Differentiating follicular thyroid carcinoma from the follicular variant of papillary thyroid carcinoma

by

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DECLARATION

I, Dr Tshikani Norman Rikhotso hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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DEDICATION

This work is dedicated with gratitude to my mother and to my three daughters.

For their encouragement and endless love.

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ABSTRACT

Introduction: Differentiating follicular adenoma (FA), follicular thyroid carcinoma (FTC) and follicular variant of papillary thyroid carcinoma (FVPTC) remain a diagnostic challenge in some cases. This is as a result of the assessment of nuclear features being highly subjective, and the threshold for confirming cytomorphological features for papillary thyroid carcinoma varying greatly among pathologists which results in poor interobserver agreement. Diagnostic challenges may be encountered with some encapsulated follicular-patterned neoplasms of the thyroid due to uncertainty about the presence of capsular or vascular invasion. Immunohistochemistry may provide a better alternative to distinguishing these entities.

Aims and objectives: To study the expression of biomarkers HBME-1, CD15, CK-19 and BRAF V600E in follicular adenoma, follicular carcinoma and follicular variant of papillary thyroid carcinoma. To ascertain the usefulness of these markers in differentiating these follicular-patterned thyroid neoplasms.

Materials and methods: This is a ten-year retrospective study in which seventy-nine cases consisting of follicular adenoma (n=26), follicular thyroid carcinoma (n=25) and follicular variant of papillary thyroid carcinoma (n=28) were retrieved and reviewed. Four immunohistochemical stains (CD15, CK-19, HBME-1 and BRAF V600E) were performed and scored in tumour tissue. Data were analysed to determine if there was any correlation between the expression of the immunomarkers and the three follicular-patterned thyroid neoplasms.

Results: The patients' ages ranged from 13 to 74 years. There was a female bias with a female-to-male ratio of 4:1. HBME-1 expression showing varying intensity and proportion was seen in 7 (28%) FA, 15 (65%) FTC and 22 (79%) FVPTC. CK-19 expression showing varying intensity and proportion was seen in 5 (19%) FA, 3 (13%) FTC and 18 (72%) FVPTC. BRAF V600E expression showing varying intensities and proportions was seen in 3 (11%) of FVPTC. FA and FTC cases were all negative for BRAF V600E. CD15 expression showing varying intensity and proportion was seen in 2 (8%) FA, 6 (24%) FTC and 9 (33%) FVPTC.

Statistical analysis detected a significant association between group and HBME-1 and CK-19 (H-score, proportion and intensity). The post-hoc comparison revealed that follicular adenoma was significantly more likely to have negative HBME-1 staining and a

lower H-score when compared to follicular thyroid carcinoma and follicular variant of papillary thyroid carcinoma. The follicular variant of papillary thyroid carcinoma was significantly more likely to have an HBME-1 intensity of three and a higher H-score compared to follicular adenoma. Post-hoc comparison revealed that follicular adenoma was significantly more likely to have a negative CK-19 and a lower H-score when compared to follicular thyroid carcinoma and follicular variant of papillary thyroid carcinoma. Follicular variant of papillary thyroid carcinoma was significantly more likely to have a CK-19 intensity of three and a higher H-score compared to follicular thyroid carcinoma.

HBME-1 had an overall specificity and sensitivity of 72% and 74% for distinguishing FA from FTC and FVPTC (benign from malignant).

CK-19 had an overall specificity and sensitivity of 80.8% and 43.8% for distinguishing FA from FTC and FVPTC (benign from malignant). CK-19 had specificity of 87% and sensitivity of 72% for distinguishing FTC from FVPTC.

Combining HBME-1 and CK-19 did not significantly increase the sensitivity and specificity of these markers.

There was statistically no significant association between group and BRAF V600E and CD15 biomarkers.

Conclusion: The study, within the small sample size power limitations, has shown that CK-19 may have a role in distinguishing follicular thyroid carcinoma from follicular variant of papillary thyroid carcinoma. In addition, HBME-1 and CK-19 may be used in differentiating benign follicular-patterned thyroid lesions from malignant follicular-patterned thyroid lesions.

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LIST OF ABBREVIATIONS

ATC	Anaplastic Thyroid Carcinoma
AUC	Area Under Curve
BRAF	B-type Raf Kinase
cPTC	Classical Papillary Thyroid Carcinoma
CD	Cluster of Differentiation
CK-19	Cytokeratin-19
FA	Follicular Adenoma
FUT-4	Fucosyltransferase 4
FTC	Follicular Thyroid Carcinoma
FVPTC	Follicular Variant of Papillary Thyroid Carcinoma
HBME-1	Hector Battifora Mesothelial -1
IHC	Immunohistochemistry
MEN	Multiple Endocrine Neoplasia
MTC	Medullary Thyroid Carcinoma
NIFTP	Non-invasive Follicular Thyroid Neoplasm with Papillary-like Nuclear Features
PTC	Papillary Thyroid Carcinoma
ROC	Receiver operating Characteristics
SSEA-1	Stage-Specific Embryonic Antigen 1
TROP-2	Trophoblastic Cell Surface Antigen 2
TTF-1	Thyroid Transcription Factor 1

CHAPTER 1

INTRODUCTION

Thyroid cancer consists of several different histopathological subtypes. The subtypes differ in terms of the cell of origin, incidence, and prognosis (1). The most common subtypes are well-differentiated carcinomas which arise from the follicular cells and comprise more than 90% of thyroid cancers. Based on the histopathological pattern, well-differentiated thyroid carcinomas are classified as papillary thyroid carcinoma (PTC) or follicular thyroid carcinoma (FTC) (2).

The basic morphologic characteristics used to diagnose tumours of follicular cell derivation are papillary and follicular architecture, presence of a tumour capsule and its invasion in the form of capsular or vascular invasion, and nuclear features typical of papillary carcinoma (3).

Many variants of PTC have been described, and include classical, follicular, tall cell, columnar cell, diffuse sclerosing, encapsulated, clear cell, oncocytic and cribriform morular (4). The follicular variant of PTC (FVPTC) is one of the most common subtypes (5).

Well-differentiated thyroid carcinomas, tend to have a more benign course and a better prognosis when compared with poorly differentiated and anaplastic thyroid carcinomas (2). However, studies have shown that the prognosis of follicular variant of papillary thyroid carcinoma (FVPTC) falls between that of classical papillary thyroid carcinoma (cPTC) and follicular thyroid carcinoma (FTC) (5). FVPTC has lower mortality and less frequent distant metastases than FTC, but higher mortality and more frequent distant metastases than cPTC. FVPTC has fewer lymph node metastases and less frequent infiltrative disease and extrathyroidal extension than cPTC, but more than FTC (6). This then raises the importance of distinguishing these entities objectively.

Diagnostic challenges may be encountered with some encapsulated follicular-patterned neoplasms of the thyroid as the presence of nuclear features may be equivocal to render a diagnosis of PTC, or when the presence of vascular or capsular invasion to render a

diagnosis of follicular carcinoma is equivocal (7). The assessment of nuclear features is highly subjective, and the threshold for rendering a diagnosis of PTC varies greatly among pathologists, resulting in poor interobserver concordance.

This study aims to investigate the diagnostic potential of the expression of Hector Battifora mesothelial-1 (HBME-1), cytokeratin-19 (CK-19), cluster of differentiation -15 (CD15) and B-type Raf kinase (*BRAF)* by immunohistochemistry (IHC) in differentiating FVPTC from FTC.

CHAPTER 2

LITERATURE REVIEW

2.1. Epidemiology

Thyroid cancer is the most pervasive endocrine cancer worldwide and accounts for the largest number of malignant neoplasms that occur in the endocrine system. The number of new cases is on the rise (8). Thyroid cancer accounts for 1-2% of all malignancies worldwide (8). This apparent increase in the incidence of thyroid cancers has been attributed to the increased use of imaging modalities like ultrasound, CT scan and MRI with cytology, and has been linked with a rise in the incidence of thyroid papillary micro-carcinomas (9).

Surgical series in Africa have also confirmed it as the most frequent endocrine malignancy (10). South Africa in 2018 is estimated (based on Globocan estimates) to have had 2 408 thyroid cancer cases and 157 thyroid cancer deaths (10, 11). In most countries around the world, thyroid carcinoma incidence has been appreciably increasing over the last few decades. Globally, there were 95 030 incident cases of thyroid cancer and 22 070 deaths in 1990 and 255 490 incident cases and 41 240 deaths in 2017(11).

Thyroid neoplasms show a female bias with a female-to-male ratio of 3:1 (1, 2). Thyroid carcinoma can occur at any age but a majority of cases are found in people between the ages of 20 and 55 years. Although they can occur at any age, they are very rare in children (1, 2, 11).

2.2. Classification of thyroid neoplasms

The World Health Organization (1) classification of thyroid tumours has introduced a new category of tumours of uncertain malignant potential (borderline neoplasms). This category encompasses encapsulated follicular patterned tumours with equivocal features of PTC or invasion.

2.2.1. Follicular adenoma and borderline tumours

FA is a benign, encapsulated thyroid neoplasm showing follicular cell differentiation in the absence of cytological features for PTC and invasion (1). Excluded from the FA category are non-invasive encapsulated follicular patterned neoplasms with PTC-like nuclear features which are now classified as non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) or well-differentiated tumours of uncertain malignant potential (WDT-UMP) (1).

The incidence of FA is difficult to investigate due to the lack of uniformity in the histopathological criteria for distinguishing hyperplastic nodules from adenomas (12). Most follicular adenomas are sporadic. Radiation exposure and iodine deficiency are known risk factors (13). Follicular adenomas have also been noted to develop in patients with Cowden syndrome or Carney complex (1,13).

Follicular adenoma is usually a well-circumscribed, encapsulated solitary round or oval nodule. The tumours usually measure 1- 10 cm. The cut surface shows a homogeneous greyish-white, tan, or brown fleshy tumour (14,15). Secondary changes such as haemorrhage and cystic degeneration may be present (15).

Follicular adenomas have a fibrous capsule, which is typically thin or thick. Capsular and vascular invasion are absent. The architectural and cytological features of the tumour are different from those of the surrounding thyroid parenchyma. Follicular adenomas show a variety of architectural growth patterns: normofollicular, macrofollicular, microfollicular, solid, and trabecular (12,15,16). More than one pattern may be seen in a single nodule. The tumour cells are cuboidal or polygonal. Mitotic figures are rare. The stromal component of the tumour is typically scant. Secondary changes such as stromal fibrosis, hyalinization, haemorrhage, oedema, cystic degeneration, calcification, and osseous or cartilaginous metaplasia may be seen (12,15).

When there is no evidence of malignancy, many pathologists do not make the diagnosis of adenoma in a multinodular gland, preferring to designate all lesions as hyperplastic nodules (12,15). Molecular studies may be required to distinguish hyperplastic nodule

from FA although this may not be relevant clinically as both lesions are benign (12). The only histological features that reliably distinguish follicular thyroid carcinoma from follicular adenoma are vascular invasion and capsular invasion. With grossly encapsulated nodules, submission of the entire capsule is recommended (15).

Mutations of the *RAS* genes occur in about 30% of follicular adenomas, *PAX8*/*PPARG* rearrangements, a feature of follicular thyroid carcinomas, are found in about 8% of follicular adenomas. Completely removed follicular adenomas pose no further risk (1,12).

2.2.1.1. Borderline thyroid tumours

Encapsulated follicular-patterned tumours of the thyroid pose diagnostic challenges when the presence of cytological features of PTC or invasion are equivocal (7). These tumours are indeterminate between FA and FTC. This makes it difficult to determine with certainty whether this represents FA or FTC. The WHO 2017 (1) refer to these tumours as “follicular tumour of uncertain malignant potential" (**Table 1**). The importance of having this category of borderline tumours is to avoid unnecessary aggressive treatment (1).

The assessment of nuclear features of PTC is highly subjective, and the threshold for rendering a diagnosis of follicular variant of PTC varies greatly among pathologists, resulting in poor interobserver concordance in the diagnosis of encapsulated FVPTC (17).

Many studies have demonstrated that non-invasive encapsulated FVPTC is associated with an excellent prognosis and has molecular features more similar to those of follicular adenoma/carcinoma than to those of conventional PTC (17,18).
The current WHO classification uses the term "non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) " which encompasses non-invasive encapsulated follicular-patterned tumours previously called encapsulated FVPTC or well-differentiated tumour of uncertain malignant potential (1). NIFTP diagnosis requires four histopathological features: (a) encapsulation or circumscription, (b) no capsular or vascular invasion, (c) a follicular pattern of growth, and (d) nuclear features

of papillary carcinoma (1,18). True papillae and psammoma bodies are not consistent with this diagnosis. Exclusion features include a component of solid, trabecular, or insular/ growth accounting for > 30% of the tumour; more than three mitotic figures per ten high-power fields); and tumour necrosis. These are features of either the solid variant of papillary carcinoma or poorly differentiated thyroid carcinoma (1,18).

The term "well-differentiated tumour of uncertain malignant potential" is reserved in this classification for tumours with nuclear features of PTC (equivocal or questionable) and questionable invasion (1).

Table 1: 2017 WHO classification: Nomenclature for encapsulated follicular-patterned tumours (Adapted from the Nikiforovi et al 2017)

		Capsular or Vascular Invasion		
		Present	**Questionable**	**Absent**
Nuclear Features of PTC	**Present**	Invasive encapsulated follicular variant of PTC	Well-differentiated tumour of uncertain malignant potential	Non-invasive follicular thyroid neoplasm with papillary-like nuclear features
	Questionable	Well-differentiated carcinoma, NOS	Well-differentiated tumour of uncertain malignant potential	Non-invasive follicular thyroid neoplasm with papillary-like nuclear features
	Absent	Follicular carcinoma	Follicular tumour of uncertain malignant potential	Follicular Adenoma

2.2.2. Follicular thyroid carcinoma (FTC)

FTC accounts for 6-10% of all thyroid carcinomas. FTC lacks the characteristic diagnostic nuclear features of PTC. A follicular epithelial cell lesion showing capsular invasion and/or angioinvasion is considered malignant (19). There is, however, still controversy with regards to the interpretation of capsular invasion. Most authors require that neoplastic cells penetrate the entire thickness of the tumour capsule (**Figure 1**). The presence of irregular contour of the inner capsular border, capsular

pushing by the tumour cells, or tumour cell nests embedded within the capsule are considered by many authors to be insufficient for the diagnosis (19).

The majority of authors agree that lymphovascular invasion should involve capsular or extra-capsular vessels. The presence of intracapsular endothelialised thrombus, tumour thrombus with fibrin, and tumour thrombus attached to the vessel wall are all acceptable as confirming lymphovascular invasion. Tumour in intra-tumoral or subcapsular vessels does not qualify for lymphovascular invasion and should not be interpreted as such (19).

The current WHO classification of endocrine tumours classifies FTC into three categories which are namely, minimally invasive FTC, encapsulated angioinvasive FTC, and widely invasive FTC. The minimally invasive FTC only has capsular invasion whilst encapsulated angioinvasive may or may not have a capsular invasion. Widely invasive FTC shows extensive invasion with extension into the surrounding soft tissue and commonly exhibits widespread vascular invasion. Widespread angioinvasion on its own is insufficient to classify FTC as being widely invasive FTC (20).

The degree of vascular invasion in FTC has some prognostic implications (21,22). In encapsulated angioinvasive FTC, tumours with extensive vascular invasion have a worse outcome when compared to those with limited vascular invasion (21). Extensive vascular invasion is defined as having more than four vessels showing vascular invasion (21). The outcome for patients with widely invasive FTC is independent of the extent of vascular invasion (21).

Metastatic FTC has been closely associated with *TERT* promoter mutation. *TERT* promoter mutation has however not been reported in atypical FA or FT-UMP (23).

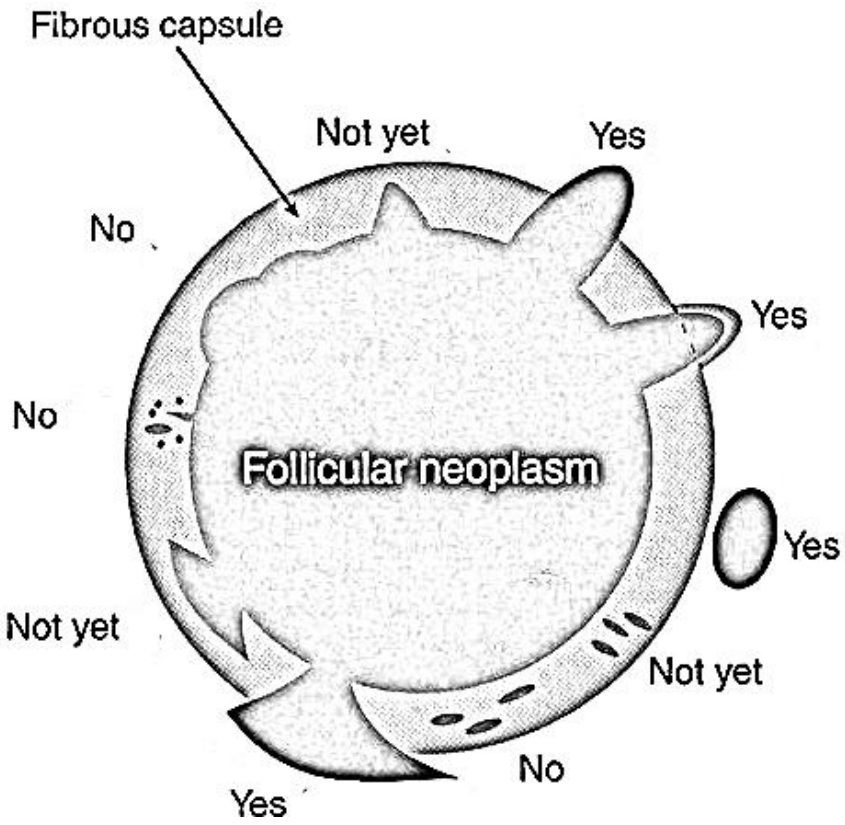

Figure 1: Schematic drawing for the interpretation of the presence or absence of capsular invasion (Adapted from Chan JCK, 2007)

2.2.3. Papillary thyroid carcinoma

PTC is a follicular cell-derived malignant tumour whose diagnosis is solely based on its characteristics nuclear features regardless of the tumour structures and growth patterns (20). Papillae, invasion or cytological features of PTC are required for the diagnosis of PTC (1,20). PTC accounts for 80% of all thyroid cancers and occurs in both adults and children. PTC is three times more common in females than in males, but this disparity decreases with increasing patient age (1).

The best-documented environmental cause of PTC is exposure to ionizing radiation. Other risk factors which have been identified include eating habits, tobacco smoking, living in a volcanic area, xenobiotics, viruses, dietary iodine excess, and genetic factors (24).

The two cardinal morphological features of classic PTC are the papillae and the nuclear changes. The papillae are composed of a central fibrovascular stalk lined by neoplastic

cells. The nuclei of the cells of PTC display a characteristic set of abnormalities, which can be grouped into three categories: (a) changes in size and shape, (b) irregularities of the membrane, and (c) chromatin characteristics (1,25).

The changes in nuclear size and shape include nuclear enlargement, overlapping, and nuclear elongation (1,25). The nuclei of PTC are typically characterised by remodelling of the nuclear envelope, with markedly irregular nuclear contours and pseudoinclusions or notable longitudinal grooves (1,25). The other consistent feature of PTC nuclei is an empty appearance of the nucleoplasm. The nuclei have been variously described as optically clear and resembling ground glass (1,25). The ground-glass appearance is evident in fixed, paraffin-embedded material but is inconspicuous or absent in frozen sections from the same cases (1,25).

Mitotic figures are rare or absent in classic PTC. Focal solid areas may be evident. Psammoma bodies are found within stroma in about 50% of cases overall and are particularly common in tumours with a predominantly papillary growth pattern (1,4,25).

2.2.3.1. Variants

There are many well-recognized histopathological variants of PTC (1). Some of the variants such as tall cell, columnar cell, and hobnail variants are of great clinical relevance since they are associated with aggressive clinical course and worse prognosis when compared with classic PTC (26,27).

2.2.3.1.1 Papillary microcarcinoma

Tumours of this variant measure < 10mm in diameter. More recently, a proposal has been advanced to call it papillary microtumour (1,28). The reported incidence in autopsy material is 5.6-35.6%. Grossly, the lesions are easily missed. Microscopically, the tumour shows an irregular, scar-like configuration. The general characteristics of PTC such as typical nuclear changes are also seen in this entity. Papillary microcarcinoma has an excellent prognosis (1,20).

2.2.3.1.2 Encapsulated variant

This variant is a type of architecturally and cytologically typical PTC that is completely surrounded by a fibrous capsule (1). This variant accounts for about 10% of all cases of PTC. Encapsulated PTC has an excellent prognosis. Regional nodal metastases may be present, but blood-borne metastases are rare. The main differential diagnosis is with follicular adenoma with papillary hyperplasia, which lacks PTC-type nuclear changes (20).

2.2.3.1.3 Follicular variant

This variant has an exclusively or almost exclusively follicular growth pattern. There are two main subtypes which are namely infiltrative and encapsulated with invasion. In its most typical form (the infiltrative subtype), this variant shares many features with conventional PTC. Most importantly, the nuclei of the cells lining the follicles have features of conventional PTC (20). The colloid within the lumina of the neoplastic follicles often has a strong and homogeneous eosinophilic quality (1).

The non-invasive encapsulated follicular variant of PTC was downgraded from carcinoma to NIFTP, and cases with incomplete invasion were downgraded from carcinoma to WDT-UMP (1,29).

2.2.3.1.4 Diffuse sclerosing variant

Diffuse sclerosing variant is most commonly seen in young females and clinically mimicks Hashimoto thyroiditis presenting with acute onset thyroid gland enlargement and increased levels of antithyroglobulin and antimicrosomal antibodies (30). Microscopically, the entity is characterized by fibrosis, lymphocytic infiltration, abundant psammoma bodies, and areas of squamous metaplasia (1,30). Lymphovascular invasion is a common finding (1). This variant commonly shows *RET*/*PTC* rearrangements whilst *BRAF* V600E mutation is infrequent (1).

The mortality rate for diffuse sclerosing variant is similar to that of classic PTC, despite it being associated with an aggressive clinical course (31).

2.2.3.1.5 Tall cell variant

Tall cells are defined as tumour cells that are three times as tall as they are wide (1, 32). The WHO (1) requires a minimum of 30% of the neoplastic cells to show tall cell morphology for this diagnosis. Studies have shown that PTCs with focal tall cell change (≥10%) should be reported as this confers a poorer prognosis (33).

Conventional PTC is more sensitive to radioactive iodine ablation and has a better outcome than the tall cell variant (27). *BRAF* V600E and *TERT* promoter mutations are commonly found in the tall cell variant (34).

2.2.3.1.6 Columnar cell variant

This variant of PTC shows columnar cells exhibiting pseudostratification of hyperchromatic nuclei in the absence of nuclear features for PTC (1,32). Immunoreactivity of the columnar cell variant with CDX2 is seen in 10% to 55% (35). The variant, however, shows TTF-1 positivity which enables differentiation from metastatic colonic adenocarcinoma (1).

Columnar cell variants which show invasion and extrathyroidal extension is associated with a worse prognosis when compared to circumscribed types that have a good prognosis (1).

2.2.3.1.7 Cribriform-morular variant

This variant which occurs exclusively in females can occur in a sporadic form or as a manifestation of familial adenomatous polyposis. Cases associated with familial adenomatous polyposis are often multifocal whereas sporadic cases are usually solitary (36).

The tumour is usually encapsulated and shows an admixture of growth patterns including cribriform and morules. Capsular and/or vascular invasion are usually present. The nuclei show grooves and pseudoinclusions but nuclear clearing is not a consistent feature (1,36).

Nuclear β-catenin is a characteristic finding in this variant. TTF1 often shows patchy positivity and thyroglobulin staining is often focal and weak (37).

2.2.3.1.8 Hobnail variant

The hobnail variant is defined by the presence of >30% of tumour cells with hobnail features (1). The variant accounts for <3% of all PTC cases. The hobnail variant shows *BRAF* V600E mutation in 50% to 94% of cases (38).

The hobnail variant must be distinguished from its close mimicker, classic PTCs showing hobnail-like morphology (39). Classic PTC showing hobnail-like morphology shows oedematous and hyalinised fibrovascular cores (39).

2.2.3.1.9 Papillary thyroid carcinoma with fibromatosis/ fasciitis-like stroma

This variant shows stroma of PTC that is abundant and cellular resulting in the tumour resembling fibromatosis or nodular fasciitis (1,40).

2.2.3.1.10 Solid variant

This variant requires that most of the tumour shows solid/trabecular/nested areas and other variants to be ruled out. The solid variant constitutes 1 -3% of adult papillary carcinomas and it is more common in patients with a history of exposure to ionizing radiation and young patients (20). A subset of tumours showing solid architecture, brisk mitotic activity and necrosis but without exhibiting nuclear features of PTC are classified as poorly differentiated thyroid carcinoma (PDTC) and are excluded from this variant (41).

In adults, this variant has a slightly more aggressive prognosis than does conventional PTC, however, other studies have not found this tumour to be aggressive in behaviour (1).

Radiation-associated paediatric and sporadic cases frequently show *RET*/*PTC3* rearrangement. Classic PTC show a higher incidence of *BRAF* V600E mutations when compared to the solid variant (42).

2.2.4 Poorly differentiated thyroid carcinoma (PDTC)

Poorly differentiated thyroid carcinoma (PDTC) is a follicular cell neoplasm that shows limited evidence of follicular cell differentiation and is morphologically and behaviourally intermediate between well-differentiated (follicular and papillary) carcinomas and anaplastic thyroid carcinoma (ATC) (1).

The Turin consensus proposal has been adopted as the diagnostic criteria for PDTC (1). The features required for diagnosis include (a) presence of a solid/trabecular/insular growth pattern, (b) absence of nuclear features of PTC, and (c) at least one of the following characteristics: convoluted nuclei; mitotic figures ≥3 per10 HPF; and tumour necrosis (1,41).

Proliferative index (Ki-67) may have a role in distinguishing PDTC from WDTC (20). The proliferative index in PDTC is usually 10% to 30% whilst in WDTC it is <10% (20).

PDTC in some cases coexists with other types of neoplasms such as PTC. The proportion of PDTC should be reported as its presence in the background of WDTC has been associated with aggressive clinical course and poor prognosis (43).

The most common genetic alteration in PDTC is *TERT* promoter mutation (44). The other genetic alterations encountered include the alterations seen in WDTC and ATC, namely *RAS, TP53* and *BRAF* V600E (44).

2.2.5 Anaplastic thyroid carcinoma (ATC)

ATC is an aggressive malignant neoplasm composed of undifferentiated follicular cells that usually presents as advanced disease which is not amenable to even radical surgery. ATC can arise directly from follicular cells or dedifferentiation of a differentiated thyroid carcinoma (20).

The proportion of ATC component found within WDTCs has implications on prognosis. Favourable patient survival has been observed in patients with small areas of ATCs found within WDTCs (45).

The common histopathological patterns encountered in ATC include sarcomatoid, giant cell and epithelial (20). Other uncommon histologic variants include paucicellular, rhabdoid, and small cell variants (1,46). Tumour-infiltrating macrophages, dense inflammatory cell infiltrate, necrosis, and a high mitotic rate are the other common findings (46).

Common thyroid-follicular cell biomarkers such as Thyroid transcription factor 1(TTF-1) and thyroglobulin are usually negative, whereas PAX8, is retained in approximately 50% of all cases (47). CD10 shows strong and diffuse cytoplasmic immunopositivity in ATC but is negative in WDTCs (1).

The common molecular alterations encountered include *TERT* promoter mutation (75%), *TP53* (63%) and *BRAF V600E* (45%). *BRAF V600E* and *TERT* promoter mutations occurring concomitantly confers a poorer outcome than when the alterations occurs in only one of the genes (1,46).

2.2.6 Medullary thyroid carcinoma

Medullary thyroid carcinoma (MTC) is a malignant epithelial tumour that shows C cell differentiation. The majority (80%) of the cases are sporadic and the other 20% occur in multiple endocrine neoplasia (MEN) syndromes subtypes 2A and 2B (1). MTC is characterised by somatic or germline mutations of the RET gene. The RET gene alterations usually involve an activating point mutation of 10q11.2(48,49).

MTC accounts for < 2-3% of all thyroid malignancies. The lower incidence has been attributed to the marked increase in the relative incidence of papillary thyroid carcinoma over the past two decades (1). Although MTC occurs in a wide age range, familial MTC cases tend to occur in significantly younger patients when compared to sporadic MTC cases (48).

MTCs are usually unencapsulated, well-circumscribed tumours showing a wide range of architectural patterns, including solid sheets, insular and organoid arrangements. Wide-ranging cytomorphology has been reported and these include round, plasmacytoid, spindled and polyhedral. The nuclei are round and oval with stippled to

coarsely clumped chromatin (1,48,50). Amyloid deposition is a common finding; often associated with calcification. Background C-cell hyperplasia is common in familial cases (1,48,50). Several histopathologic variants are recognised, but this does not alter management or outcome (1,48). The variants are more important for differential diagnosis. The tumour cells are immunoreactive with calcitonin, monoclonal carcinoembryonic antigen, chromogranin, synaptophysin and TTF-1; the cells are negative with thyroglobulin (1,50).

2.3. Role of immunohistochemical markers

For the majority of thyroid carcinomas, a diagnosis can be reached by morphological assessment alone. Diagnostic challenges may be encountered with some encapsulated follicular-patterned neoplasms of the thyroid due to equivocal nuclear features of PTC, or questionable presence of capsular or vascular invasion. The assessment of nuclear features is highly subjective, and the threshold for rendering a diagnosis of PTC varies greatly among pathologists, resulting in poor interobserver concordance.

The distinction may be difficult, but critical because the differential diagnosis is benign or malignant. In some instances, cases that were concluded as benign follicular adenoma may have the diagnoses changed to malignancies because of recurrence or metastasis. As a result, immunohistochemical studies may become essential to establish a diagnosis.

Currently, the WHO (1) does not recommend the use of immunohistochemistry in distinguishing encapsulated follicular-patterned neoplasms (FA, FTC and FVPTC). Many immunohistochemical (IHC) markers have been proposed, including galectin-3, HBME-1, CK-19, CD15 and *BRAF* (51,52).

2.3.1 Hector Battifora mesothelial-1 (HBME-1)

HBME-1 (Hector Battifora mesothelial 1) is a monoclonal antibody that recognizes uncharacterised antigen in the microvilli of mesothelioma cells. Subsequently, the same antigen has also been identified in normal tracheal epithelium, and adenocarcinoma of the lung, pancreas, and breast (53,54). HBME-1 is negative in gastrointestinal tract epithelium, and normal thyroid tissue (51,55,56).

HBME-1 is a thyroid marker of follicular origin that shows preferential staining for malignant tumours when compared to benign lesions (54). HBME-1 shows positive staining in most PTCs (88%) and FTCs (75%) (53). PDTC and ATC also often express HBME-1 (67%–91% and 0%–50%, respectively) (53). HBME-1 expression in a focal pattern has also been noted in benign thyroid lesions such as FA and nodular goitre, with an overall positive rate of 26% and 12%, respectively (54).

HBME-1 has been found to have an overall specificity of 82% and overall sensitivity of 78% for thyroid carcinoma, 65% for FTC and 87% for PTC (54). A meta-analysis of 21 IHC studies on histopathological specimens found a sensitivity of 77% and specificity of 83% for thyroid carcinoma (51). Other studies using fine needle aspirate (FNA) specimens have shown HMBE-1 with similar overall sensitivity and specificity of 78% and 85% for thyroid malignancy (55, 56).

HBME-1 has been researched in thyroid tumours to determine its usefulness in distinguishing benign from malignant lesions. FVPTC has been shown to have a significantly higher expression of HBME-1 than in FA or FTC (57). Other studies have also shown a higher expression of HBME-1 in FTC than in FA (57).

HBME-1 displays cytoplasmic expression with some membrane accentuation in thyroid carcinomas (51,56,58).

2.3.2 Cluster of differentiation 15 (CD15)

CD15 (LeuM1, Lewis X, or stage-specific embryonic antigen 1), is a carbohydrate antigen with the common trisaccharide structure 3-fucosyl-N-acetyl-lactosamine. The CD15 gene is located on the long arm of chromosome 11 at position q12 (59). The main function of CD15 is in cell-to-cell adhesion mediated by carbohydrate-specific ligands (59).

CD15 antigen is expressed in eosinophils, neutrophils, and to a lesser degree in monocytes. CD15 is also immunoreactive with Reed-Sternberg cells of Hodgkin's lymphoma (60). CD15 is expressed in varying proportions of some carcinomas from lung, ovary, breast and colorectal region. Mesothelial cells are negative (57,61–64).

CD15 has been proposed as a biomarker for thyroid cancer stem cells (65). CD15 expression has been reported in thyroid carcinomas but not in benign thyroid neoplasms or normal thyroid tissue (66). CD15 shows higher positivity in anaplastic thyroid carcinoma (80%) and conventional papillary thyroid carcinoma (cPTC) (60.7%) when compared to the follicular variant of papillary thyroid carcinoma (FVPTC) (20.6%) and follicular carcinoma (FCA) (32.1%) (66). CD15 has a higher chance of being immunoreactive in neoplasms with metastases than in those without metastases (24,66).

Although CD 15 expression is specific for PTC, it has a low sensitivity (67). CD15 immunoreactivity may have a role in distinguishing well-differentiated thyroid carcinomas from benign lesions (54). Concomitant use of CD15 immunostaining with CK-19 or HBME-1 has been shown to have a sensitivity of 95% and specificity of 90% for the diagnosis of PTC and can assist in challenging cases (54). Although CD15 is inferior in terms of sensitivity for FVPTC, its excellent specificity supports the definitive diagnosis of thyroid malignancy (67).

2.3.3 Cytokeratin 19 (CK-19)

Cytokeratin 19 (CK-19) is a member of type 1 acidic subfamily of intermediate filaments. The human gene encoding CK-19 is localized on chromosome 17q21-22 (68). The keratins are intermediate filament proteins responsible for the structural integrity of epithelial cells. The CK-19 antibody labels many types of simple and non-keratinising epithelia, including ductal and glandular epithelia like the thyroid gland. Normal thyroid follicular cells do not produce CK-19 and its upregulation is associated with malignant transformation (68).

CK-19 expression in thyroid lesions is strong and diffuse in PTC and variable in FTC and FA (69). Normal thyroid follicular epithelium is often negative, although focal staining for CK-19 is usually identified in the compressed thyroid parenchyma surrounding nodules and in follicular cells within lymphocytic thyroiditis (69,70).

CK-19 shows strong and diffuse membranous ± cytoplasmic immunoreactivity in papillary carcinoma (70). Positive CK-19 staining is recognized as a reliable tumour

marker of PTC (71,72). Studies have shown that immunostaining by CK-19 is effective for distinguishing between follicular variant of papillary thyroid cancer and follicular thyroid carcinoma (71,72).

2.3.4 B-type Raf kinase (BRAF)

The B-type Raf kinase (*BRAF*) gene is located on the long arm of chromosome 7 at position q34 and it encodes a cytoplasmic serine-threonine kinase which in turn is responsible for activation of the mitogen-activated protein kinase (MAPK) signalling pathway (73). In the event of oncogenic BRAF V600E mutation, constitutive activation MAPK signalling pathway occurs which leads to downstream increased cell proliferation, anti-apoptosis and ultimately tumour progression (73,74).

BRAF and *RAS* mutations are considered to be mutually exclusive although concomitant mutations have been reported recently (73,74). *BRAF* mutations commonly occur at amino acid V600 with the V600E mutation being the most frequent. The result of this mutation is the substitution of valine (V) to glutamic acid (E) at position 600 of the amino acid sequence (73,74).

Mutation of the *BRAF* gene is detected in approximately 20% to 80% of sporadic cases of PTC. Conventional PTC has a higher prevalence of *BRAF* gene mutation than FVPTC (75). BRAF mutation is not found in cases that show follicular growth pattern exclusively (74,75). The *BRAF* V600E mutation is generally negative in normal thyroid tissue, FA and FTC (54).

The gold standard for detecting *BRAF* V600E is by gene sequencing, but it is a relatively expensive procedure requiring suitably equipped laboratory and sufficient quality of DNA. Immunohistochemistry is a more rapid cost-effective method to identify the *BRAF* V600E mutation. Studies have confirmed immunohistochemical detection of the mutated BRAF V600E protein as a reliable, highly specific method for detection of the *BRAF* V600E mutation in PTC (76,77).

The mutation-specific monoclonal anti-BRAF V600E (VE1 clone) antibody targets the mutated amino acid sequence from amino acids 596 to 606 (78). Immunohistochemical

studies have shown *BRAF V600E* mutations to be more associated with conventional, tall cell variants and oncocytic variants of PTC (76,77).

Follicular thyroid carcinoma does not carry BRAF V600E mutations. A case report with a BRAF (K601E) mutation has been reported. This however would not be detected by immunohistochemistry when using the mutation-specific immunohistochemistry like anti-BRAF V600E (VE1 clone) (79).

2.4 Aim and objectives

To study the expression of immunohistochemical markers HBME-1, CD15, CK-19 and BRAF V600E in follicular adenoma, follicular carcinoma and the follicular variant of papillary thyroid carcinoma.

To assess the significance of expression of these markers in follicular patterned neoplasms of the thyroid gland.

To ascertain the usefulness of these markers in differentiating between these tumours. Finding the best immunohistochemical panel to diagnose follicular variant papillary thyroid carcinoma.

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethics approval

The study protocol and methodology for this study was approved by the Human Research Ethics Committee (HREC) of the University of Cape Town (Ethics approval number: 770/2015).

3.2 Study design and sample selection

This was a laboratory based retrospective study. A search of the NHLS DISA database in the Division of Anatomical Pathology, Groote Schuur Hospital, for all cases of thyroid follicular adenoma, follicular carcinoma and papillary carcinoma diagnosed between 2004 and 2014 was performed.

The slides of the selected cases were retrieved from storage. The diagnoses of all cases were re-evaluated, and cases with adequate residual tissue were chosen for the study. All the cases of follicular variant papillary thyroid carcinoma were also identified from the papillary thyroid carcinoma group. A total of 79 cases, 26 follicular adenomas, 25 follicular carcinomas and 28 follicular variant of papillary carcinomas were identified. The slides were reviewed, and a block was selected for immunohistochemistry.

Clinical and patient information was recorded from the DISA database and Trakcare laboratory information systems (LIS) (age and sex). Data for the selected seventy-nine cases were entered into a Microsoft Excel (2016) spreadsheet. Names were removed and study numbers assigned to ensure patient confidentiality.

This research project was funded by the National Health Laboratory Service (NHLS) Research Trust.

3.3 Histochemical and immunohistochemical staining

A section was cut on each case for haematoxylin and eosin (H&E) staining.

Mayers Haematoxylin and eosin method:

- Sections were deparaffinised using xylene, brought down through alcohols and rinsed in running tap water.
- The tissue sections were then stained for 5minutes with Mayers haematoxylin.
- The tissue sections were rinsed in tap water and then placed in bluing reagent (Ammoniated water). The slides were rinsed again in tap water.
- The cytoplasm was stained with 1% phloxine/eosin for 2 mins.
- Slides were rinsed in water, dehydrated in alcohol and cleared in xylene.
- Mounting with coverslip in Entellan mount medium.

Sections for immunohistochemical staining were cut at 3µm. Each case had four immunohistochemical stains (**Table 2**) performed using two kits (**Table 3**).

Table 2: Primary antibody information

Primary Antibody	**Clone**	**Supplier**	**Antigen Retrieval**	**Dilution**	**Incubation Time**	**Positive Control**
CD15	MMA/ Mono	Cell Marque	TEDTA	1:50	1Hr	Hodgkin Lymphoma
CK-19	RCK108 /Mono	Dako	TEDTA	1:100	1Hr	Liver- bile ducts
BRAF V600E	VE1/Mono	Roche	HIER-CC1	RTU	28min	Papillary Thyroid Carcinoma
HBME-1	HBME-1 /Mono	Dako	TEDTA	1:100	1Hr	Mesothelioma

Key: CC1 - Cell Conditioning 1 HIER - Heat Induced Epitope Retrieval
Mono - Monoclonal RTU - Ready to use

Supplier information:

Cell Marque – California, USA
Dako – Denmark
Roche – Switzerland

Table 3: Kits used in the study

Kits	Supplier
Envision HRP System Labelled Polymer Anti-mouse	Dako - CA, USA
Liquid DAB + Substrate chromogen system	Dako - CA, USA

3.4 Immunohistochemical protocol (for CD15, CK-19 and HBME1)

- Three-micron tissue sections were cut, picked up onto Histobond slides (Marienfeld-Germany) and heat-fixed on a hotplate for 10-15 minutes.
- Sections were deparaffinised using xylene, cleared in ethanol and rehydrated in water.
- The slides were treated with 3% hydrogen peroxide (H_2O_2) solution for 10 minutes to block endogenous peroxidase activity.
- Slides were rinsed in water.
- Antigen retrieval was carried out in a specialized pressure-cooker with Tris EDTA (TEDTA) for 90 seconds at full pressure.
- This was followed by rinsing in tap water.
- Thereafter, slides were rinsed with phosphate-buffered saline solution (PBS pH 7.6), (Oxoid-Hampshire, England).
- The slides were treated with 5% Goat Serum Solution (DAKO- Denmark) to block non-specific binding.
- The Goat serum was then drained off and sections were incubated with primary antibody at room temperature at specified times and dilutions (**Table 2**).
- The slides were then rinsed with PBS Buffer.
- This was followed by incubation with the respective (Monoclonal) DAKO Envision labelled Polymer, HRP (DAKO- USA) (**Table 3**) for 30 minutes at room temperature.
- Sections were rinsed with PBS buffer.
- Chromogenic substrate 3.3 –diaminobenzidine (DAB), (DAKO- USA) was then applied for 5-10 minutes to develop positivity.
- Slides were rinsed in running tap water and counterstained with Mayers haematoxylin for approximately three minutes.

- After rinsing in running tap water, sections were then placed in bluing reagent (Ammoniated water).
- Finally, the slides were then dehydrated, cleared and mounted with Entellan.

Controls:

A positive control and a negative reagent control in which the primary antibody was replaced with PBS was used (**Table 2**) for each IHC run.

3.5 Immunohistochemical protocol (for BRAF V600E)

- Three-micron tissue sections were cut, picked up onto Histobond slides (Marienfeld-Germany) and heat-fixed on a hotplate for 10-15 minutes.
- BRAF stain was run on an automated immunostainer (Ventana Benchmark Ultra, Tucson, AZ) using the manufacturer's instructions.
- Slides were rinsed in running tap water and counterstained with Mayers haematoxylin for approximately 3 minutes.
- After rinsing in running tap water, sections were then placed in bluing reagent (Ammoniated water).
- Finally, the slides were then dehydrated, cleared and mounted with Entellan.

3.6 Scoring of immunohistochemical stains

All 79 cases had four immunohistochemical stains (**Table 2**) performed on them. An Olympus BX43 light microscope was used for scoring immunohistochemical stains. A four-tiered scoring system (0 = negative; 1+ = weak; 2+ = moderate and 3+ = strong) was used for scoring intensity staining.

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The proportion was assessed by determining the percentage of positive staining cells after counting a total of one hundred cells at high power. More than one set of a hundred cells was counted in areas of heterogeneity and the average was calculated as the proportion.

The tumour cell staining for BRAF V600E, CD15, CK-19 and HMBE was recorded using an H-score assessment. The evaluations are recorded as percentages of positively stained tumour cells in each of the intensity categories. Then, the final score should be

taken from the summation of the percentage of cells at each intensity level using the following formula:
Total score (H-score): ∑ (1× [% cells with weak staining] +2 × [% cells with moderate staining] +3× [% cells with strong staining]).
The final score ranged from 0 to 300.

3.7 Statistical analysis

All data were entered into a Microsoft Excel 2016® spread sheet. Statistical analysis was done using SPSS Version 27 (SPSS Inc., Chicago, IL, USA).

Data were reported as mean and standard deviation for continuous variables, and as proportions for categorical variables. One-way ANOVAs compared between-group differences (Follicular adenoma, Follicular thyroid carcinoma and Follicular variant of papillary thyroid carcinoma) for the proportion and H-score variables. Chi-square analyses compared the association between group and Intensity score. For all of the above analyses, post-hoc comparisons were performed to confirm where significant differences lay. A receiver operating curve (ROC) analysis was performed to determine the best antibody to detect a benign vs malignant tumour. All the results were considered statistically significant if the p-value was less than 0.05.

CHAPTER 4

RESULTS

4.1. General

Sixty-three (79.7%) patients were females and only sixteen (20.3%) were males. There was a female bias with a female-to-male ratio of 4:1. The female predominance was maintained among the three neoplasms.

The patients' ages ranged from 13 to 74 years with a mean age of 45 years and a standard deviation of 14.9 years (**Figure 2**). Mean age among the three neoplasms are summarised in **Table 4**.

Of the 79 cases, roughly one-third were of each type of tumour (Follicular adenoma: 33%, n = 26; Follicular thyroid carcinoma: 32%, n = 25; Follicular variant of papillary carcinoma: 35%, n = 28) (**Figure 3**).

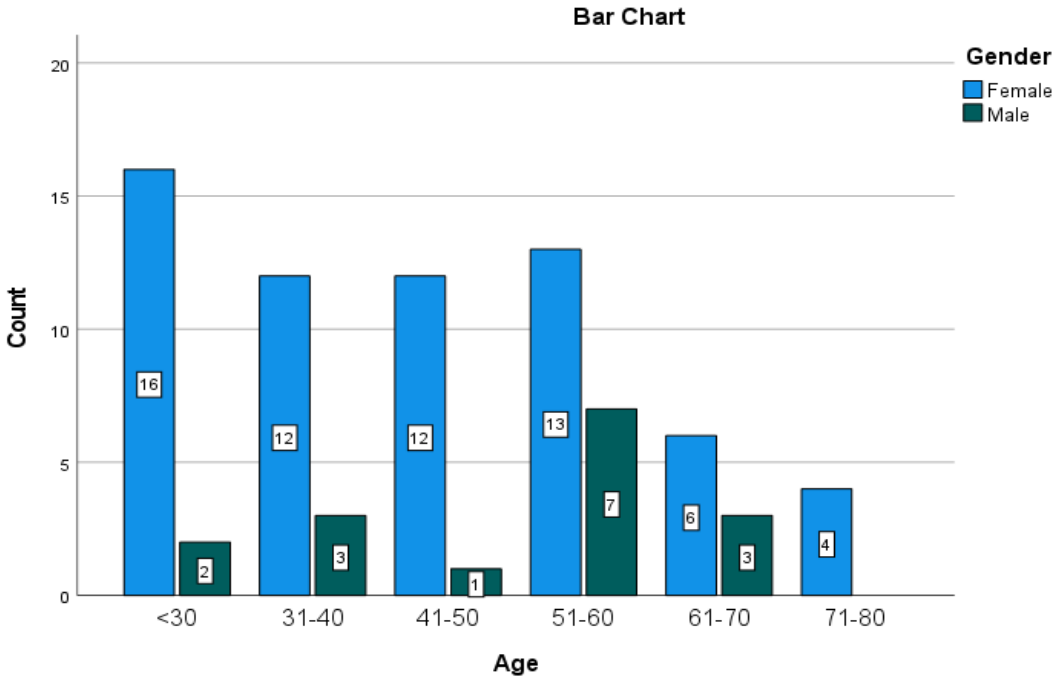

Figure 2: Age distribution of participants by gender

Table 4. Comparison of mean age among the three neoplasms.

Diagnosis	Number	Age (Years)		
		Minimum	Maximum	Mean ± SD
FA	26	16	62	40.9±13
FTC	25	26	76	50.6±13.3
FVPTC	28	13	74	44±16.8
Total	79	13	74	45±14

No statistically significant differences were observed in mean age when comparing between FA, FTC and FVPTC (p=0.240).

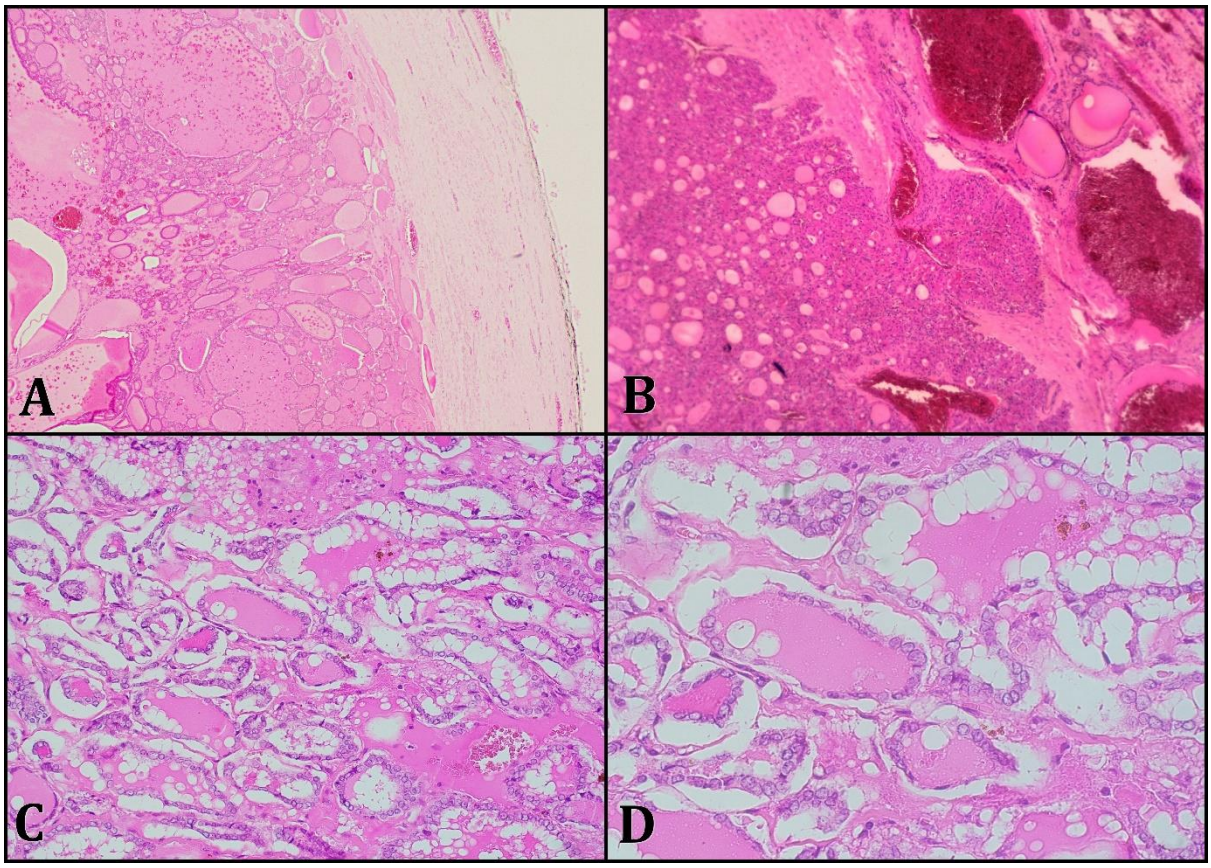

Figure 3: Histology of encapsulated follicular thyroid neoplasms A. follicular adenoma showing a fibrous capsule and variable-sized follicles (Haematoxylin and eosin, × 40). B. Follicular thyroid carcinoma demonstrating neoplastic cells invading through the tumour capsule (Haematoxylin and eosin, × 40). C. Follicular variant of papillary carcinoma (Haematoxylin and eosin, × 200). D. Follicular variant of papillary carcinoma showing nuclear features of PTC-nuclear crowding, clearing, and nuclear grooves (Haematoxylin and eosin, × 400).

4.2. HBME-1 immunohistochemical findings

HBME-1 expression showing varying intensity and proportion was seen in 7 (28%) FA, 15 (65%) FTC and 22 (79%) FVPTC (**Figure 4** and **5**).

There was a significant main effect of group for HBME-1 proportion (**Table 5**). Post-hoc comparison revealed that for HBME-1, the proportion was significantly different between FA and FVPTC (**Table 6).** The proportion was higher in FVPTC. There was no statistically significant difference between FA and FTC (p=0.363) and FTC and FVPTC (p= 0.326).

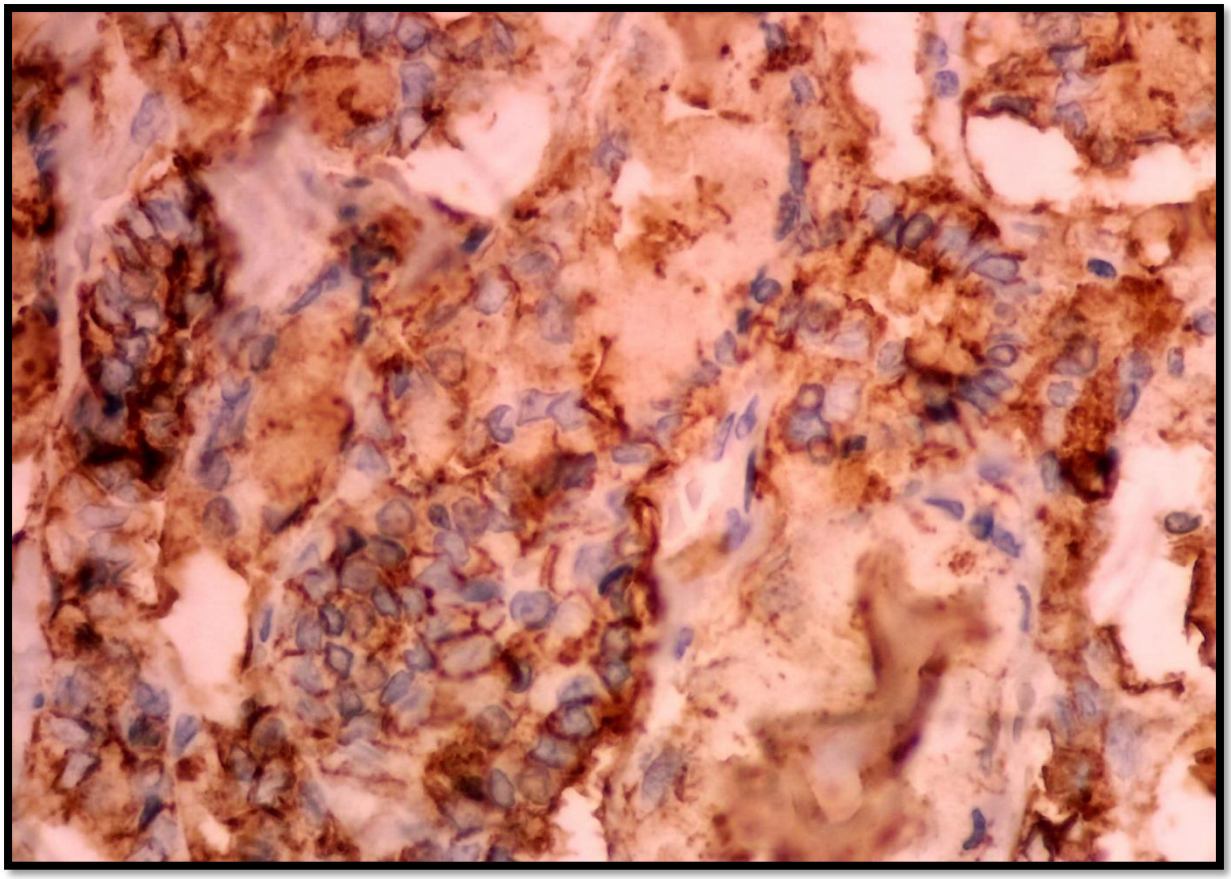

Figure 4: Immunohistochemical expression of HMBE-1 in encapsulated follicular thyroid neoplasms. Follicular variant of papillary carcinoma showing about 100% tumour cells reactive: 3+(x400).

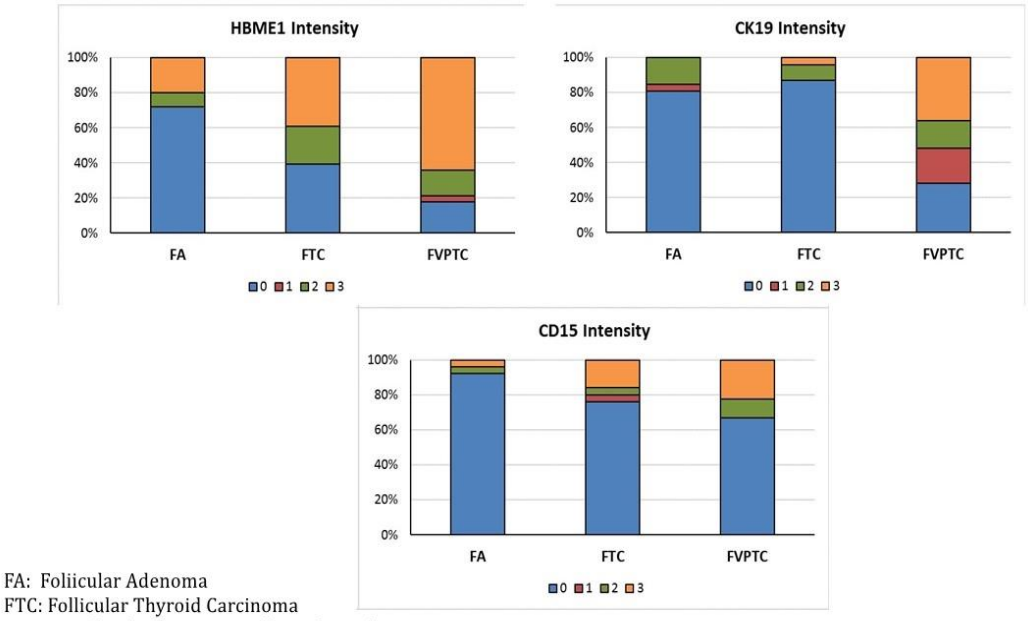

Figure 5: Immunohistochemistry staining intensity in three groups of tumours

Table 5. One-way ANOVA results for proportion

	FA N = 26		**FTC** N = 25		**FVPTC** N = 28		*F*	*p*	η^2
	M	SD	M	SD	M	SD			
HBME1	0.13[a]	0.26	0.27[b]	0.37	0.42	0.33	5.48	.006	0.13
CK-19	0.03	0.09	0.08[b]	0.25	0.32[a]	0.34	10.23	<.001	0.22
BRAF	0.004[a]	0.02	0.05[b]	0.21	0.11[c]	0.30	1.53	.224	0.04
CD15	0.04	0.19	0.04	0.11	0.10[c]	0.25	0.88	.420	0.02

Note. [a]Data based on 25 cases. [b]Data based on 23 cases. [c]Data based on 27 cases.

Table 6. Post-hoc comparisons (Bonferroni corrected) for proportion

	FA vs FTC	**FA vs FVPTC**	**FTC vs FVPTC**
HBME1	.363	.004	.326
CK-19	1.00	<.001	.003

Chi-square analyses detected a significant association between group and HBME-1 intensity (**Table 7**). Post-hoc comparison revealed that FA was significantly more likely

to have negative HBME-1 staining compared to FTC and FVPTC (**Figure 5** and **Table 5).** FVPTC were significantly more likely to have an HBME-1 intensity of three compared to FA. There was no significant difference in staining intensity between FTC and FVPTC.

Table 7. Chi-square analyses for intensity

	p	*V*
HBME1	.001	0.35
CK-19	<.001	0.45
BRAF	.613	0.15
CD15	.180	0.23

Table 8 Post-hoc comparisons for intensity

	FA vs FTC	**FA vs FVPTC**	**FTC vs FVPTC**
HBME-1			
0	.045	<.001	.168
1	-	-	-
2	.348	.774	.744
3	.255	.003	.131
CK-19			
0	.843	<.001	<.001
1	1.00	.175	.073
2	.782	1.00	.743
3	.951	.003	.019

There was a significant main effect of group for HBME-1 H-score (**Table 9**). Post-hoc comparisons (**Table 10**) revealed that for HBME-1, the H-score was significantly different between FA and FVPTC (p=0.010), with a higher H-score for FVPTC. There was no statistically significant difference between FA and FTC (p=0.343) and FTC and FVPTC (p= 0.554).

Table 9. One-way ANOVA results for H-score

	FA N = 26		FTC N = 25		FVPTC N = 28		*F*	*p*	η^2
	M	SD	M	SD	M	SD			
HBME1	36.2[a]	75.52	79.59[b]	110.98	115	93.56	4.65	.013	0.11
CK-19	5.08	17.73	19.26[b]	65.69	75.44[a]	91.81	8.10	<.001	0.19
BRAF	0.36[a]	1.80	4.78[b]	20.84	14.41[c]	44.24	1.60	.209	0.04
CD15	11.00	55.89	11.92	32.56	29.19[c]	74.66	0.83	.441	0.02

Note. [a]Data based on 25 cases. [b]Data based on 23 cases. [c]Data based on 27 cases.

Table 10. Post-hoc comparisons (Bonferroni corrected) for H-score

	FA vs FTC	FA vs FVPTC	FTC vs FVPTC
HBME1	.343	.010	.554
CK-19	1.00	<.001	.012

Receiver operating characteristic (ROC) curve analysis

ROC curve analyses showed that HBME-1 was good at predicting benign vs malignant tumours (**Table 11**). For HMBE-1 the area under the curve was significantly better than chance (AUC = 0.724; *p* = .002). Coordinates on the curve (**Figure 6**) indicated that the best H-score cut-off value should be 0.75 to optimise sensitivity and specificity. At this cut-off value, sensitivity is 0.723 and specificity 0.720.

Table 11. ROC curve analyses

	Positive	Negative	Missing	Area	Std. Error	*p*	Asymptotic 95% Confidence Interval	
							Lower Bound	Upper Bound
HBME-1	51	25	3	.724	.063	.002	.601	.847
CK-19	48	26	5	.649	.063	.036	.525	.772
BRAF	50	25	4	.551	.069	.472	.417	.686
CD15	51	26	2	.609	.065	.118	.481	.737

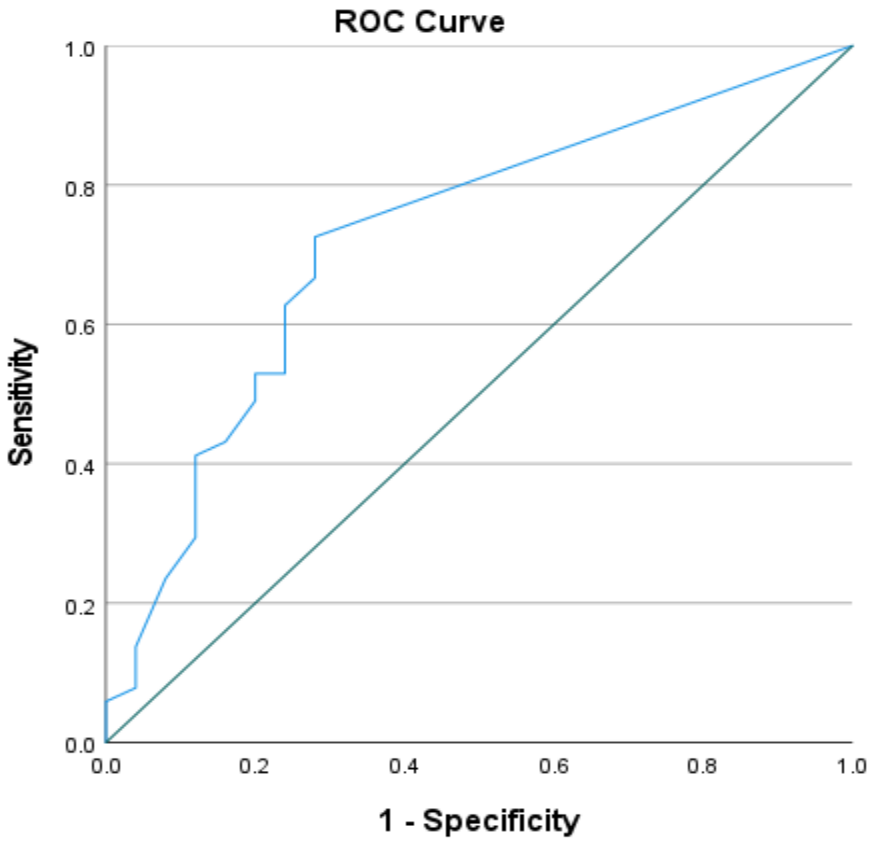

Figure 6: HBME-1 receiver operating characteristic (ROC) curve

4.3. CK-19 immunohistochemical findings

CK-19 expression showing varying intensity and proportion was seen in 5 (19%) FAs, 3 (13%) FTC and 18 (72%) FVPTC (**Figure 5** and **7**).

There was a significant main effect of group for CK-19 proportion (**Table 5**). Post-hoc comparison revealed that for CK-19, the proportion was significantly different between FA and FVPTC, and between FTC and FVPTC (**Table 6**). In all cases, the proportion was higher in FVPTC.

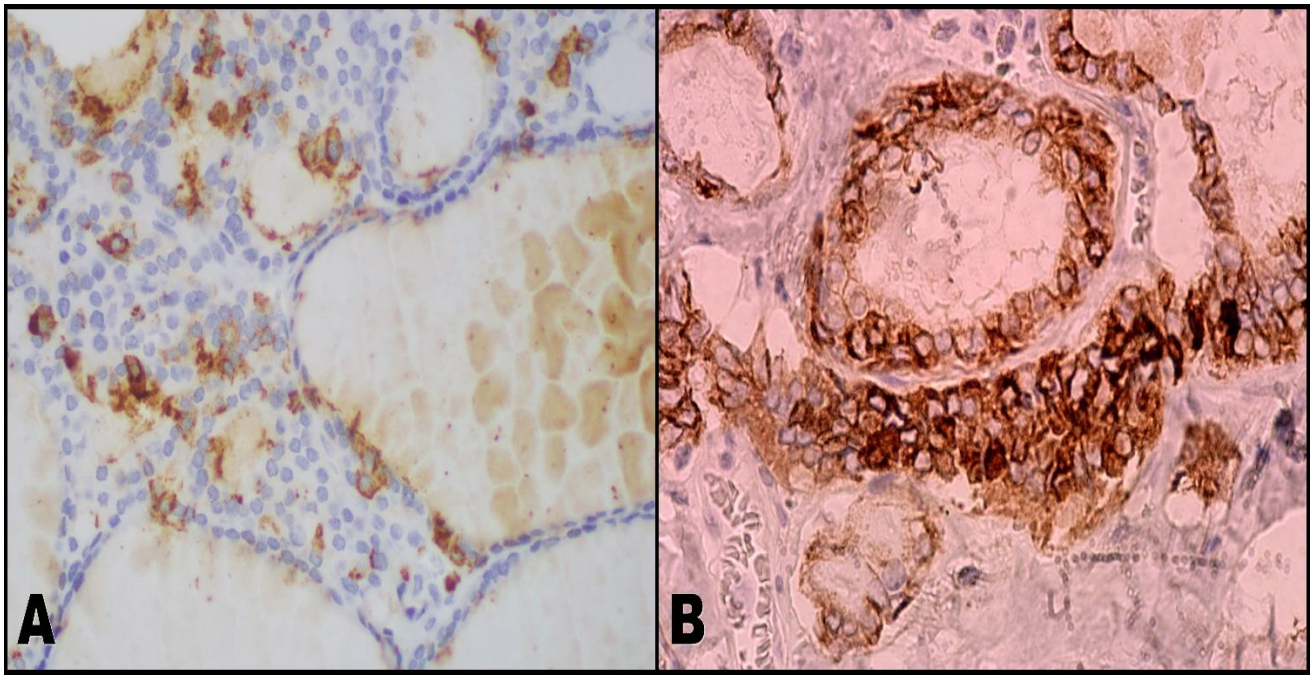

Figure 7: Immunohistochemical expression of CK-19 in encapsulated follicular thyroid neoplasms. A. Follicular adenoma showing about 20% of lesional cells reactive: 3+ (x400). B. Follicular variant of papillary carcinoma showing about 90% of lesional cells reactive: 3+ (x400).

Chi-square analyses detected a significant association between group and CK-19 intensity (**Table 7**). Post-hoc comparison revealed that FA and FTC were significantly more likely to have negative CK-19 staining when compared to FVPTC (**Figure 5** and **Table 8**). FVPTC were significantly more likely to have a CK-19 intensity of three compared to FA and FTC (**Table 8**).

There was a significant main effect of group for CK-19 H-score (**Table 9**). Post-hoc comparisons (**Table 10)** revealed that for CK-19, the H-score was significantly different between FA and FVPTC, with a higher H-score in FVPTC. In addition, post-hoc comparisons revealed that for CK-19, the H-score was significantly different between FTC and FVPTC, with a higher H-score in FVPTC.

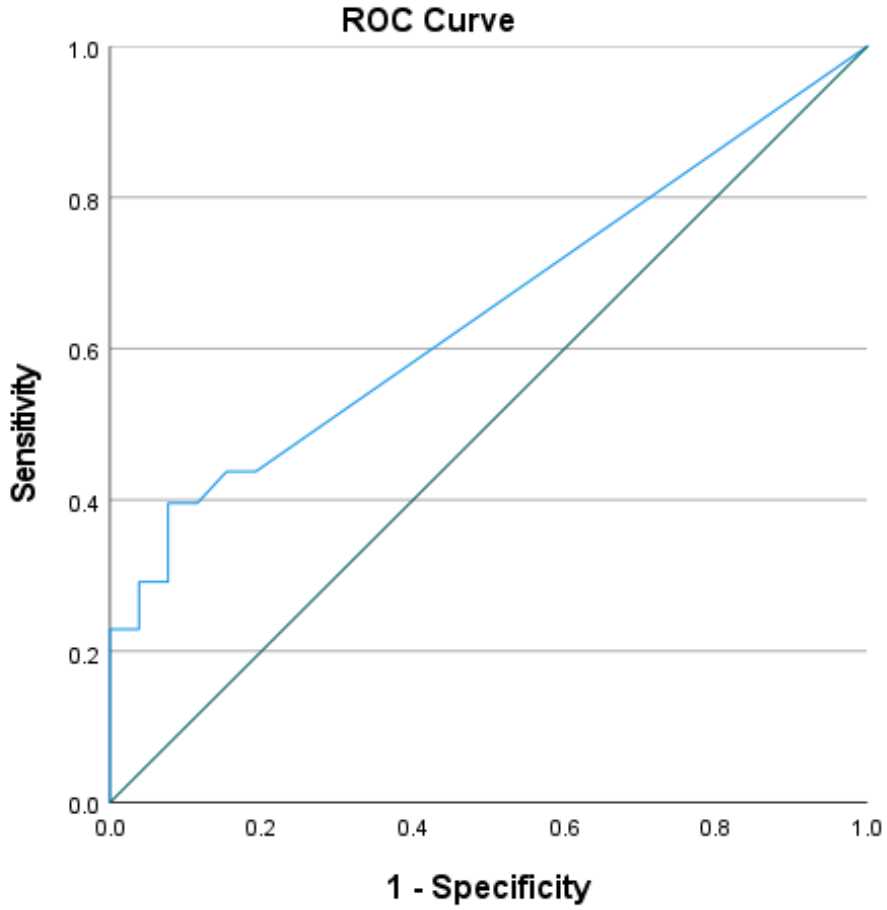

Figure 8: CK-19 receiver operating characteristic (ROC) Curve

ROC curve analysis

ROC curve analyses showed that CK-19 was good at predicting benign vs malignant tumours (**Table 11**). For CK-19 the area under the curve was significantly better than chance (AUC = 0.649; *p* = .036). Coordinates on the curve (**Figure 8**) indicated that the best H-score cut-off value should be 150 to optimise sensitivity and specificity. At this cut-off value, sensitivity is 43.8% and specificity 80.8%.

4.4. BRAF immunohistochemical findings

BRAF expression showing varying intensities and proportions was seen in 3 (11%) of FVPTC. FA and FTC cases were all negative.

There was no significant main effect of group for BRAF V600E proportion (One-way ANOVA: p-value =0.224) or intensity (Chi-square, p=0.613) (**Table 5**). There was also no significant main effect of group for BRAF V600E H-score (One-way ANOVA; p=0.209)

(**Table 9**). ROC curve analyses showed that BRAF V600E was not suitable for predicting benign vs malignant tumours (p-value =0.472) (**Table 11**). BRAF V600E staining was also not suitable for distinguishing FTC from FVPTC.

4.5. CD15 immunohistochemical findings

CD15 expression showing varying intensity and proportion was seen in 2 (8%) FAs, 6 (24%) FTC and 9 (33%) FVPTC (**Figure 5** and **9**).

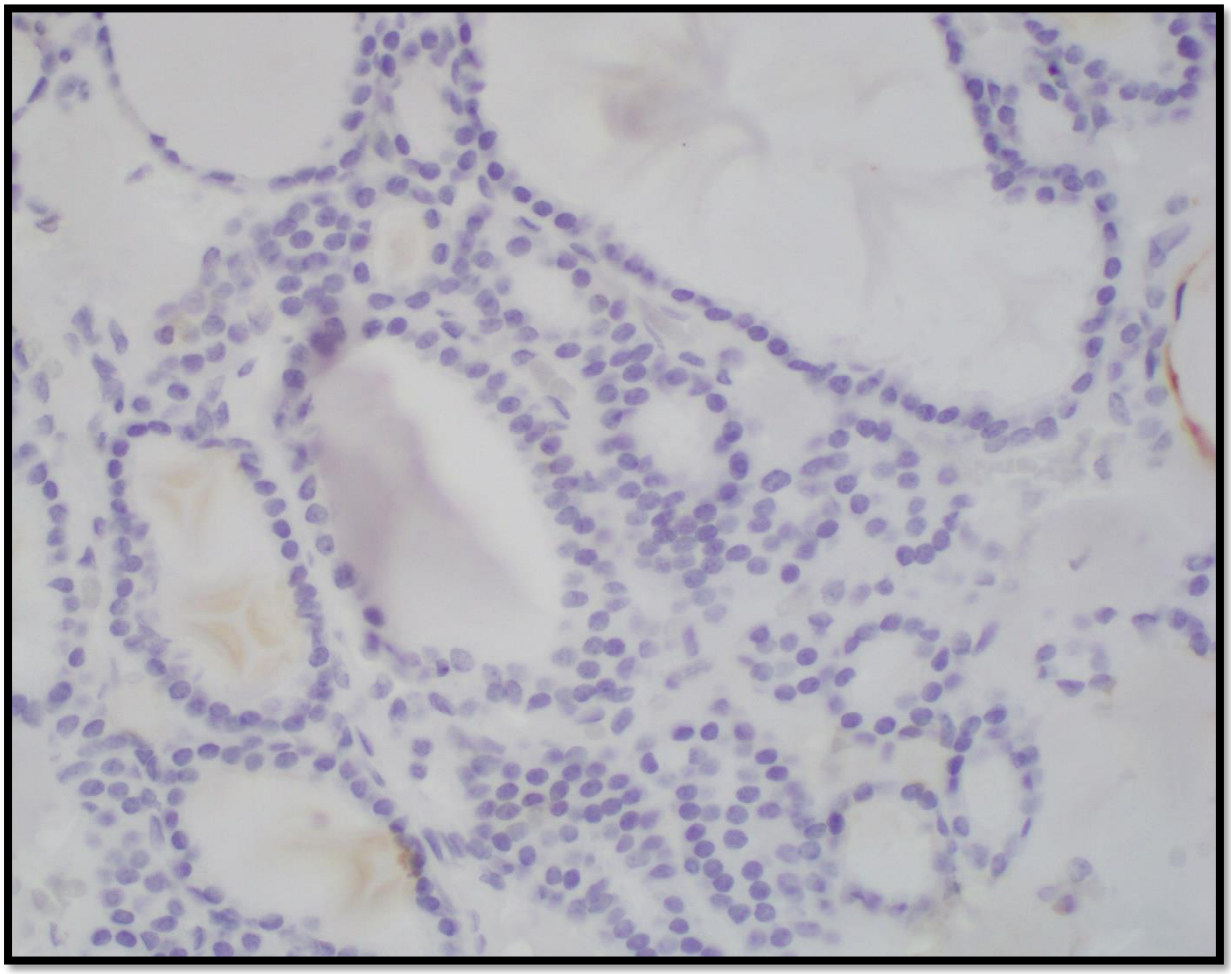

Figure 9: Immunohistochemical expression of CD15 in encapsulated follicular thyroid neoplasms. Follicular thyroid adenoma showing no reactive cells: 0 (x400).

There was no significant main effect of group for CD15 proportion (One-way ANOVA: p-value =0.420) or intensity (Chi-square, p=0.18013) (**Table 5**). There was also no significant main effect of group for CD15 H-score (One-way ANOVA; p=0.441 (**Table 9**). ROC Analyses showed that CD15 was not suitable for predicting benign vs malignant

tumours (p-value =0.118) (**Table 11**). CD15 staining was also not suitable for distinguishing FTC from FVPTC.

4.6. Sensitivity, specificity, positive and negative predictive value

Using the H-score cut-offs derived from the ROC curves, a lesion was considered positive when the H-score was > 225 for HBME-1 and > 150 for CK-19. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each of the markers and for different combinations of these markers in the benign vs. malignant, benign vs. malignant (FTC only) and benign vs. malignant (FVPTC only) and malignant (FTC) vs. malignant (FVPTC) groups.
HBME-1 was the best marker at differentiating benign from malignant tumours (**Table 12-15**).

CK-19 was the only marker for which sensitivity and specificity for distinguishing FTC from FVPTC were calculated as it had an H-score, staining intensity and proportion that were statistically significant between the two groups.

In terms of marker combinations, HBME-1 + CK-19, HBME-1 + BRAF, and HBME-1 + CD15 were all equivalently good combinations at differentiating benign from malignant tumours but did not significantly increase the sensitivity and specificity of these markers (**Table 16-18**).

Table 12. Sensitivity, specificity, PPV and NPV of benign vs. all malignant thyroid neoplasms

Markers	Sensitivity	Specificity	PPV	NPV
HBME1	74%	72%	84.1%	58.1%
CK-19	43.8%	80.8%	80.8%	43.8%
BRAF	14%	96%	87.5%	35.8%
CD15	29.4%	92.3%	88.2%	40%

*Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

Table 13. Sensitivity, specificity, PPV and NPV of benign vs. FTC

Markers	Sensitivity	Specificity	PPV	NPV
HBME1	60.9%	42.3%	31.8%	71%
CK-19	13%	54.69%	11.5%	58.3%
BRAF	13%	90.3%	37.5%	70.1%
CD15	25%	79.2%	35.3%	70%

*Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

Table 14. Sensitivity, specificity, PPV and NPV of benign vs. FVPTC

Markers	Sensitivity	Specificity	PPV	NPV
HBME1	85.2%	56.3%	52.3%	87.1%
CK-19	72%	83.7%	69.2%	85.4%
BRAF	14.8%	91.7%	50%	65.7%
CD15	33.3%	84%	52.9%	70%

* Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

Table 15. Sensitivity, specificity, PPV and NPV of FTC vs. FVPTC for CK-19

Marker	Sensitivity	Specificity	PPV	NPV
CK-19	72%	87%	85.7%	74%

Table 16. Comparison of sensitivity, specificity, PPV and NPV of combined markers expression in benign vs. all malignant thyroid lesions.

Marker Combination	Sensitivity	Specificity	PPV	NPV
HBME-1 + CK-19	80.9%	69%	82.6%	65.3%
HBME-1 + BRAF	76%	68%	82.6%	58.6%
HBME-1 + CD15	74%	72%	84.1%	58.1%
CK-19 + BRAF	47.9%	76%	79.3%	43.2%
CK-19 + CD15	64.6%	76.9%	83.8%	54.1%
BRAF + CD15	38%	88%	86.4%	41.5%

* Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

Table 17. Comparison of sensitivity, specificity, PPV and NPV of combined markers expression in FA vs. FTC

Marker Combination	Sensitivity	Specificity	PPV	NPV
HBME-1 + CK-19	68.2%	38%	32.6%	73.1%
HBME-1 + BRAF	69.6%	42.3%	34.8%	75.9%
HBME-1 + CD15	65.2%	44.2%	34.1%	74.2%
CK-19 + BRAF	22.7%	52.9%	17.2%	61.4%
CK-19 + CD15	34.8%	43.1%	21.6%	59.5%
BRAF + CD15	34.8%	73.1%	36.4%	71.7%

* Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

Table 18. Comparison of sensitivity, specificity, PPV and NPV of combined markers expression in FA vs. FVPTC

Marker Combination	Sensitivity	Specificity	PPV	NPV
HBME-1 + CK-19	92%	51.1%	50%	92.3%
HBME-1 + BRAF	81.4%	50%	47.8%	82.8%
HBME-1 + CD15	81.4%	54.2%	50%	83.9%
CK-19 + BRAF	72%	77.1%	62.1%	84.1%
CK-19 + CD15	92%	71.4%	62.2%	94.6%
BRAF + CD15	40.7%	77.1%	50%	69.8%

* Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

CHAPTER 5

DISCUSSION

Thyroid carcinoma accounts for the largest number of malignant neoplasms that occur in the endocrine system and the number of new cases is on the rise (8). This apparent increase in the incidence of thyroid carcinomas has been attributed to the increased use of radiological modalities and easier diagnostic screening using cytology.

In most cases of thyroid carcinomas, a definite diagnosis can be reached based on morphological assessment. Diagnostic challenges may be encountered with some encapsulated follicular-patterned neoplasms of the thyroid as the presence of nuclear features may be equivocal to render a diagnosis of PTC, or when the presence of vascular or capsular invasion to render a diagnosis of follicular carcinoma is equivocal. The assessment of nuclear features is highly subjective, and the threshold for rendering a diagnosis of PTC varies greatly among pathologists, resulting in poor inter-observer concordance.

The World Health Organisation currently does not recommend the use of immunohistochemistry for differentiating encapsulated follicular patterned neoplasms (1). The use of immunohistochemical markers has been proven in many studies to aid in distinguishing follicular thyroid neoplasms and papillary thyroid carcinoma (51,80). A lot of research to identify possible immunohistochemical markers which may be used for distinguishing these entities has been undertaken. Liu et al (54) analysed various studies evaluating the role of IHC in diagnosing thyroid lesions and concluded that no single antibody is sufficient to differentiate between benign and malignant thyroid lesions. Some of the immunohistochemical markers suggested in the review to assist in the diagnosis of PTC include TROP2, HBME-1, CK-19 and Galectin-3. Other markers which have been identified as possible markers include CD15 and BRAF.

This study was thus done to assess the possibility of using HMBE-1, CK-19, CD15 and BRAF to distinguish various thyroid neoplasms. We examined the expression of these markers in follicular adenoma, follicular thyroid carcinoma and follicular variant of papillary thyroid carcinoma.

Seventy-nine cases of follicular variant of papillary thyroid carcinoma, follicular adenoma and follicular carcinoma diagnosed between January 2004 and December 2014 in the Division of Anatomical Pathology, Groote Schuur Hospital, were analysed for HMBE-1, CK-19, CD15 and BRAF immunomarker expression.

The wide age distribution and female preponderance observed in the current study is consistent with the literature (1).

HBME-1, a biomarker of mesothelial cells has been demonstrated to have significant expression in malignant thyroid carcinomas (53).

Our findings of HBME-1 expression showing high expression in malignant neoplasms compared to benign neoplasms (28% FAs, 63% FTC and 79% FVPTC) mirrors findings in other studies (81). Cheung et al (82) also showed a higher HMBE-1 positivity in cPTC and FVPTC with no expression in FA. FVPTC has a significantly higher expression of HBME-1 than FA or FTC. Other studies have also shown FTC to have a higher expression of HBME-1 than in FA (57). Our findings of high expression of HBME-1 in FVPTC confirm previous studies that have found this antibody to be diagnostically useful (67,80,81).

Miettinen et al (81) and Cheung et al (82) have reported low expression of HMBE-1 in FA and higher expression in cPTC and FVPTC. Our study also showed similar findings with FVPTC significantly showing higher H-scores when compared to FA. FVPTC in the current study also showed that it was statistically more likely to have an HBME-1 intensity of three compared to FA. FA was also significantly more likely to have a negative HBME-1 staining compared to FTC and FVPTC.

HBME-1 has been reported as having an overall specificity and sensitivity for thyroid malignancy of 82.1% and 78.8% respectively. Studies have also shown HBME-1 to have a sensitivity of 87.3% for cPTC, and 65.2% for follicular thyroid carcinomas (50, 53, 54, 55,82). Other studies have shown higher sensitivity and specificity values for HBME-1 for FVPTC of 92% and 89% respectively (67). On the other hand, other studies have shown low sensitivity of 45% for HBME-1 for FVPTC (53). Our study showed HMBE-1

to have sensitivity and specificity values of 74% and 72% respectively for distinguishing benign from malignant tumours (FA vs. FTC/FVPTC). HBME-1 in the current study was noted to have sensitivity and specificity values of 85.2% and 56.3% respectively for distinguishing FA from FVPTC which are similar to findings in other studies (53, 82).

Cytokeratin 19 (CK-19) is a member of type 1 acidic subfamily of intermediate filaments. Normal thyroid follicular cells do not produce CK-19 and its upregulation is associated with malignant transformation (68).

Liu et al (54) showed that CK-19 is more frequently expressed in PTC when compared to benign lesions. Similarly, the current study showed 72% CK-19 expression in FVPTC in comparison to 19% (FA) and 13% (FTC).

Bose et al (71) and Sahoo et al (72) have shown that focal CK-19 staining may be found in benign diseases such as FA, but diffuse and strong positivity is characteristic of PTC, which can be used in the diagnosis of PTC in lesions of equivocal morphological appearances. Rorive et al (84) were able to show a similar trend by demonstrating low expression of CK-19 in FA and higher expression in encapsulated FVPTC. Studies in cytological specimens have also reported similar findings (85, 86). Therefore, the proportion and intensity of the expression of these stains are also of considerable value in the differentiation of tumours. This is consistent with observations in the current series where FVPTC was significantly more likely to have higher H-scores when compared to FA. FVPTC was also more likely to show strong and diffuse staining with CK-19 compared to FA. FTC was also significantly more likely to have a lower H-score and less intensity of CK-19 compared to FVPTC.

Nasser et al (86) and Khuran et al (85) have reported on sensitivity and specificity of CK-19 being as high as 93% and 97% respectively. The current study showed CK-19 to have sensitivity and specificity of 43.8% and 80.8% for distinguishing benign from malignant tumours (FA vs. FTC/FVPTC). Dunderovic et al. (87) used similar antibody to the one used in the current study and reported on sensitivity and specificity of CK-19 to discriminate benign from malignant thyroid lesions as 75.41% and 70.89% respectively.

Although we reported lower sensitivity compared to Dunderovic et al. (87) the current study found close specificity and PPV. These differences might be due to different IHC methods used in the different studies. The study by Dunderovic et al. (87) used a similar antibody but used different antigen retrieval method, shorter incubation period and different visualisation kit.

CK-19 in the current study was noted to have sensitivity and specificity values of 72% and 83.7% respectively for distinguishing FA from FVPTC. The results also showed that CK-19 can be used to distinguish FTC from FVPTC with a sensitivity and specificity of 72% and 87% respectively. These findings are congruent with findings by Dunderovic et al. (87).

Palo et al (88) reported on HBME-1 and CK-19 being the most sensitive markers of in differentiating FVPTC from FA and FTC. Their report also indicated that combining HMBE-1 and CK-19 increased sensitivity for differentiating benign thyroid lesions from malignant lesions {75% (CK-19) and 86% (HBME1) vs 94% (CK-19+ HMBE-1}. Our cohort found no significant increase in the sensitivity when combining the two antibodies vs single use of either antibody. Findings similar to ours have been reported by Saleh et al (89) who also did not find any significant increase in sensitivity or specificity with the combined use of CK-19 and HBME-1.

Mutation of the *BRAF* gene is detected in approximately 20% to 80% of sporadic cases of PTC. Conventional PTC has a higher prevalence of *BRAF* gene mutation than FVPTC (75). BRAF mutation is not found in cases which show only follicular growth pattern (74,75). The *BRAF* V600E mutation is generally negative in normal thyroid tissue, FA and FTC (54). A case report with a BRAF (K601E) mutation in FTC has been reported (79). This however would not be detected by immunohistochemistry when using the mutation-specific immunohistochemistry like anti-BRAF V600E (VE1 clone). Johnson et al (90) reported BRAF V600E expression in a series that showed 0% for NIFTP, 14.6% for FVPTC and 54.5% for cPTC. Their study did not investigate BRAF expression in FA and FTC.

Our current study showed BRAF expression in 3 (11%) FVPTC. None of the FA or FTC cases showed positive staining with BRAF. This staining profile is consistent with findings in the literature (75, 78, 90). The mutation-specific monoclonal anti-BRAF V600E (VE1 clone) antibody targets the mutated amino acid sequence from amino acids 596 to 606 (78). FA and FTC which do not harbour this mutation are thus expected to be negative.

CD15 has been proposed as an immunomarker for thyroid cancer stem cells (65). Miettinen et al (81) have reported strong immunoreactivity for CD15 in PTC and, to a lesser degree, in FTC, contrasting with the absence of immunoreactivity for such antibodies in adult thyroid parenchyma and the absence or only focal positivity in benign thyroid lesions.

Ohta et al (67) reported the sensitivity and specificity of CD15 for distinguishing benign thyroid lesions from thyroid malignancies including FVPTC as 23% and 100%, respectively. Although CD15 is apparently inferior in terms of sensitivity for FVPTC, its excellent specificity will support the definitive diagnosis of thyroid malignancies (67).

Our current study showed CD15 expression in 8% FAs, 24% FTC and 33% FVPTC. There was no statistically significant difference in H-score, intensity and proportion of CD15 between the different neoplasm groups (malignant vs. benign). The most probable reason as to why our study could not reproduce the results by Ohta et al (67) and Miettinen et al (81) could be due to the differences in antibody clones used. The current study used the MMA monoclonal antibody whilst the other previous studies used clone Carb-3 and 3CD-1.

5.1. Limitations of study

The study had some limitations. This study had a relatively small sample size and was conducted at a single-centre in Cape Town, South Africa. The findings in this study thus need further validation. Due to financial constraints, other immunohistochemical markers such as Galectin-3, CD117 and CITED1 could not be assessed.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Encapsulated thyroid lesions with follicular architecture may pose diagnostic challenges in differentiating follicular variant of papillary thyroid carcinoma from follicular adenomas or follicular thyroid carcinomas. This study explored the possibility of using immunohistochemical markers in differentiating these entities.

The study showed that HMBE-1 and CK-19 are sensitive markers that may be used to predict benign vs malignant thyroid tumours. HBME-1 and CK-19 staining may have a role in distinguishing follicular adenoma from follicular variant of papillary thyroid carcinoma. In addition, CK-19 staining may have a role in distinguishing follicular thyroid carcinoma from follicular variant of papillary thyroid carcinoma. Combining HMBE-1 and CK-19 did not show any significant increase in sensitivity and specificity for distinguishing encapsulated thyroid lesions with follicular architecture.

There was no statistically significant differences in staining for CD15 between follicular adenoma, follicular thyroid carcinoma and follicular variant of papillary thyroid carcinoma. Although BRAF immunohistochemical stain was only positive in FVPTC cases, only three cases were positive.

Although from our study, it is demonstrated that HMBE-1 and CK-19 may be used to differentiate encapsulated follicular-patterned thyroid tumours, further research is recommended to validate the diagnostic utility of these immunomarkers.

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APPENDIX 1: Ethics Clearance

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

16 November 2015

HREC REF: 770/2015

Prof D Govender
Anatomical Pathology
Room 4.10, Level 4
Falmouth Building
Clinical Laboratory Sciences

Dear Prof Govender

PROJECT TITLE: DIFFERENTIATING FOLLICULAR THYROID CARCINOMA FROM THE FOLLICULAR VARIANT OF PAPILLARY THYROID CARCINOMA (MMed-candidate-Dr T Rikhotso)

Thank you for your response to the Faculty of Health Sciences Human Research Ethics Committee dated 6 November 2015.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th November 2016.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

We acknowledge that the MMed student, Dr Tshikani Norman Rikhotso will also be involved in this study.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical

HREC 770/2015

APPENDIX 2: Data Collected

ID	Diagnosis	Age	Gender	HBME1-I*	HBME1-P*	HBME1-H	CK19-P*	CK19-I*	CK19-H*	BRAF-I*	BRAF-P*	BRAF-H*	CD15_I*	CD15-P*	CD15-H*
1	FTC	37	F	3	0,80	240,0	0,00	0	0	0	0,00	0	2	0,11	22
10	FVPTC	37	F	3	0,70	210,0	0,00	0	0	0	0,00	0	2	0,05	10
11	FA	40	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
12	FTC	37	F	3	0,80	240,0	0,00	0	0	0	0,00	0	3	0,20	60
13	FTC	28	F	2	0,09	18,0	.	.	.	0	0,00	0	1	0,09	9
14	FVPTC	44	F	3	0,95	285,0	.	.	.	0	0,00	0	0	0,00	0
15	FVPTC	72	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
16	FA	16	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
17	FA	25	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
18	FA	25	F	3	0,60	180,0	0,00	0	0	0	0,00	0	0	0,00	0
19	FTC	26	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
2	FA	40	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
20	FTC	28	F	2	0,13	26,0	0,00	0	0	0	0,00	0	0	0,00	0
21	FTC	40	F	3	0,20	60,0	0,00	0	0	0	0,00	0	0	0,00	0
22	FA	41	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
23	FA	41	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
24	FVPTC	27	M	3	0,50	150,0	1,00	1	100	0	0,00	0	0	0,00	0
25	FTC	48	F	0	0,00	0,0	0,00	0	0	0	0,00	0	3	0,50	150
26	FA	51	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
27	FTC	52	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
28	FTC	56	F	3	0,01	1,5	0,00	0	0	0	0,00	0	0	0,00	0
29	FVPTC	41	F	3	0,30	90,0	0,00	0	0	0	0,00	0	2	0,05	10
3	FA	37	M	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
30	FVPTC	43	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
31	FTC	51	M	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
32	FA	51	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
33	FA	55	M	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
34	FA	57	M	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
35	FTC	57	F	2	0,10	20,0	0,00	0	0	0	0,00	0	3	0,05	15
36	FTC	58	F	3	0,70	210,0	0,00	0	0	0	0,00	0	0	0,00	0
37	FA	57	M	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
38	FTC	57	M	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
39	FTC	57	F	2	0,05	10,0	0,00	0	0	0	0,00	0	3	0,10	30
4	FA	35	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
40	FTC	58	F	3	0,70	210,0	0,00	0	0	0	0,00	0	0	0,00	0
41	FTC	67	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
42	FA	59	F	3	0,15	45,0	0,00	0	0	0	0,00	0	0	0,00	0
43	FTC	65	M	3	1,00	300,0	0,00	0	0	0	0,00	0	0	0,00	0
44	FA	60	M	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
45	FTC	65	M	3	0,60	180,0	0,00	0	0	0	0,00	0	0	0,00	0
46	FTC	61	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
47	FVPTC	64	F	3	0,60	180,0	0,00	0	0	0	0,00	0	3	0,85	255
48	FVPTC	64	F	1	0,90	90,0	0,00	0	0	0	0,00	0	3	1,00	300
49	FTC	61	F	3	0,60	180,0	0,00	0	0	0	0,00	0	0	0,00	0
5	FA	33	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
50	FVPTC	62	M	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
51	FA	62	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
52	FA	27	F	2	0,05	10,0	0,40	2	80	0	0,00	0	0	0,00	0
53	FTC	71	F	3	0,90	270,0	0,00	0	0	0	0,00	0	0	0,00	0
54	FA	40	F	3	0,90	270,0	0,01	2	2	0	0,00	0	0	0,00	0
55	FVPTC	39	M	3	0,30	90,0	0,02	1	2	0	0,00	0	0	0,00	0
56	FVPTC	72	F	0	0,00	0,0	0,05	3	15	0	0,00	0	0	0,00	0
57	FA	31	M	0	0,00	0,0	0,23	2	46	0	0,00	0	0	0,00	0
58	FVPTC	13	F	3	0,02	6,0	0,02	2	4	0	0,00	0	0	0,00	0
59	FVPTC	74	F	3	0,55	165,0	0,00	0	0	0	0,00	0	3	0,15	45
6	FA	33	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0
60	FVPTC	24	F	3	0,70	210,0	0,01	2	2	0	0,00	0	0	0,00	0
61	FA	56	F	2	0,50	100,0	0,01	2	1	0	0,00	0	0	0,00	0
62	FTC	60	F	0	0,00	0,0	0,02	2	4	0	0,00	0	0	0,00	0
63	FA	30	F	3	0,30	90,0	0,03	1	3	0	0,00	0	2	0,01	1
64	FVPTC	35	F	0	0,00	0,0	0,10	2	20	0	0,00	0	0	0,00	0
65	FVPTC	21	F	3	0,20	60,0	0,30	3	90	1	0,09	9	0	0,00	0
66	FVPTC	22	M	2	0,50	100,0	0,30	2	60	0	0,00	0	0	0,00	0
67	FVPTC	56	F	2	0,05	10,0	0,35	1	35	0	0,00	0	0	0,00	0
68	FVPTC	30	F	3	0,70	210,0	0,53	1	53	0	0,00	0	0	0,00	0
69	FVPTC	30	F	3	0,70	210,0	0,50	1	50	0	0,00	0	2	0,09	18
7	FA	32	F	3	0,70	210,0	0,00	0	0	0	0,00	0	3	0,95	285
70	FVPTC	52	F	3	0,55	165,0	0,55	3	165	1	0,80	80	0	0,00	0
71	FVPTC	47	F	2	0,02	4,0	0,55	3	165	0	0,00	0	3	0,15	45
72	FTC	52	F	3	0,95	285,0	0,93	3	279	0	0,00	0	0	0,00	0
73	FVPTC	25	F	3	0,05	15,0	0,60	3	180	0	0,00	0	0	0,00	0
74	FVPTC	50	M	3	0,85	255,0	0,90	3	270	0	0,00	0	0	0,00	0
75	FVPTC	56	M	3	0,55	165,0	0,60	3	180	1	1,00	100	3	0,05	15
76	FVPTC	43	F	2	0,65	130,0	0,90	3	270	2	1,00	200	3	0,30	90
77	FVPTC	47	F	3	0,55	165,0	0,75	3	225	0	0,00	0	0	0,00	0
78	FVPTC	43	F	3	0,85	255,0	0,80	2	160	1	1,00	1	0	0,00	0
79	FTC	43	F	0	0,00	0,0	0,80	2	160	1	1,00	100	0	0,00	0
8	FTC	30	F	2	0,15	30,0	0,00	0	0	0	0,00	0	0	0,00	0
9	FA	30	F	0	0,00	0,0	0,00	0	0	0	0,00	0	0	0,00	0

I* - Intensitity P* - Proportion H *- H-Score