cardiovascular, autoimmune, and overweight did not show a direct correlation.

Among papillary thyroid carcinomas, the most common histological subtype of papillary thyroid carcinoma was the follicular variant of papillary carcinoma, found in more than 25% of cases, a percentage consistent with the literature. The diagnosis in these cases could be easily established on the basis of the specific features of the nuclei, which were optically clear, with a "matte glass" appearance.

Histopathological diagnosis of unencapsulated carcinoids remains one of the most controversial surgical thyroid pathologies. Correct diagnosis in these cases has been very important because these tumors may have lymphatic and distant metastatic potential. It was also considered that interpreting a follicular adenoma as follicular carcinoma may expose the patient to unhelpful aggressive surgery.

In current practice, many confounding situations can occur in follicular lesions with atypical cytological features. These may be encountered in benign lesions such as hyperplastic adenomatosis or follicular adenomas. Often overdiagnosed these lesions may undergo aggressive surgery and treatment with radioactive iodine.

In Chapter 7 we examined smears obtained by fine-needle aspiration cytroscopy of the thyroid gland which were correlated with histopathological examinations in order to identify as precisely as possible the variants of papillary carcinoma known to be biologically aggressive, especially in cases of poorly differentiated carcinoma. Aspiration followed by cytological examination has proved to be a useful tool used in the evaluation of thyroid tumours, but it cannot replace histopathological diagnosis of certainty. This has allowed early treatment with a significant rate of improvement in cure rate and prognosis of thyroid neoplasms.

Recognition of aggressive variants of papillary carcinoma has been of major importance in the prognosis and clinical management of patients.

The association of morphometry in histological smears for follicular lesions suspected of malignancy leads to increased acuity in establishing suspicious diagnoses of malignancy for follicular lesions.

In Study III, Chapter 8, nuclear morphometry provided an unbiased view that can be considered of real use in differentiating cases with borderline lesions bordering between malignancy and benignity, but the method is limited because it is based only on correlations obtained by statistical studies.

We used a semi-automated method in the morphometric study to eliminate possible errors that may have artifactually occurred through processing techniques, as histological images are complex and often the precise delineation of nuclear margins can be easily intruded with the brightness of the cytoplasm. Thus the edges of the nuclei were outlined with great care and attention manually. The data obtained are consistent with some studies in the literature and confirm the value of accuracy in nuclear morphometry.

This elaborate t ehnics could be recommended in particular for clinically controversial cases in patients with unbalanced metabolism who may develop post-surgical complications.

The measured morphometric parameters characterizing benign and malignant lesions in the smears evaluated in this study are in line with the literature data.

Computerized morphometry is proving to be a useful tool that can be implemented in the pathological management of thyroid investigations due to its simplicity of execution, safety and cost-effectiveness. It can positively influence diagnostic accuracy, allowing better correlation with clinical and imaging data.

Conclusions

Despite the increasing incidence of thyroid disease, thyroid cancer mortality has been on a downward trend in recent decades due to better diagnosis and better thyroid treatment strategy expected on the development of knowledge in the field.

Thyroid carcinomas are a compact group with heterogeneous morpho-phenotypic and immuno-biological expression that are sometimes overlooked, especially small tumours such as papillary microcarcinomas. Most of these carcinomas are identified incidentally during routine screening or at autopsy.

Detailed study of the thyroid gland collected during autopsies, as performed in the present study, has shown that a thyroid, apparently normal on macroscopic examination, can sometimes be the site of pathological or tumoural manifestations. The increased incidence of papillary microcarcinomas often creates dilemmas in terms of the application of

appropriate therapeutic management, sometimes delaying the application of surgical interventions, which may be followed by a more aggressive course.

In complex and difficult cases, cytomorphological features correlated with clinical data, immunohistochemical markers and molecular profiling can lead to rapid elucidation of diagnoses .

The study highlights the importance of examining the thyroid gland in autopsy series as well as the importance of including molecular and metabolic markers in future autopsies.

Computerized morphometry can be considered as a useful tool that can be implemented in the pathological management of thyroid investigations due to its simplicity of execution, safety and cost-effectiveness. The results obtained can positively influence diagnostic accuracy, allowing a better correlation with clinical and imaging data.

T he elaborate technique of nuclear morphometry could be performed on series of thyroid tumours and in particular could be applied to clinically controversial cases, to patients with unbalanced metabolism so that they would not undergo laborious surgery that would unbalance them by developing post-surgical complications.

Further application of more in-depth studies, on larger groups of patients, would open new horizons in the application of quantitative assessment in the pathology of thyroid lesions, especially for cases with aggressive progression and difficult degree of diagnosis.

In conclusion, there is a high prevalence of thyroid nodules detected in the general population which can often hide occult thyroid carcinomas and therefore we consider it necessary to create screening programmes for early detection of thyroid pathology.

Novelty/Originality

Identification of clinical, histological and immunohistochemical correlations in histological subtypes of incidentally detected thyroid carcinomas in serial autopsies.

Nuclear morphometry has provided an unbiased viewpoint, with the aim of achieving greater diagnostic accuracy, and is of real use, especially in differentiating cases bordering on malignancy and benignity, with lesions that are difficult to interpret.

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