UNIVERSITY OF KWAZULU-NATAL
COLLEGE OF HEALTH SCIENCES
SCHOOL OF LABORATORY MEDICINE AND MEDICAL SCIENCES

A MORPHOLOGIC AND IMMUNOHISTOCHEMICAL APPRAISAL OF INVASIVE BREAST CARCINOMAS WITH NEUROENDOCRINE DIFFERENTIATION

by

NIMALLEN NAICKER
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DECLARATION

I, Dr Nimallen Naicker, declare as follows:

1. The work described in this dissertation has not been submitted to UKZN or any other institution for the purposes of an academic qualification, whether by myself or any other party.
2. The research reported in this dissertation, except where otherwise indicated, is my original research.
3. The dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. Where other sources have been quoted:
    - Their words have been re-written, but the general information attributed to them has been referenced.
    - Where their exact words have been used, their writing has been placed inside quotation marks, and referenced.
5. My contribution to the project is that of the principal investigator. I have been involved in every aspect of the project including slide review, data collection and tabulation, critical review of the results and synthesis of the discussion.
6. The contribution of others to the project are as follows:
    - Dr Gamalenkosi Nhlonzi - Supervisor
    - Dr Absalom Mwazha - Co-supervisor

This work is dedicated to my wife Cherise and my sons Caiden and Aaron.

ACKNOWLEDGMENTS

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I owe a deep sense of gratitude to my parents, Vennie Naicker and Meggie Naicker, who supported me through medical school and to whom I am indebted for the many sacrifices made for my benefit.

I would also like to thank my father-in-law, Gordon Timothy, who provided invaluable support and assistance throughout my training.

ABSTRACT

Background: Invasive breast carcinomas with neuroendocrine differentiation (IBCNED) are a heterogenous group of tumours first recognised, as a distinct entity, by the World Health Organisation (WHO) in 2003. The classification of these tumours has undergone significant changes since they were first described, and the diagnostic criteria has been inconsistent amongst reporting authors. IBCNED have not been studied in the South African context, and this study aims to review the incidence, demographic profile, histopathology and immunohistochemical profile of IBCNED.

Materials and Methods: A three-month retrospective study of cases with the diagnosis of invasive breast carcinomas was undertaken to determine the clinicopathologic profile of IBCNED.

Results: The mean age of female patients with IBCNED was 55 years. Thirty-five (35/91, 38%) cases were positive for synaptophysin and/or chromogranin A. The tumours showed a histomorphology comparable with invasive breast carcinoma of no special type and were predominantly (33/35, 94%) moderately to poorly differentiated. The predominant molecular subtype, with 91% (33/35), was luminal B .

Conclusion: IBCNED show a diverse range of histomorphologic features, similar to those seen in conventional breast carcinomas of no special type, however they do have distinct cytomorphological characteristics and show a predilection for luminal B molecular subtype. A larger cohort is necessary to confirm these findings and to expand knowledge and treatment options.

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LIST OF ABBREVIATIONS

ASCO	American Society of Clinical Oncologists
CAP	College of American Pathologists
DCIS	Ductal carcinoma in situ
DDSA	Dodecenyl Succinic Anhydride
DMP30	2,4,6-Tris(dimethylaminomethyl)phenol
DNA	Deoxyribonucleic acid
ER	Oestrogen receptor
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
IARC	International Agency for Research on Cancer
IBCNED	Invasive breast carcinoma with neuroendocrine differentiation
LCNEC	Large cell neuroendocrine carcinoma
LVI	Lymphovascular invasion
NEC	Neuroendocrine carcinoma
NEN	Neuroendocrine neoplasm
NET	Neuroendocrine tumour
nm	Nanometre
NST	No special type
PNI	Perineural invasion
PgR	Progesterone receptor
SOP	Standard operating procedure
TEM	Transmission electron microscope
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Breast cancer is the second commonest cancer in the world and the most common cancer in women(1). In 2012, there were 1.67 million new cases of breast cancer, accounting for 25% of all malignancies diagnosed. Data shows the highest incidence to be in North America, however, there is an increasing number of cases being diagnosed in the developing world. The 2017 cancer registry in South Africa showed 9624 new breast cancer diagnoses in female patients(2). Breast cancer had the highest incidence, comprising 22.58% of the total cancer burden in women. A study of breast cancer survival in sub-Saharan Africa showed that at three years after diagnosis, 31% of patients had demised(3). Breast cancer thus represents a major health care burden in South Africa with a high incidence and mortality rate.

It is therefore not surprising that a plethora of research has been conducted with regards to classifying and treating breast cancer, specifically invasive breast carcinomas, which are the most common type of invasive breast malignancy. “Invasive breast carcinomas” refers to a large and heterogenous group of malignant epithelial neoplasms of the glandular elements of the breast(4). Invasive breast carcinomas are categorised by histologic subtype and biomarker-defined subtypes. These biomarker-defined subtypes show distinct clinical outcomes and responses to therapy as well as differences in their genomic and transcriptomic profiles(4). Additionally, histologic subtyping provides further prognostic information(5).

In 2003, the World Health Organization introduced “neuroendocrine carcinoma of the breast” as a distinct entity, in the third edition of the WHO classification of tumours series(6). Since this inclusion, the literature shows a wide range in reported frequency of neuroendocrine breast carcinomas or neuroendocrine differentiation in breast carcinomas, which ranges from 5-30%(7). As neuroendocrine markers are not routinely implemented when assessing invasive breast carcinomas and the definition of such varies in different classification

systems, there is much disparity in the reported incidences. Also, there is currently no consensus as to the prognostic value of identifying neuroendocrine differentiation in breast carcinomas(7).

As mentioned earlier, breast carcinoma is on the rise in South Africa and shows a high mortality rate in this population. A search of the literature revealed no studies conducted in South Africa, with regards to the incidence, morphology, and biomarker classification of invasive breast carcinomas with neuroendocrine differentiation. In this study, we aim to fill this gap, as a starting point for further investigation into this unique and underreported subset of invasive breast carcinomas.

1.2 DEFINING THE CLINICAL PROBLEM

Invasive breast carcinomas with neuroendocrine differentiation (IBCNED) are an increasingly more recognised pathologic entity, the exact clinical significance of which is yet to be fully elucidated. Although many studies have described the clinicopathologic features of these neoplasms, treatment protocols specific to their unique biologic features have not yet been synthesised(4). Hence further studies are necessary to determine epidemiological data (no South African studies have been conducted) and clinical parameters.

1.3 LITERATURE REVIEW

1.3.1 Introduction and Development of the WHO Classification

1.3.1.1 Pathology and genetics of tumours of the breast and female genital organs (2003, 3rd edition)(8)

Breast carcinomas with neuroendocrine differentiation are a relatively uncommon and heterogenous group of tumours which show variable expression of neuroendocrine markers. In 2003, the World Health Organization first introduced this category into their classification system under the heading "neuroendocrine tumours" and defined them as follows: *"Primary neuroendocrine carcinomas of the breast are a group, which exhibits morphological features similar to those of neuroendocrine carcinomas of both the gastrointestinal tract and the lung. They express neuroendocrine markers in more than 50% of the cell population."* The 50% cut off described by Sapino et al(9) was utilised and they excluded invasive breast carcinomas, not otherwise specified, that show focal neuroendocrine differentiation by expression of neuroendocrine markers using immunohistochemistry. The subcategories in this classification scheme comprised solid neuroendocrine carcinoma, small cell/oat cell carcinoma and large cell neuroendocrine carcinoma.

1.3.1.2 WHO classification of tumours of the breast (2012, 4th edition)(10)

In 2012, the 4th edition of the WHO classification of tumours of the breast(11) defined *carcinomas with neuroendocrine features,* as follows: *"Carcinomas with neuroendocrine differentiation exhibit morphological features similar to those of neuroendocrine tumours of the gastrointestinal tract and of the lung. All tumours express neuroendocrine markers to a greater or lesser degree. Other invasive breast carcinomas of no special type, and some special variants, may show neuroendocrine differentiation."* Here there was a significant

change in the classification from a cut-off value of 50% to any amount of staining with neuroendocrine markers. Additionally, subcategories described included: neuroendocrine tumour, well differentiated; neuroendocrine carcinoma, poorly differentiated/small cell carcinoma; invasive breast carcinoma with neuroendocrine differentiation. The latter category included invasive breast carcinomas of no special type and special subtypes such as solid papillary carcinoma and the hypercellular subtype of mucinous carcinoma.

1.3.1.3 WHO classification of tumours: breast tumours (2019, 5th edition)(4)

In the latest, 5th edition, of the WHO classification of tumours of the breast, the WHO/IARC have revised the classification of neuroendocrine tumours once again. In an effort to unify the categorisation of neuroendocrine neoplasms across all organ systems, the newly devised scheme starts by classifying all tumours under the umbrella term "*neuroendocrine neoplasm (NEN)".* These are further divided into two main categories: well-differentiated neuroendocrine tumour (NET) and poorly differentiated neuroendocrine carcinoma (NEC). Furthermore, the classification outlines the following: *"Although in other organ systems, such as the lung or gastroenteropancreatic system, NETs are graded as G1, G2 and G3 based on mitotic count and Ki67 proliferation index, this does not apply to tumours in the breast. Despite this, mitotic count remains the main parameter in determining grade via the Nottingham grading system. According to this grading system, the majority of NETs should be G1 or G2."* NECs comprise small cell neuroendocrine carcinomas (SCNEC) and large cell neuroendocrine carcinomas (LCNEC). Morphology and the expression of markers of neuroendocrine differentiation are used to identify these tumours. The reason for this uniform classification framework was to reduce inconsistencies and contradictions among the various systems currently in use. The key feature here is the distinction between well-differentiated

NETs and poorly differentiated NECs, both of which share the common expression of neuroendocrine markers. It was also noted that, in the breast, neuroendocrine neoplasms are malignant by definition. It was also acknowledged that overlap with neuroendocrine neoplasms and other breast carcinomas showing neuroendocrine differentiation (specifically solid papillary carcinoma and the hypercellular subtype of mucinous carcinoma) could fulfil the diagnosis as mammary neuroendocrine neoplasm. However, because both of these neoplasms are distinct breast carcinomas with specific morphologies and biological characteristics, they should not be classified as NET or NEC. **Therefore, invasive breast carcinomas of no special type with neuroendocrine differentiation** should be diagnosed if neuroendocrine histologic features and neuroendocrine marker expression are not distinct or uniform enough to classify the tumour as a NEN. The same would apply for those of other special types. This is the "third" category of neuroendocrine neoplasms of the breast. **The difference in classifying a tumour as NEN versus invasive breast carcinoma with neuroendocrine differentiation is based on the presence and extent of histologic features characteristic of neuroendocrine differentiation in the tumour.** In essence, there has been no material change in the category of invasive breast carcinoma of no special type (or special subtype) with neuroendocrine differentiation. However, there has been improvement in defining these tumours and delineating them from NETs.

The typical light microscopic features of neuroendocrine tumours show cells arranged in nests and trabeculae with "salt and pepper" chromatin and granular eosinophilic cytoplasm(12, 13).

According to this latest edition of the WHO classification of tumours of the breast, most NETs and NECs show a component of conventional-type mammary carcinoma. Hence if SCNEC makes up 10-90% of the tumour area, the terminology for mixed invasive (NST or other special type) and SCNEC may be used and the NEC percentage should be reported.

Carcinomas with less than 10% NEN pattern should be classified as invasive carcinoma NST or other types with an option to describe the focal specialised neuroendocrine pattern in the comment of the report. Carcinomas with greater than 90% NEN pattern should be reported as NET or NEC.

1.3.2 Current Literature

Due to the lack of uniformity and inadequacy of definitions applied over the years, the prevalence of these tumours, as cited in the literature, varies from 5-30%(7). Numerous studies have analysed the morphologic(14-16), immunohistochemical(14, 15, 17-19) and prognostic features(19-21) of neuroendocrine breast carcinomas. Most of these studies have only analysed true primary neuroendocrine carcinomas of the breast (carcinoid and small cell/large cell carcinomas) and those showing more than 50% expression with neuroendocrine markers using immunohistochemistry. Few have used a lower threshold for neuroendocrine marker positivity in defining tumours with neuroendocrine differentiation(19, 22, 23). All the investigations showed that neuroendocrine breast carcinomas preferentially exhibit a luminal molecular phenotype and that they tend to appear in older women and exhibit a more aggressive behaviour. No study has been conducted in South Africa, in conjunction with the new WHO classification, to categorise (histologic subtype, histologic grade, molecular subtype) invasive breast carcinomas with neuroendocrine differentiation, a specific subtype that is defined in the most recent WHO classification of breast tumours(4)

1.3.3 Neuroendocrine Cells in the Breast

Since the first identification of argyrophilic cells in breast carcinomas in 1963(24), numerous studies have failed to demonstrate the definite presence of resident neuroendocrine cells

within normal breast parenchyma(25). Additionally, neuroendocrine hyperplasia or benign neuroendocrine tumours have not been described in the breast. As a result, it is proposed that breast carcinomas with neuroendocrine differentiation develop by divergent differentiation during the neoplastic process, rather than originating from a neuroendocrine cell precursor(19, 20, 26, 27). A study in 2010 showed that certain regulatory peptides associated with neuroendocrine cells in other organs, were present in breast duct epithelial cells. These peptides included vesicular monoamine transporter, chromogranin B, obestatin, ghrelin, adrenomedullin and apelin. These cells did not express chromogranin A or synaptophysin(28), at least one of which is always expressed in breast carcinomas with neuroendocrine differentiation and as a result, the significance of this finding has not been validated. Chromogranin A and synaptophysin are currently considered the most specific immunohistochemical markers for neuroendocrine neoplasms(29). Neural cell adhesion molecule (NCAM), also known as CD56, is a sensitive marker for neuroendocrine differentiation, however it not specific and stains many other non-neuroendocrine neoplasms(29). In the WHO classification of tumours of the breast, the prerequisite for diagnosis of IBCNED is the expression of either chromogranin A, synaptophysin or both(4).

1.3.4 Electron microscopy

Electron microscopy has been available for diagnostic use in pathology since the early 1900s(30). Its main use was initially limited to the diagnosis and interpretation of renal biopsies. Subsequently, the indications were expanded to tumour diagnosis, including that of neuroendocrine tumours(30). The characteristic ultrastructural feature of neuroendocrine tumours is identification of dense core secretory granules(30, 31). These often have a surrounding halo of varying width(30) with a peripheral limiting membrane(30, 31). The

granules range in size from 50-450nm(32), however, depending on cellular metabolism and the secretory product, diameters of up 1000nm can be present(33). The granules are usually fairly uniform within any given tumour(32). Although electron microscopy has been superseded by the advent of immunohistochemistry, it still provides some utility in difficult cases where a specific line of differentiation cannot be proven using immunohistochemistry(34).

1.3.5 Categorisation of Invasive Breast Carcinomas

1.3.5.1 Histologic grade

Description of histologic grade is routine in the reporting of invasive breast carcinomas(4). The most widely used system, recommended by numerous professional bodies, is the Nottingham (Elston-Ellis) modification of the Scarff-Bloom-Richardson grading system, also known as the Nottingham grading system(35). The prognostic value of this system was first demonstrated in 1991 and has since been validated by multiple independent studies(5). This system has an independent and equally powerful prognostic value and has been combined with lymph node stage and tumour size to form prognostic indices(35). The following interpretation is outlined in the latest edition of the WHO tumours of the breast(4):

- Tubule and gland formation:
    - Majority of tumour (>75%) = 1.
    - Moderate degree (10-75%) = 2.
    - Little or none (<10%) = 3.
- Nuclear pleomorphism:
    - Small, regular uniform cells = 1.
    - Moderate increase in size and variability = 2.

    - Marked variation = 3.
- Mitotic counts:
    - Based on standardised area in mm^2 / ten high power fields.

In addition, the following notes should be adhered to:

- Gland formation:
    - Assessed over the whole tumour at low power.
    - Only structures exhibiting clear central lumina surrounded by polarized neoplastic cells are counted.
- Nuclear pleomorphism:
    - Assessed by reference to the regularity of nuclear size and shape of normal epithelial cells in adjacent breast tissue.
    - Score 1:
        - Nuclei are very similar in size (<1.5x) to those of benign pre-existing epithelial cells.
        - Minimal pleomorphism.
        - Even chromatin pattern.
        - Nucleoli are not visible or very inconspicuous.
    - Score 2:
        - Nuclei are larger (1.5-2x of benign epithelial cell nuclei).
        - Mild to moderate pleomorphism.
        - Visible but small and inconspicuous nucleoli.
    - Score 3:
        - Larger nuclei (>2x size of benign epithelial cell nuclei).
        - Marked increase in size and shape.
        - Vesicular chromatin.

- Prominent nucleoli.

The three values are added together to produce scores of 3 to 9 to which the grade is assigned as follows:

- 3-5 points: grade I, well-differentiated.
- 6-7 points: grade II, moderately differentiated.
- 8-9 points: grade III, poorly differentiated.

1.3.5.2 Molecular subtype

Invasive breast carcinomas are categorised using various clinicopathologic criteria. This includes tumour size, lymph node status, histologic grade and HER2 status(36). However apart from these, it has been shown that specific intrinsic biologic differences affect clinical outcomes and response to treatment(37). This refers to the molecular subtypes of breast cancer. In a seminal study by Perou et al in 2000, five main subtypes were described: luminal A, luminal B, basal-like, HER2-enriched and normal breast-like(38). Subsequent studies have shown that the normal breast-like category is likely an artefact due to overrepresentation or disproportionately high content of normal breast ducts or stroma(36, 37, 39). Furthermore, additional rare subtypes that have been identified include molecular apocrine, claudin-low and interferon-rich(37, 39), but these do not have immunohistochemical surrogates. The initial identification of these subtypes was discovered using complementary DNA microarrays and hierarchical clustering in order to isolate groups of similar genes and determine their expression in the various tumours(38). Hence this original classification was based on complex, time-consuming and expensive molecular techniques not suitable for integration into daily practice. Because of this,

immunohistochemistry-based surrogate molecular classification has been advocated(39). Although there is good concordance between the two, discrepancies do exist(36, 39).

The St Gallen International Expert Consensus endorses the following classification using immunohistochemical markers(40):

- Luminal A-like: ER+, PgR≥20%, HER2-, Ki67<20%.
- Luminal B-like: ER+, PgR>20% and/or HER2+ and/or Ki67 ≥ 20%.
- HER2-overexpression: ER-, PgR-, HER2+.
- Basal-like: ER-, PgR-, HER2- (triple negative).

Cheang et al advocated a Ki67 staining cut-off of 14% to distinguish luminal A from luminal B(41) whilst the most recent St Gallen Consensus recommends using 20% as the discriminating value(42). According to recommendations from the WHO(4), Ki67 is not universally used or officially recommended due to lack of international consensus about scoring and cut-off values. Despite these reservations, they do concede that the Ki67 proliferation index can be of clinical value as a supplement to histologic grade in determining prognosis and potential chemotherapy benefit.

1.3.5.3 Ki67 proliferation index

The interpretation of the Ki67 proliferation index is a pivotal factor in assigning a tumour to luminal A-like versus luminal B-like when using immunohistochemistry as a surrogate for gene expression profile. Its importance is due to the fact that there are differences in treatment and prognosis between these two hormone receptor-positive subtypes(40). There are numerous automated and manual techniques currently being used and no method has been universally accepted as the gold standard(42).

There is an array of different counting methods currently in use for interpreting the Ki67 proliferation index. The recent St Gallen consensus conference held in 2015 outlined a number of hot-spot only and average methods that are currently in use internationally(42). There is currently no internationally accepted methodology that has been prescribed for use in daily practice.

1.3.5.4 Hormone receptors

The current American Society of Clinical Oncology/ College of American Pathologists (ASCO/CAP) guidelines for oestrogen receptor (ER) and progesterone receptor (PgR) testing in breast cancer recommends the following(43):

- Breast cancer samples with 1% to 100% of tumour nuclei positive should be interpreted as positive.
- A sample is considered ER negative if <1% or 0% of tumour cell nuclei are immunoreactive.
- Those with 1% to 10% should be reported as ER low positive, as there is little data on endocrine therapy benefit for these.
- The status of controls should be reported for cases with 0-10% staining.
- PgR is interpreted in the same fashion.

The most recent update on the ASCO/CAP guidelines for reporting HER2 states the following(44):

Negative
- 0 = absence of staining or barely perceptible membrane staining in ≤10% of invasive tumour cells.
- 1+ = incomplete feint membrane staining in >10% of invasive tumour cells.

Equivocal
- 2+ = weak to moderate complete membrane staining in >10% of invasive tumour cells.

Positive
- 3+ = complete, intense, circumferential membranous staining in >10% of invasive tumour cells.

If a case is equivocal, further testing is necessary and an algorithmic guideline is provided in order to resolve the case.

The importance of determining the molecular subtype, as alluded to earlier, is due to the difference in intrinsic biologic potential of the different subtypes, which differ in their incidence(39), response to therapy and prognosis(37). Therefore, the information provided by the intrinsic subtype complements and expands the information provided by classic clinicopathological markers(45). Luminal A tumours are the commonest, have the best prognosis and show a good response to endocrine therapy. However, their response to chemotherapy is poor(36, 37, 39). Conversely, luminal B tumours have a higher histologic grade and less favourable prognosis but respond better to chemotherapy(36, 37, 39). HER2-enriched carcinomas (HER2-overexpressed) tend to have a more aggressive clinical course similar to that of triple negative tumours yet have the advantage of the potential for targeted therapy using trastuzumab(36, 37, 39). As a result, the ER-positive tumours portend a better prognosis than the ER-negative tumours.

1.3.6 Molecular Subtype in IBCNED

All the published work on breast carcinoma with neuroendocrine differentiation shows that the majority of tumours are of luminal subtype(14, 16, 17, 19, 46, 47). In all but one of these studies, only tumours which expressed neuroendocrine markers (synaptophysin or chromogranin A) in more than 50% of the cells were included. Both true neuroendocrine breast carcinomas and breast carcinomas of other morphologic subtypes (including no special type) with neuroendocrine differentiation were included. One study showed 91% of the specimens to be of luminal A subtype, 6% to be luminal B and 3% to be triple negative(14). However, this study did not specify the cut-off value used for the Ki67 proliferation index to discriminate between luminal A and luminal B subtypes. Another study showed oestrogen receptor and progesterone receptor positivity in 93% and 73% of cases, respectively, indicating a luminal subtype(47). Only 10 of the cases were evaluated for Ki67, 4 of which displayed a proliferative index of more than 14%, thus defining a luminal B subtype according to the criteria used. A much larger, and more uniform report described 128 cases of breast carcinoma with neuroendocrine features(19). A Ki67 cut-off value of 14% was used and showed 42% and 58% as luminal A and luminal B, respectively. A further 7% were denoted HER2+ luminal B (ER +, HER2+). In addition, 2% were HER2-enriched (ER -, HER2+) and 3% were basal-like (triple negative). In this study, focal expression of neuroendocrine markers was taken as 10-49% and diffuse as greater than or equal to 50%. There were no significant differences with regards to categorisation into the different molecular subtypes between the focal and diffuse groups. Other studies showed a similar preponderance of cases falling into the luminal subtype(16, 17, 46).

1.3.7 Histologic Grade in IBCNED

Three papers which further evaluated histologic grade showed the following similar findings(14, 17, 19): most tumours were classified as grade 2 (moderately differentiated), followed by grade 3 (poorly differentiated) and very few or none as grade 1 (well-differentiated).

1.3.8 Treatment

Treatment options for neuroendocrine neoplasms of the breast are currently the same as for any invasive breast carcinoma. Due to the heterogenous nature of these tumours, rare occurrence, and poor recognition, specific treatment protocols or standardised therapeutic schemes have not been developed(48, 49). Surgery is the mainstay of treatment for early-stage tumours. Depending on the size of the tumour, wide local excision, or mastectomy with or without axillary dissection may be indicated. Neoadjuvant or adjuvant therapy follows the same indications as for other invasive breast carcinomas.

1.4 THE RESEARCH QUESTION

What is the incidence and pathologic profile (histologic grade, histologic subtype and molecular subtype) of invasive breast carcinomas with neuroendocrine differentiation?

CHAPTER 2

AIMS AND OBJECTIVES

2.1 AIMS

- To determine the incidence of invasive breast carcinomas with neuroendocrine differentiation in the study population, Kwazulu-Natal, South Africa.
- To elucidate whether these tumours have a specific pathologic profile.
- To compare the clinicopathologic features to invasive breast carcinomas of no special type.

2.2 OBJECTIVES

- Identify breast carcinomas with neuroendocrine differentiation using immunohistochemical stains.
- Assessment of histologic grade, histologic subtype and molecular subtype of breast carcinomas with neuroendocrine differentiation, using routine haematoxylin and eosin-stained tissue sections and immunohistochemistry.

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY DESIGN

This was a laboratory-based, retrospective, observational study. All breast core needle biopsies signed out as invasive breast carcinoma between 1st May 2020 and 31st July 2020 were included in the study cohort.

3.2 SETTING

This study was conducted in the laboratory of the Department of Anatomical Pathology of the National Health Laboratory service and University of Kwazulu-Natal, Inkosi Albert Luthuli Central Hospital (IALCH), Durban, South Africa.

3.3 PARTICIPANT SELECTION AND SAMPLING STRATEGY

The population of interest were those patients who had undergone a core needle biopsy procedure with a resultant pathological diagnosis of invasive breast carcinoma.

The following cases were excluded from the study: excision specimens, patients who received neoadjuvant therapy and recurrent tumours.

3.4 DATA COLLECTION METHODS AND TOOLS

All breast core needle biopsies diagnosed as invasive breast carcinoma between 1st May 2020 and 31st July 2020 were retrieved. These underwent morphological re-evaluation by three pathologists (NN, GN, AMW) to determine morphology, histologic grade and histologic subtype. Immunohistochemical slides used to establish the initial diagnosis by the primary pathologist were reviewed. The molecular findings were reviewed on all cases which had

fluorescence in situ hybridisation conducted for assessment of HER2 amplification. Furthermore, immunohistochemical studies were undertaken to determine the presence of neuroendocrine differentiation. To prevent bias, previous reports for the cases were not reviewed . Each pathologist undertook independent analysis of every case. Thereafter, the results were compared and whenever a discrepancy occurred, all investigators re-examined the slides to reach a consensus. Data was collected using the data collecting tool cited in **APPENDIX 1**.

3.4.1 Histomorphology

When assessing the histomorphology of invasive carcinomas, the following qualitative criteria were used as previously described by Talu et al(14):

- Architecture:
    - Large-sized solid cohesive groups of tumour cells.
    - Small- to medium-sized solid cohesive groups of tumour cells as well as trabeculae/ribbons and glandular structures.
    - Mixed patterns of growth.
- Stromal reaction:
    - Stromal desmoplasia.
    - Sclerotic stroma.
    - Fibroelastotic stroma.
    - Mixed.
- Presence or absence of coagulative tumour necrosis.
- Cytomorphology:
    - Cell shape:

        - Polygonal-oval.
        - Plasmacytoid.
        - Spindled.
    - Cytoplasm:
        - Eosinophilic / granular eosinophilic.
        - Foamy / vacuolated.
    - Nucleus:
        - Even chromatin distribution / stippled.
        - With prominent nucleoli.
        - Combination of the above two.

The cases were also assessed for the presence of carcinoma in situ, lymphovascular invasion and perineural invasion.

3.4.2 Histologic grade

The histologic grade was calculated using the most recent recommendation by the World Health Organization, as follows(4):

- Tubule and gland formation:
    - Majority of tumour (>75%) = 1.
    - Moderate degree (10-75%) = 2.
    - Little or none (<10%) = 3.
- Nuclear pleomorphism:
    - Small, regular uniform cells = 1.
    - Moderate increase in size and variability = 2.
    - Marked variation = 3.

- Mitotic counts:
    - Based on mm^2 / ten high power fields.

In addition, the following notes should be adhered to:

- Gland formation:
    - Assessed over the whole tumour at low power.
    - Only structures exhibiting clear central lumina surrounded by polarized neoplastic cells are counted.
- Nuclear pleomorphism:
    - Assessed by reference to the regularity of nuclear size and shape of normal epithelial cells in adjacent breast tissue.
    - Score 1:
        - Nuclei are very similar in size (<1.5x) to those of benign pre-existing epithelial cells.
        - Minimal pleomorphism.
        - Even chromatin pattern.
        - Nucleoli are not visible or very inconspicuous.
    - Score 2:
        - Nuclei are larger (1.5-2x of benign epithelial cell nuclei).
        - Mild to moderate pleomorphism.
        - Visible but small and inconspicuous nucleoli.
    - Score 3:
        - Larger nuclei (>2x size of benign epithelial cell nuclei).
        - Marked increase in size and shape.
        - Vesicular chromatin.
        - Prominent nucleoli.

The three values were added together to produce scores of 3 to 9 to which the grade was assigned as follows:

- 3-5 points: grade I, well-differentiated.
- 6-7 points: grade II, moderately differentiated.
- 8-9 points: grade III, poorly differentiated.

3.4.3 Neuroendocrine marker expression

To determine the status of neuroendocrine differentiation, any amount of cytoplasmic staining with neuroendocrine markers (synaptophysin and chromogranin A) was deemed a positive result. Additionally, according to the most recent WHO classification for neuroendocrine tumours of the breast(4), focal neuroendocrine neoplasm pattern is defined as less than 10%, mixed as 10-90% and >90% as pure. Hence, a similar disposition has been adopted in this study, when defining invasive breast carcinomas with neuroendocrine differentiation, as follows:

- <10% staining with neuroendocrine markers = focal.
- 10-90% staining with neuroendocrine markers = significant.
- >90% staining with neuroendocrine markers = diffuse.

3.4.4 Hormone receptor status

The most recent update of the ASCO/CAP guidelines for evaluation of oestrogen receptor and progesterone receptor was utilised as follows:

- Breast cancer samples with 1% to 100% of tumour nuclei positive should be interpreted as positive.

- A sample is considered ER negative if <1% or 0% of tumour cell nuclei are immunoreactive.
- Those with 1% to 10% should be reported as ER low positive, as there is little data on endocrine therapy benefit for these.
- The status of controls should be reported for cases with 0-10% staining.
- PgR is interpreted in the same fashion.

3.4.5 HER2 amplification

The most recent update on the ASCO/CAP guidelines for reporting HER2 was utilised as follows(44):

Negative
- 0 = absence of staining or barely perceptible membrane staining in ≤10% of invasive tumour cells.
- 1+ = incomplete feint membrane staining in >10% of invasive tumour cells.

Equivocal
- 2+ = weak to moderate complete membrane staining in >10% of invasive tumour cells.

Positive
- 3+ = complete, intense, circumferential membranous staining in >10% of invasive tumour cells.

- Equivocal cases require further testing using molecular diagnostic techniques. In this study, dual probe fluorescence in situ hybridization was used with the following categories for interpretation:
    - Negative / not amplified: HER2:Ch17 < 1.8.
    - Equivocal / borderline: HER2:Ch17 = 18.-2.2.
    - Positive / amplified: HER2:Ch17 ≥ 2.2.

3.4.6 Ki67

In this study, the base laboratory has optimized the MIB1 antibody for a Ki67 proliferation index cut-off value of 14%(41), hence this value was used to define luminal A versus luminal B molecular subtypes.

The method of Ki67 interpretation that was utilised in this study is one of the hot spot-only methods described at the St Gallen conference in 2015(42). This method, called the Ki67-Eye10 method, requires an eyeball estimation of the tumour's hot spot (area of Ki67 highest staining intensity) at 20x magnification. The score is divided at 10% intervals to determine an estimate of the proliferation index of the tumour.

3.4.7 Immunohistochemical and histochemical stains

The immunohistochemistry monoclonal antibodies were performed on an automated immunostainer (Ventana Benchmark Ultra, Tucson, AZ) using formalin-fixed paraffin-embedded 2-µm sections:

TABLE 1. List of antibodies used for immunohistochemical analysis.

Antibody	**Supplier**	**Clone**	**Concentration**
Oestrogen receptor	Ventana	SP1	1µg/ml
Progesterone receptor	Ventana	1E2	1µg/ml
HER2	Ventana	4B5	6µg/ml
Ki67	Ventana	30-9	2µg/ml
Synaptophysin	Cell Marque	MRQ-40	0.04µg/ml
Chromogranin A	Ventana	LK2H10	1µg/ml
p63	Ventana	4A4	0.14µg/ml
Calponin	Cell Marque	EP798Y	0.09µg/ml

Anti-keratin	Ventana	34βE12	1.4µg/ml
E-cadherin	Cell Marque	EP700Y	0.73µg/ml

Appropriate positive controls were used throughout.

Calponin, p63 and anti-keratin (34βE12) were used to identify the presence of myoepithelial cells when diagnosing carcinoma in situ. Loss of membranous E-cadherin staining was used as an indicator of lobular carcinoma, together with morphology.

Additionally, the following histochemical stains were utilized when necessary:

- Period acid-Schiff.
- Periodic acid-Schiff with diastase.
- Mucicarmine.

The above-mentioned histochemical stains were used in cases, to confirm adenocarcinoma by the presence of intracytoplasmic mucin, where glandular differentiation was not overtly evident.

3.4.8 Electron Microscopy

Electron microscopy was performed on two cases during routine working as part of registrar training. These cases were selected for electron microscopy at the time of reporting by the attending pathologist. The formalin-fixed, paraffin embedded tissue blocks were utilized for ultrastructural examination. Although not ideal for viewing and diagnostic purposes, this method is adequate(30). The following lab-developed SOP was used for tissue preparation for viewing with the transmission electron microscope (TEM):

- Tissue was removed from the wax block.

- Immersed in xylene for 60 minutes.
- Immersed in absolute alcohol for 30 minutes.
- Immersed in 95% alcohol for 30 minutes.
- Immersed in 70% alcohol for 30 minutes.
- Rinsed with distilled water.
- Immersed in 0.2 mole concentration sodium cacodylate for 10 minutes.
- Tissue submerged in 4% osmium tetroxide overnight at room temperature.
- Tissue rinsed in sodium cacodylate (3 times) and then allowed to stand for 10 minutes.
- Tissue rinsed with 70% alcohol and then submerged for 30 minutes.
- Tissue rinsed with 95% alcohol and then submerged for 30 minutes.
- Tissue rinsed with 100% alcohol and then submerged for 30 minutes.
- Tissue submerged in fresh 100% alcohol for another 30 minutes.
- Specimen immersed in propylene oxide for 30 minutes.
- Specimen immersed in a solution of propylene oxide and araldite for 3 hours.
- Tissue immersed in pure araldite overnight.
- Tissue was embedded using epoxy resin with plasticiser (dibutyl phthalate), hardener (DDSA) and accelerator (DMP30).
- Semi-thin sections were cut and stained with toluidine blue.
- Ultra-thin sections were cut and stained using metals dyes (potassium permanganate, uranyl acetate and lead citrate) on copper grids.
- Viewing was undertaken on the FEI Tecnai Spirit transmission electron microscope.

3.5 VARIABLE SELECTION AND DATA ANALYSIS

The extrapolated data was used to determine continuous and categorical variables. The study cohort was analysed for demographic characteristics (age, race, gender, HIV status). Additionally, comparison of the histologic features, histologic grade and molecular subtype was undertaken. This data was analysed using SPSS statistical software (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). Pearson Chi^2 and Fisher's exact tests were used to determine p values to assess whether there were statistically significant differences between IBCNED and NST. In all instances, a p-value of less than 0.05 was considered statistically significant.

3.6 ETHICAL CONSIDERATIONS

Biomedical Research Ethics Committee (BREC) of the University of Kwazulu-Natal approval was obtained (BREC/00001851/2020) (**APPENDIX 2)**. Permission was also granted by the National Health Laboratory Service (NHLS) to retrieve and use archived material and data **(APPENDIX 3)**.

3.7 METHOLOGICAL CHALLENGES AND STUDY LIMITATIONS

A representative data pool of the entire population of Kwazulu-Natal was initially intended. However, due to pre-existing contracts, biopsies from numerous hospitals were not obtainable as they were processed at external laboratories. Apart from skewing representativity, this also diminished the number of cases in the sample population.

3.8 FUNDING

As this is a retrospective study, using archived materials and routine staining protocols, estimated incurred costs approximates nil for the routine diagnostic work. NHLS funding was utilised for running neuroendocrine markers (synaptophysin and chromogranin A) which are not routinely done. The electron microscopy was undertaken as part of the registrar training budget used in routine work by the NHLS.

3.9 STUDY SIGNIFICANCE

This has been the first study in South Africa to attempt to determine the incidence of invasive breast carcinomas with neuroendocrine differentiation. This study may provide a steppingstone for further clinical trials with regards to treatment protocols for invasive breast carcinomas with neuroendocrine differentiation.

CHAPTER 4

RESULTS

4.1 EPIDEMIOLOGY AND DEMOGRAPHICS

4.1.1 Incidence of IBCNED

A total of 91 cases diagnosed as invasive breast carcinoma were retrieved from the archives of the Department of Anatomical Pathology, National Health Laboratory Service, University of Kwazulu-Natal, Durban over a three-month period between 1st May 2020 and 31st July 2020. Thirty-five (35) of the ninety-one (91) cases (38%) showed neuroendocrine differentiation, according to the current WHO definition for invasive breast carcinomas with neuroendocrine differentiation. All of these tumours showed synaptophysin positivity whilst only 6 (17%) were positive for chromogranin A. Three cases (8.5%) showed focal expression of neuroendocrine markers, 28 (80%) showed significant expression and 4 (11.4%) showed diffuse expression. Both synaptophysin and chromogranin A showed a range of staining between 5-100% **(FIGURES 1-3)**.

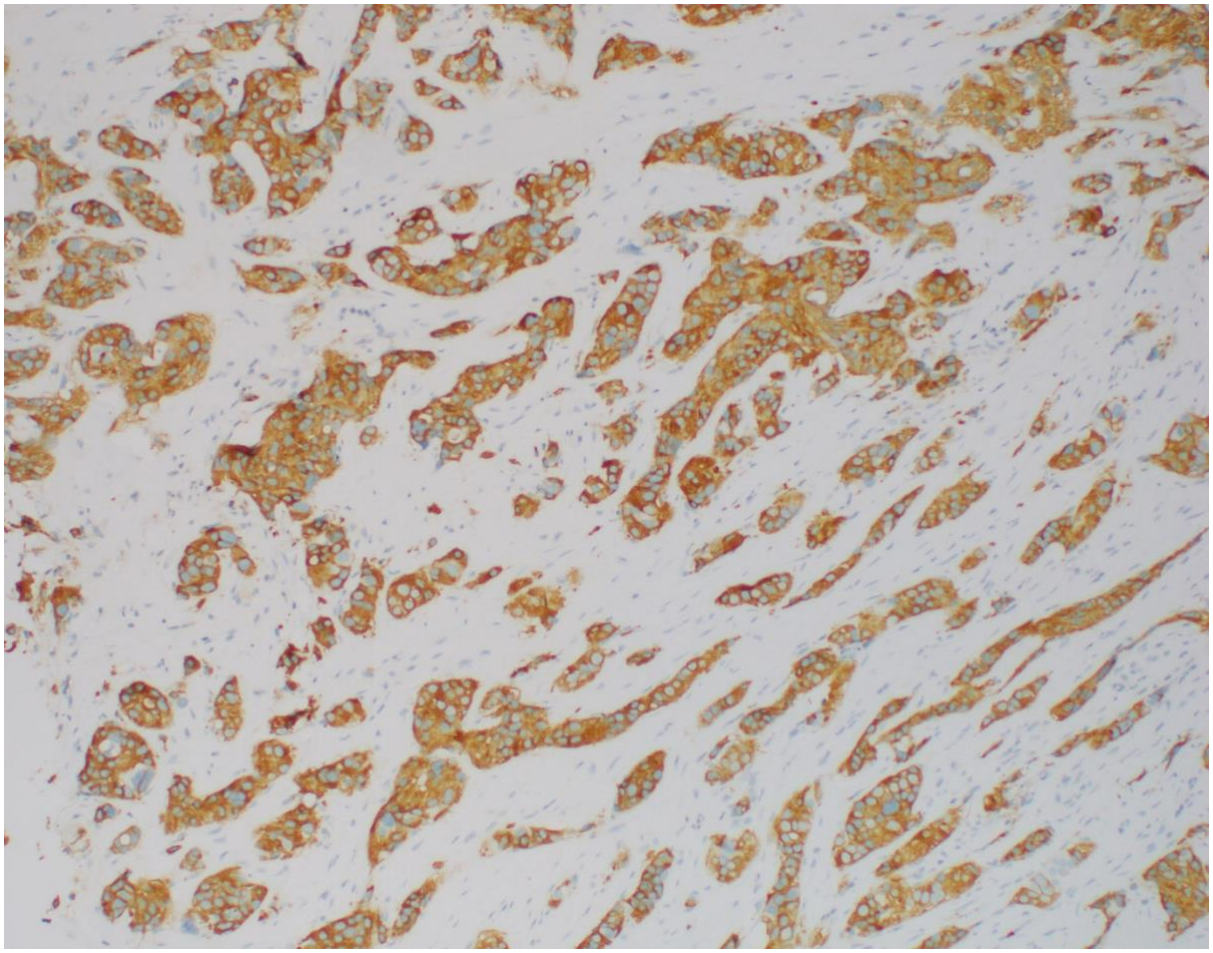

FIGURE 1. Diffuse cytoplasmic staining with synaptophysin. (x100 magnification)

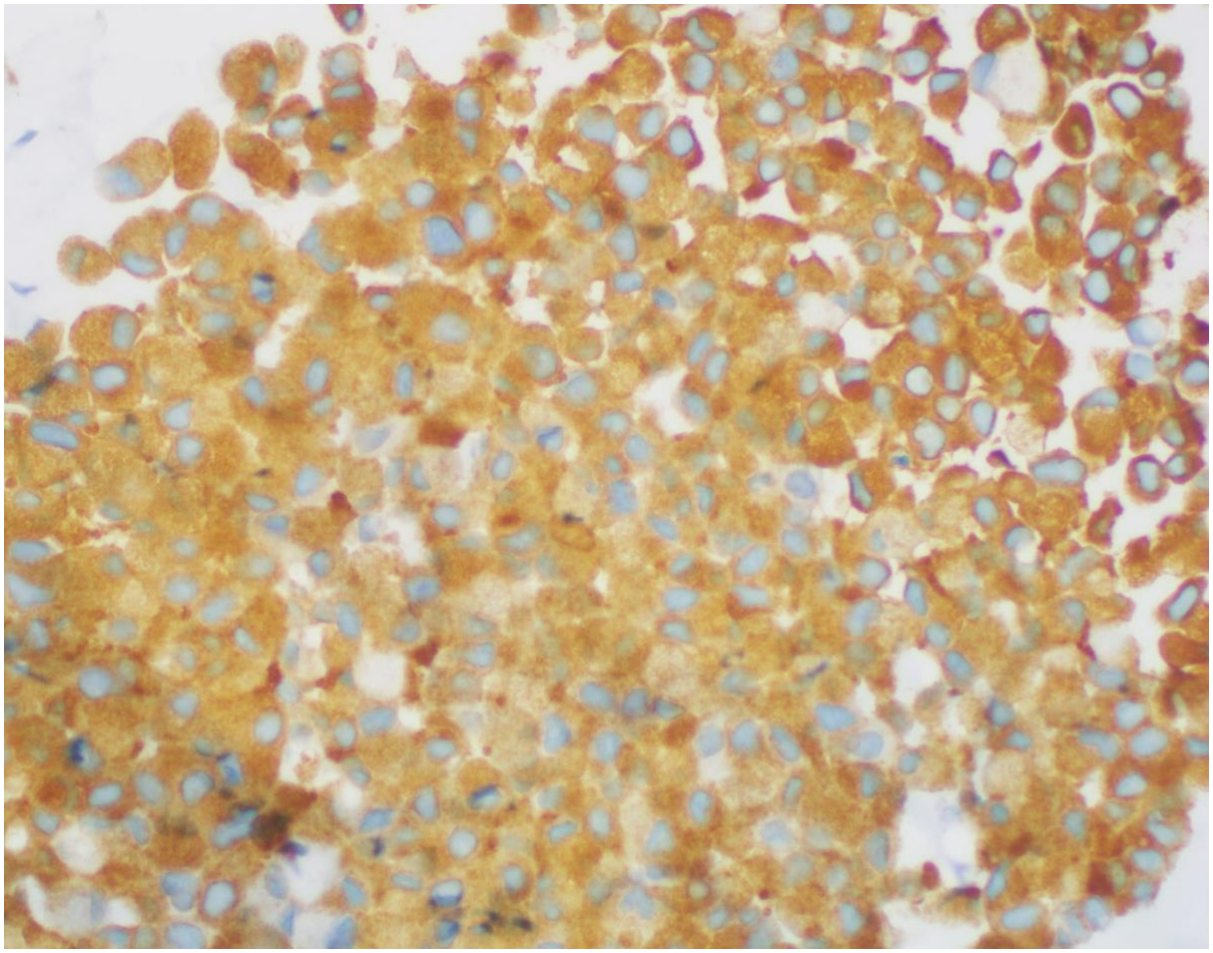

FIGURE 2. Another case showing a high-power view of diffuse cytoplasmic synaptophysin positivity. (x400 magnification).

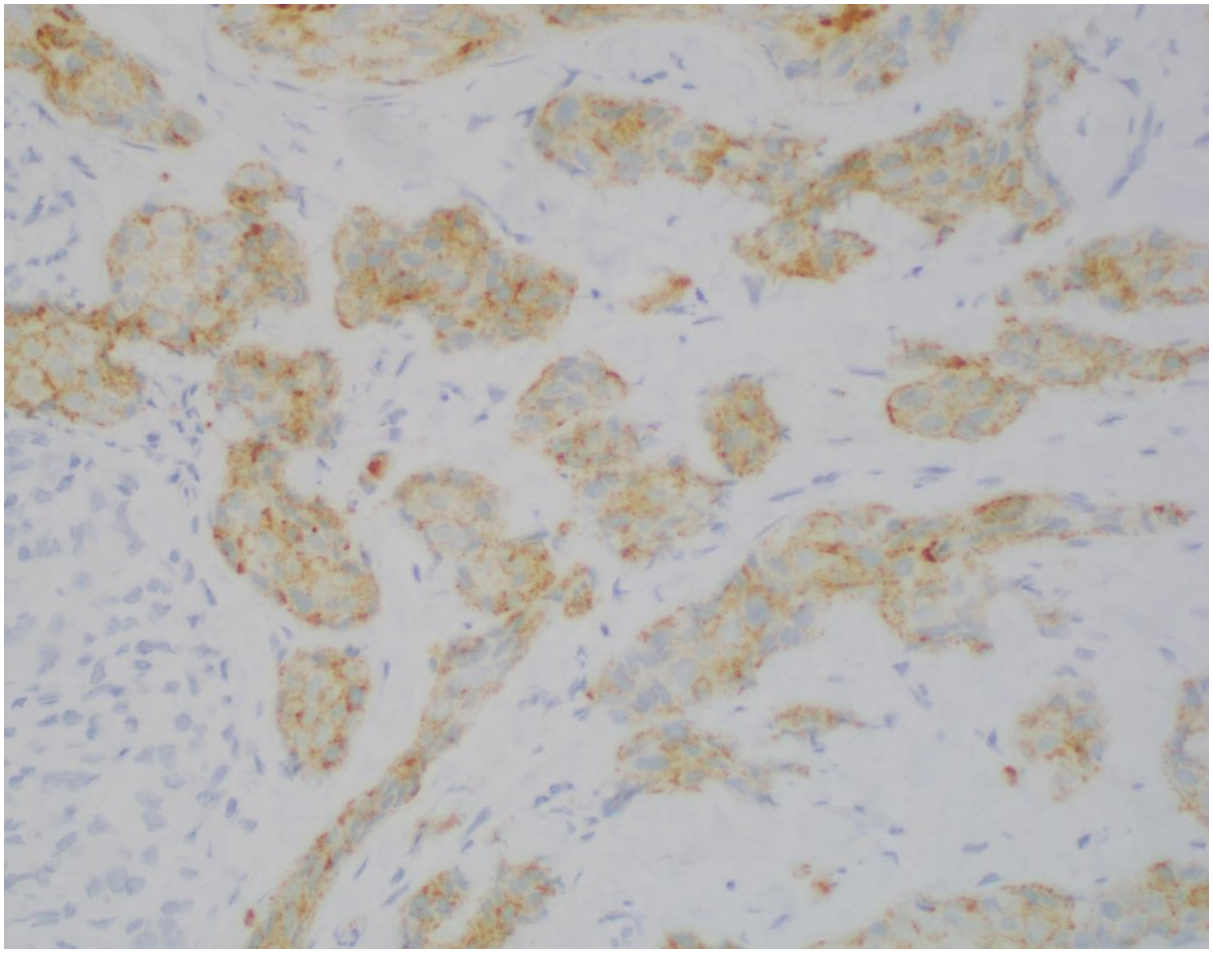

FIGURE 3. Chromogranin A stain showing cytoplasmic granular and dot-like positivity.

(x400 magnification)

4.1.2 Gender

All patients in the study cohort were female.

4.1.3 Age

Most patients diagnosed with IBCNED were in the 5th to 7th decades **(TABLE 2)**. There was no significant difference in the age distribution between invasive breast carcinomas of no special type and those with neuroendocrine differentiation, both showing a similar mean and median **(FIGURE 4)** (Pearson Chi²; $p = 0.5$):

TABLE 2. Age range of IBCNED and NST.

	IBCNED	**NST**
0-39 years	7 (20%)	11 (19%)
40-69 years	17 (49%)	33 (59%)
≥ 70 years	11 (31%)	12 (21%)
Mean	55	55
Median	54	55

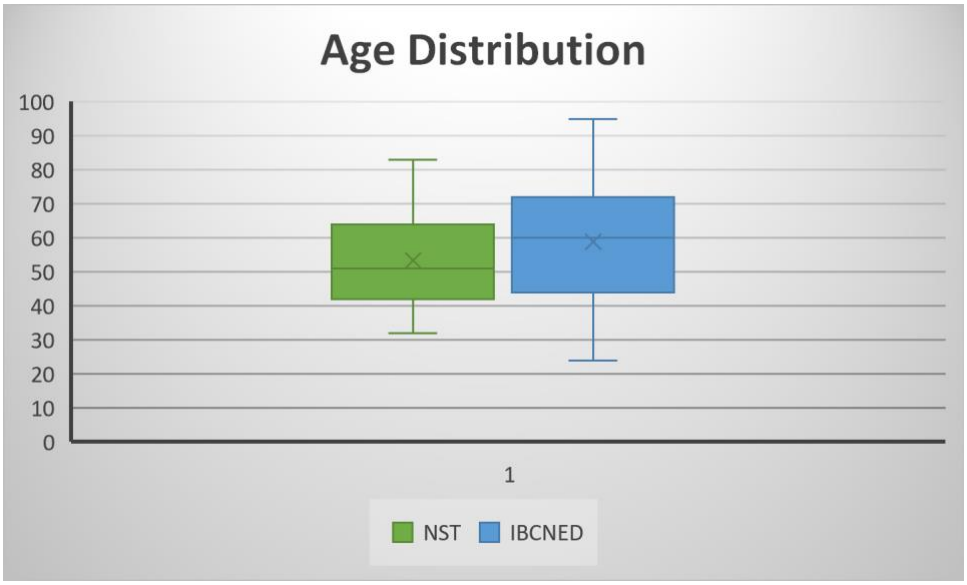

FIGURE 4. Box and whisker plot depicting age distribution of IBCNED and NST.

4.1.4 Race/ethnicity

Asian female patients showed a high percentage of carcinomas with neuroendocrine differentiation, with six (60%, 9/15) of cases showing neuroendocrine marker expression. This is almost double of that seen in the African cohort (34%, 25/46). A 20% (1/5) incidence was noted in the Caucasian subpopulation **(FIGURE 5)**. Despite the apparently higher incidence of neuroendocrine differentiation in the Asian subpopulation, due to the few number of cases, there was no statistically significant difference amongst the various ethnic groups (Fisher's exact; $p = 0.11$).

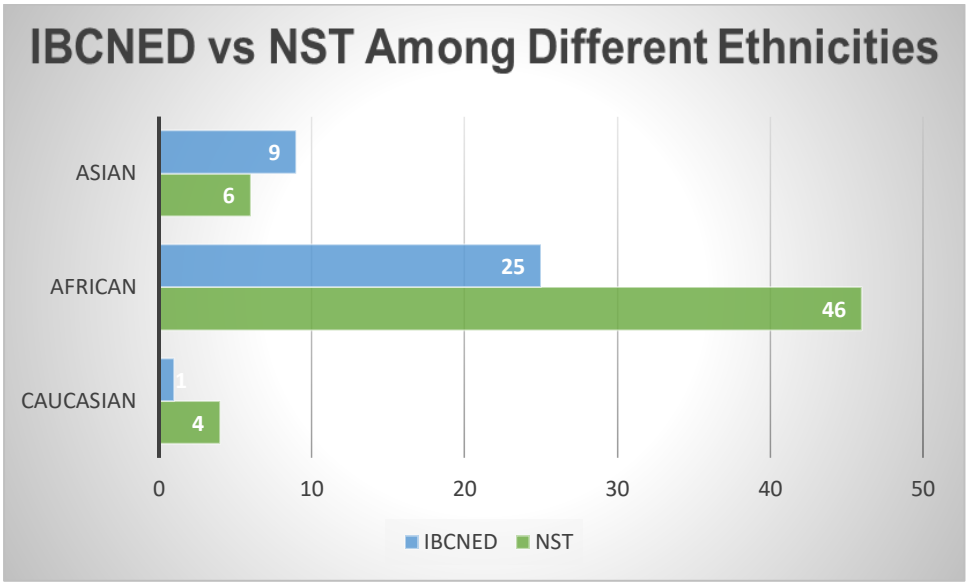

FIGURE 5. Bar graph depicting ethnic distribution between IBCNED and NST.

4.1.5 HIV status

Within the IBCNED group, twenty-five (71%, 25/35) patients were documented as HIV negative, one (3%, 1/35) as HIV positive and nine (26%, 9/35) as unknown. The NST group had twenty-six (46%, 26/56) HIV-negative cases, fifteen (27%, 15/56) HIV positive and

fifteen (27%, 15/56) as unknown **(TABLE 3)**. Most patients with IBCNED were HIV negative (Fisher's exact; $p = 0.006$).

TABLE 3. HIV status of IBCNED and NST.

HIV Status	**IBCNED**	**NST**
Positive	1	15
Negative	25	27
Unknown	9	25

4.2 HISTOMORPHOLOGY

4.2.1 Architecture patterns

The architecture patterns in IBCNED were categorised as follows: three (9%, 3/35) cases showed large-sized solid cohesive groups of tumour cells, twenty (57%, 20/35) cases showed small- to medium-sized solid cohesive groups of tumour cells as well as trabeculae/ribbons and glandular structures **(FIGURES 6-8)**, and twelve (34%, 12/35) cases showed mixed patterns of growth.

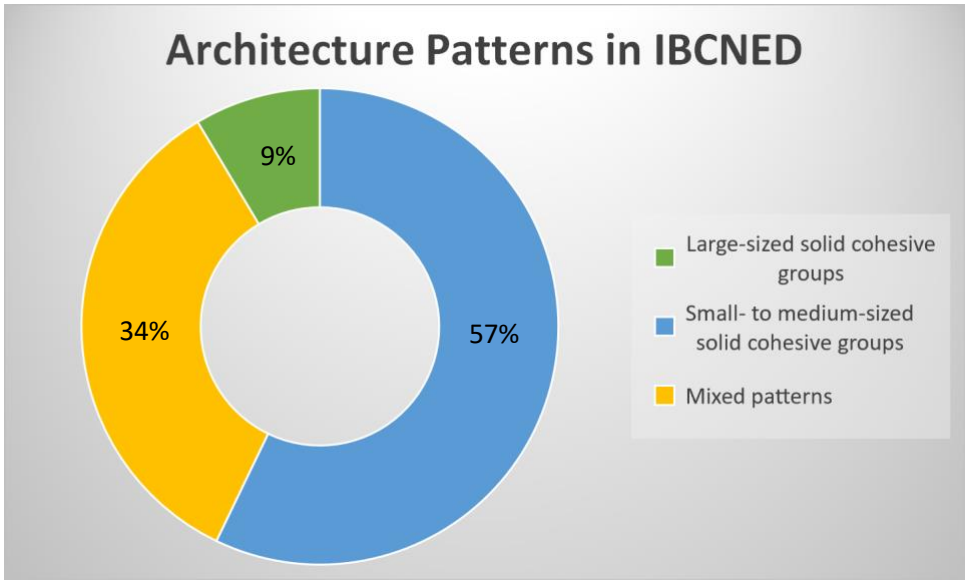

FIGURE 6. Architecture patterns in IBCNED.

The architecture patterns in NST were categorised as follows: seven (12%, 7/56) cases showed large-sized solid cohesive groups of tumour cells, forty-three (77%, 43/56) cases showed small- to medium-sized solid cohesive groups of tumour cells as well as trabeculae/ribbons and glandular structures, and six (11%, 6/56) cases showed mixed patterns of growth.

There was a significant difference in the architecture patterns between IBCNED and NST (Fisher's exact; $p = 0.03$). NST showed a higher percentage of tumours with an exclusively small groups/glands pattern whilst IBCNED tended to contain large cohesive groups in at least a proportion of the tumour.

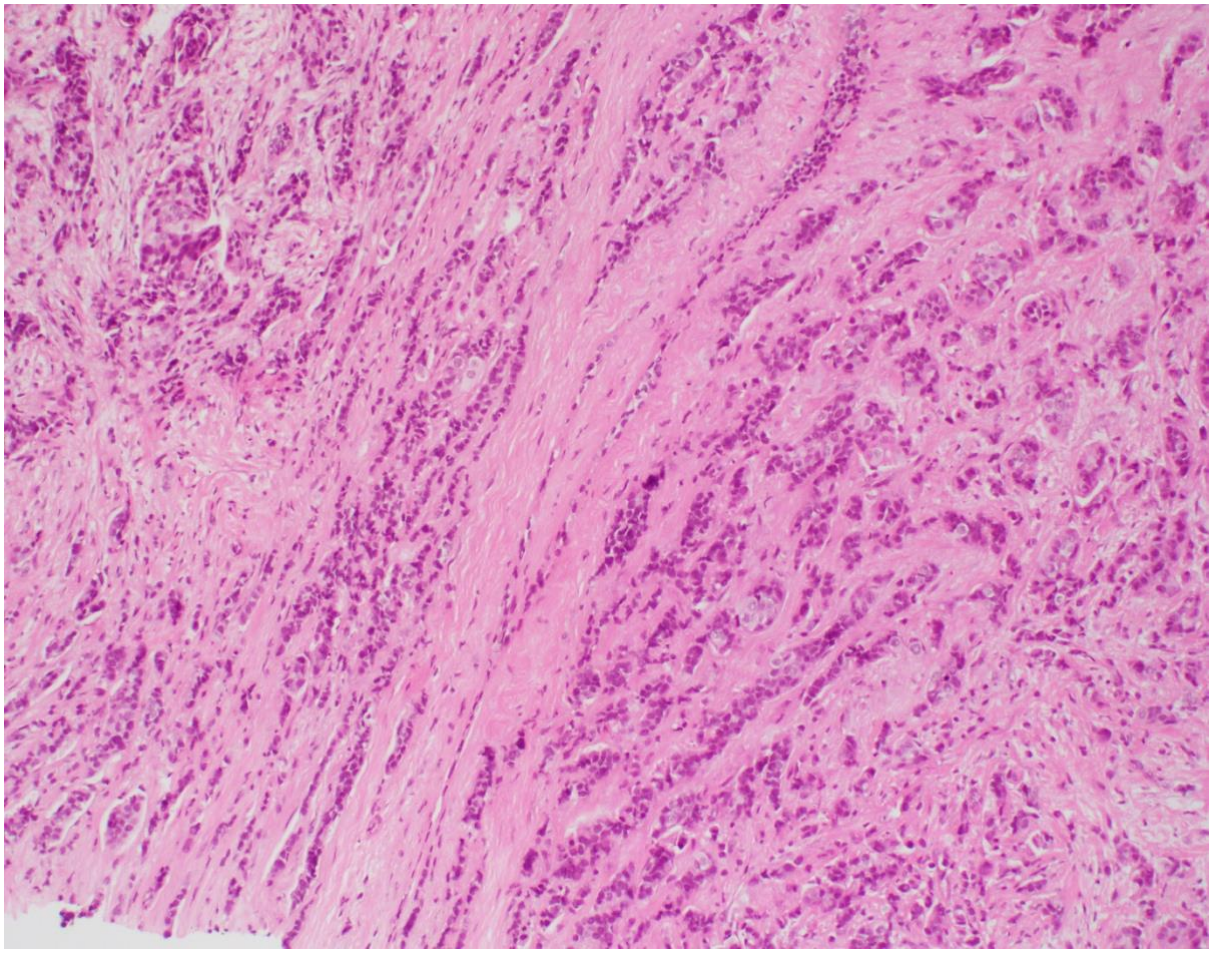

FIGURE 7. IBCNED showing nests and trabeculae with central cords, the latter reminiscent of lobular carcinoma. This case showed retained membranous E-cadherin staining. (H&E, x100 magnification)

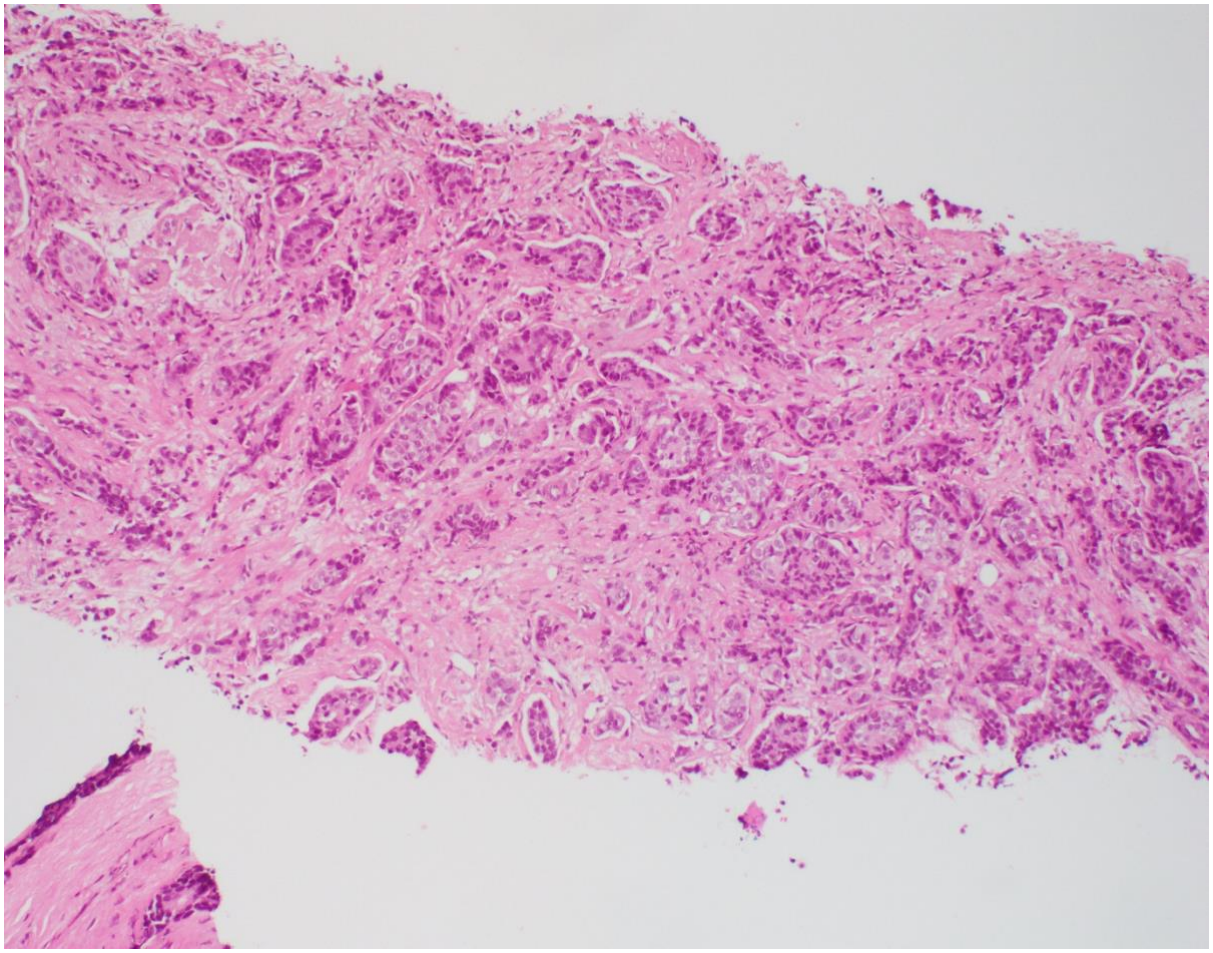

FIGURE 8. IBCNED displaying predominantly nested growth pattern with prominent retraction artefact simulating lymphovascular invasion. (H&E, x100 magnification)

4.2.2 Cytomorphology

The cytomorphology for IBCNED was assessed as follows: twenty-seven cases (77%, 27/35) had cells which were polygonal-oval in shape, seven (20%, 7/35) had cells with plasmacytoid morphology **(FIGURE 10),** and one (3%, 1/35) had spindled cells **(FIGURE 9)**.

The cytomorphology for NST was assessed as follows: fifty-two cases (93%, 52/56) had cells which were polygonal-oval in shape, three (5%, 3/56) had cells with plasmacytoid morphology, and one (2%, 1/56) had spindled cells.

Distinct differences in cell shape were not identified (Fisher's exact; *p = 0.06).*

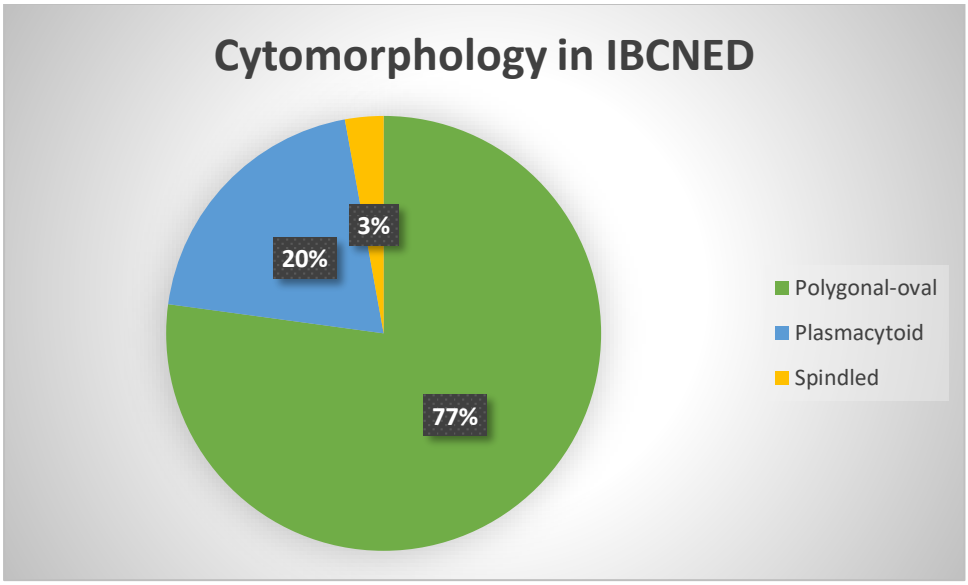

FIGURE 9. Bar graph depicting cytomorphological features of IBCNED.

In IBCNED, twenty-seven (77%, 27/35) cases had eosinophilic/granular eosinophilic cytoplasm **(FIGURES 10,13)** and eight (23%, 8/35) cases had foamy/vacuolated **(FIGURES 12,14,15)**.

NST had twenty-one (38%, 21/56) cases with eosinophilic/granular eosinophilic cytoplasm and thirty-five (62%, 35/56) cases with foamy/vacuolated cytoplasm.

There was a significant different in the quality of the cytoplasm between IBCNED and NST (Pearson Chi²; *p <0.001)*. IBCNED showed predominantly eosinophilic granular cytoplasm whilst NST displayed predominantly vacuolated cytoplasm.

Nuclear morphology in IBCNED showed the following: nineteen (54%, 19/35) cases had even chromatin distribution/stippled chromatin, whilst nine (26%, 9/35) cases showed prominent nucleoli and a combination of both was seen in seven (20%, 7/35) cases.

Nuclear morphology in NST showed the following: twenty-seven (48%, 27/56) cases had even chromatin distribution/stippled chromatin, whilst twenty-one (38%, 21/56) cases showed prominent nucleoli and eight (14%, 8/56) cases displayed a combination of both.

There was no substantial difference in nuclear morphology (Pearson Chi^2; $p = 0.5$)

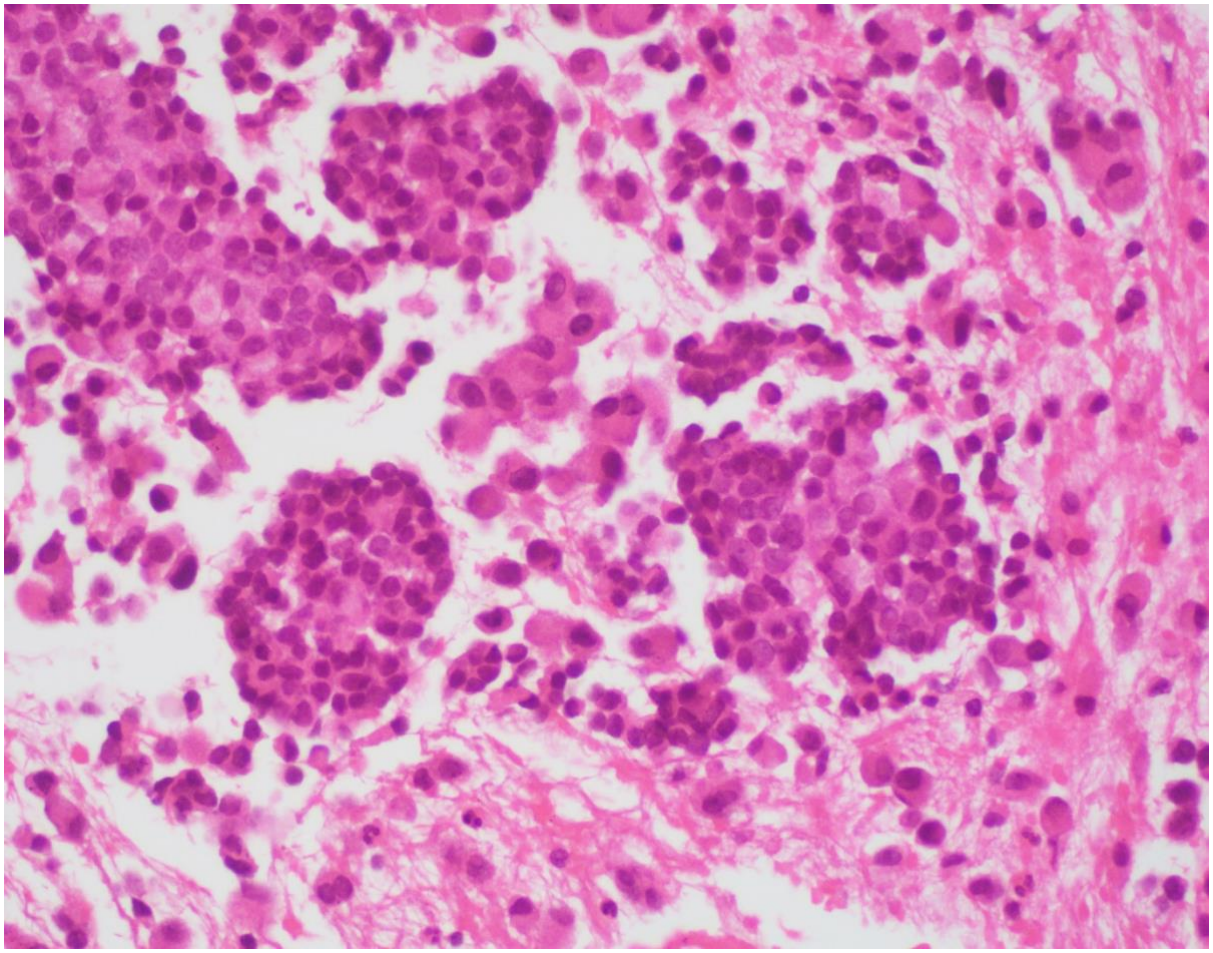

FIGURE 10. Invasive breast carcinoma with neuroendocrine differentiation showing cellular monomorphism and dense eosinophilic cytoplasm. A plasmacytoid morphology is also seen. (H&E, x200 magnification)

4.2.3 Stromal reaction

With regards to the background stromal reaction in IBCNED, the following alterations were noted (**FIGURE 11)**: stromal desmoplasia was seen in thirteen (37%, 13/35) cases, sclerotic stroma was noted in ten (29%, 10/35) cases **(FIGURE 16)**, fibroelastotic stroma was present in two (6%, 2/35) cases and mixed patterns were identified in ten (29%, 10/35) cases. Six (17%, 6/35) cases demonstrated coagulative necrosis **(FIGURE 17)**.

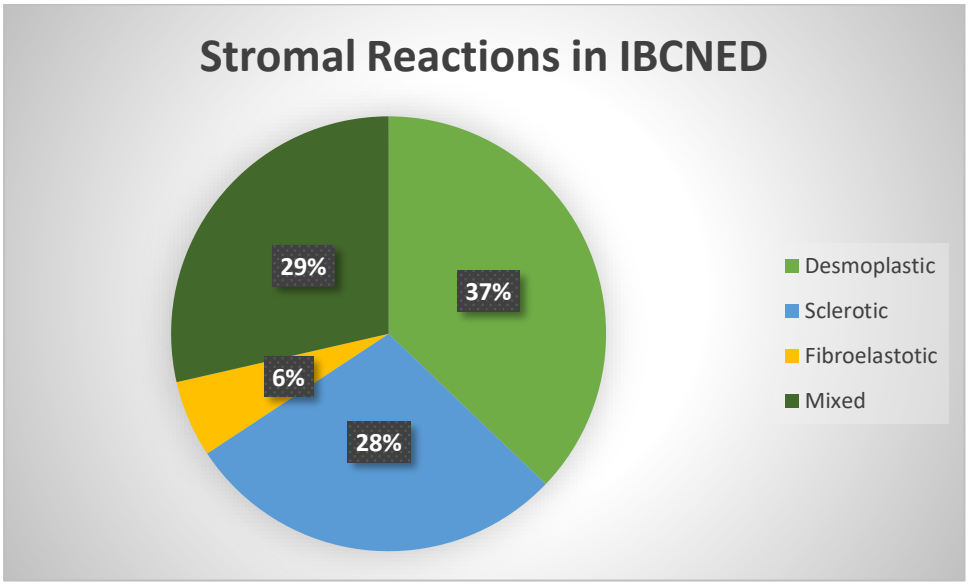

FIGURE 11. Stromal reactions in IBCNED.

The stromal reaction in NST showed the following: stromal desmoplasia was seen in twenty-seven (48%, 27/56) cases, sclerotic stroma was noted in fifteen (27%, 15/56) cases, fibroelastotic stroma was present in one (2%, 1/56) case and mixed patterns were identified in thirteen (23%, 13/56) cases. Eleven (20%, 11/56) cases demonstrated coagulative necrosis.

The stromal reaction between the two cohorts were similar (Fisher's exact; $p = 0.6$)

The commonest overall morphology in IBCNED was that of small- to medium solid cohesive groups of tumour cells as well as trabeculae/ribbons and glandular structures with polygonal-

oval cells showing granular eosinophilic cytoplasm and stippled chromatin. Stromal desmoplasia and sclerosis were common whilst fibroelastosis was seen less frequently.

The commonest overall morphology in NST was small- to medium-sized solid cohesive groups of tumour cells as well as trabeculae/ribbons and glandular structures with oval-polygonal cells showing vacuolated cytoplasm with even chromatin distribution. Desmoplastic and sclerotic stroma were common whilst fibroelastotic was less common.

4.2.5 Ductal carcinoma in situ, lymphovascular invasion, perineural invasion

IBCNED showed ductal carcinoma in situ in nine (3%, 9/35) cases, LVI in five (14%, 5/35) cases and PNI in one (3%, 1/35) case.

NST showed ductal carcinoma in situ in eight (14%, 8/56) cases, LVI in six (11%, 6/56) cases and PNI in one (2%, 1/56) case.

There was no statistically significant difference between IBCNED and NST in relation to DCIS (Fisher's exact; $p = 0.2$), LVI (Fisher's exact; $p = 0.6$) or PNI (Pearson Chi^2; $p = 0.9$).

TABLE 4. Histopathological features of IBCNED and NST.

	IBCNED	**NST**
Architecture		
Large / solid + cohesive	3 (9%)	7 (12%)
Small/medium/ribbons/glands	20 (57%)	43 (77%)
Mixed	12 (34%)	6 (11%)

Cell shape		
Oval-polygonal	27 (77%)	52 (93%)
Plasmacytoid	7 (20%)	3 (5%)
Spindled	1 (3%)	1 (2%)
Cytoplasm		
Eosinophilic/granular	27 (77%)	21 (38%)
Vacuolated	8 (23%)	35 (62%)
Chromatin		
Even / stippled	19 (54%)	27 (48%)
Prominent nucleoli	9 (26%)	21 (38%)
Mixed	7 (20%)	8 (14%)
Stroma		
Desmoplastic	13 (37%)	27 (48%)
Sclerotic	10 (29%)	15 (27%)
Fibroelastotic	2 (6%)	1 (2%)
Mixed	10 (29%)	13 (23%)
Necrosis present	6 (17%)	11 (20%)
DCIS present	9 (26%)	8 (14%)
LVI present	5 (14%)	6 (11%)

Perineural invasion present	1 (3%)	1 (2%)

4.3 Histologic subtype

All carcinomas with neuroendocrine differentiation showed morphology consistent with no special type and only two showed focal mucinous differentiation. Carcinomas without neuroendocrine differentiation were also predominantly of no special type, with 2 showing exclusively mucinous features, and one showing focal mucinous differentiation.

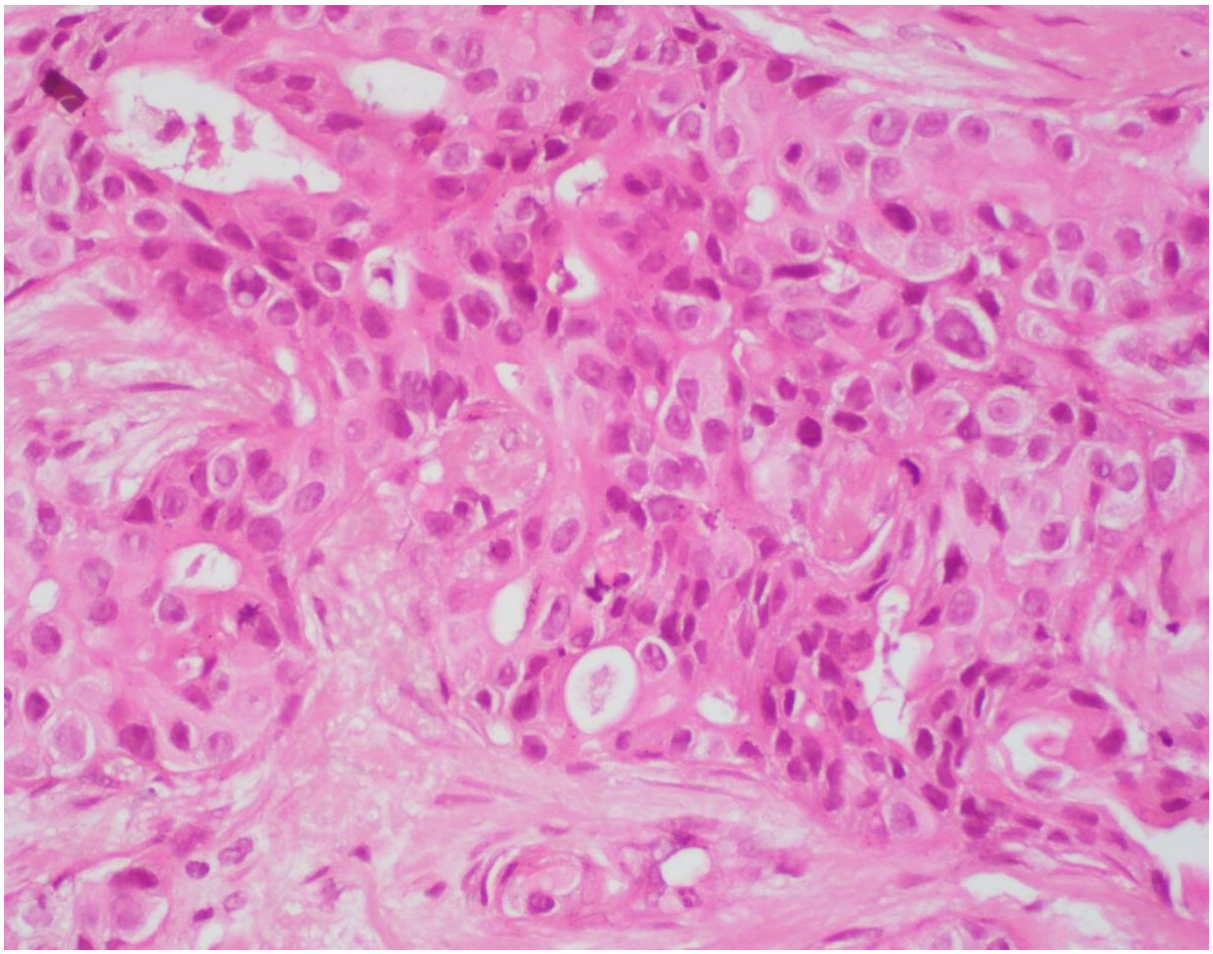

FIGURE 12. Invasive breast carcinoma of no special type with neuroendocrine differentiation. Glands and nests of invasive tumour surrounded by fibroelastotic stroma. The cytoplasm is abundant, eosinophilic and vacuolated. (H&E, x400 magnification)

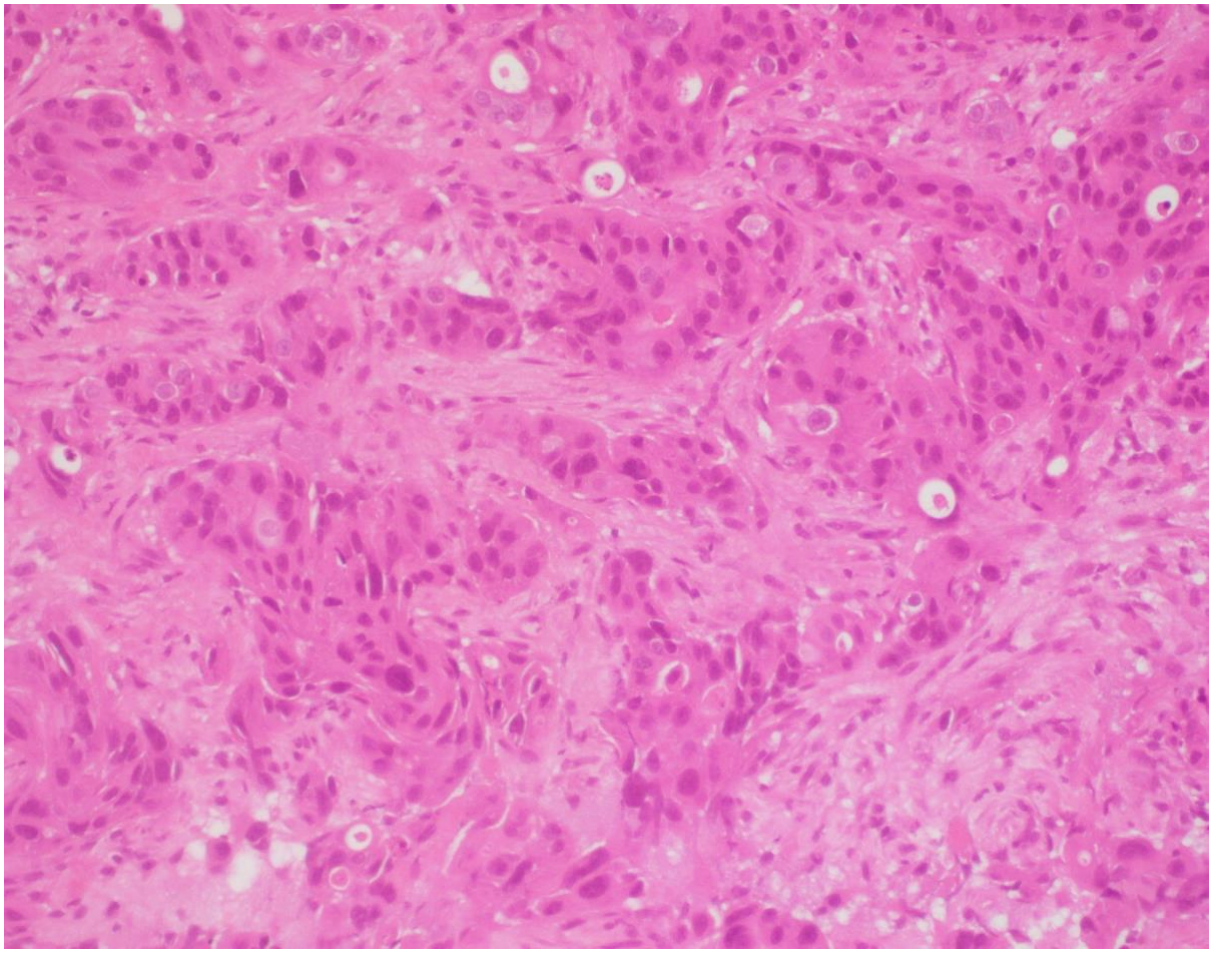

FIGURE 13. IBCNED with invasive nests and islands showing eosinophilic cytoplasm and numerous apoptotic bodies imparting a pseudoglandular appearance. (H&E, x200 magnification)

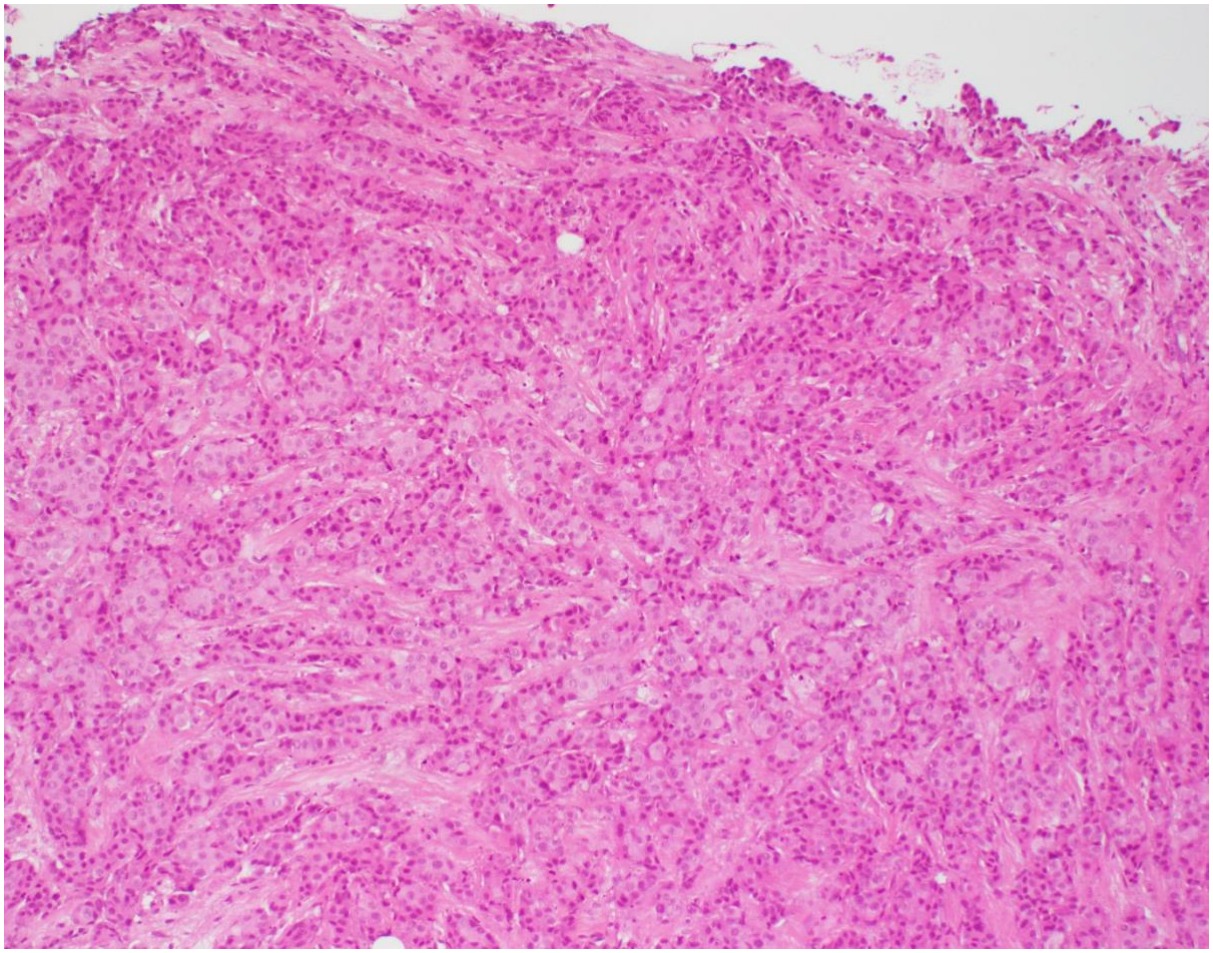

FIGURE 14. IBCNED showing cells with abundant vacuolated eosinophilic cytoplasm and surrounding desmoplastic stroma. (H&E, x100 magnification)

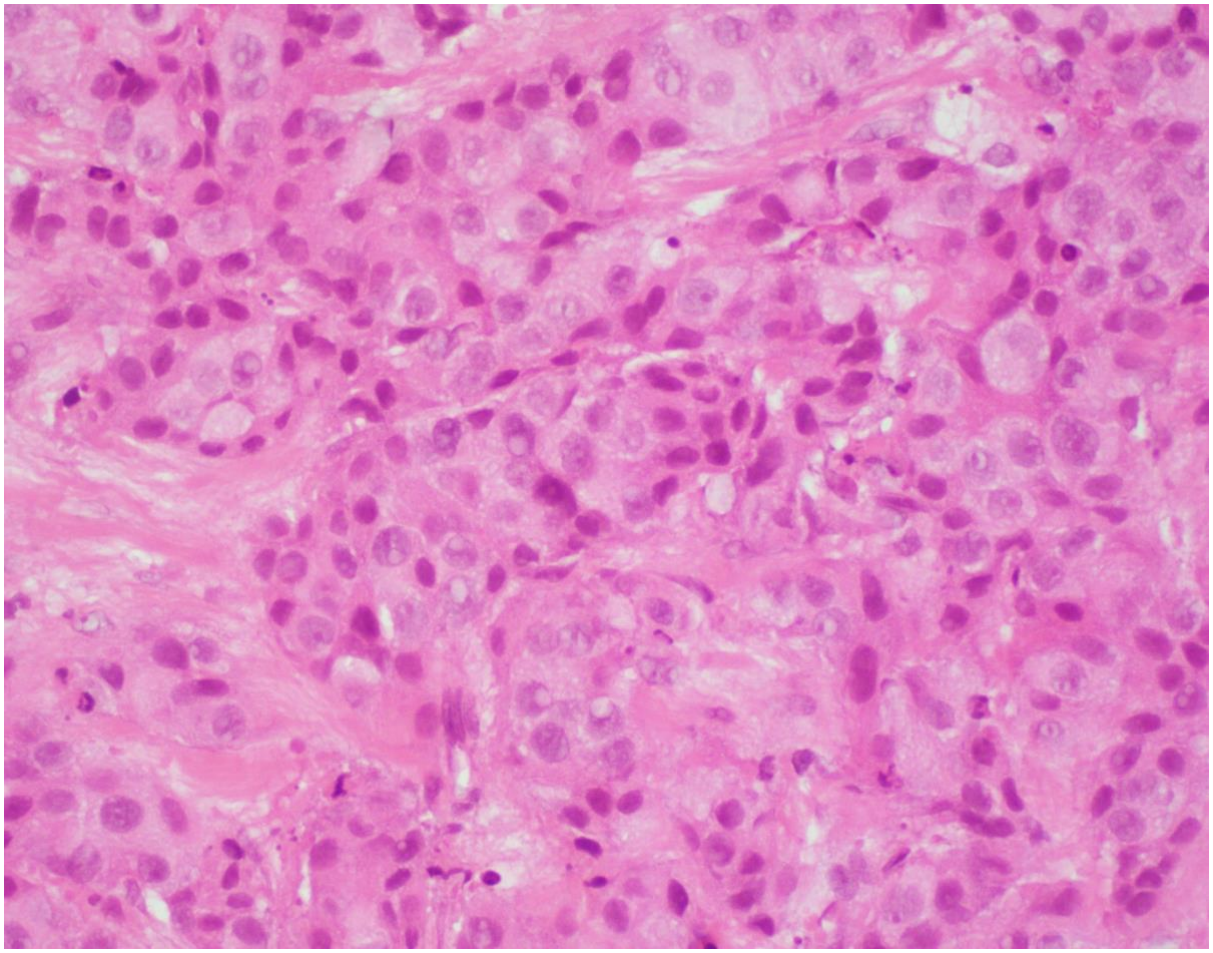

FIGURE 15. High power view of tumour from Figure 14. (H&E, x400 magnification)

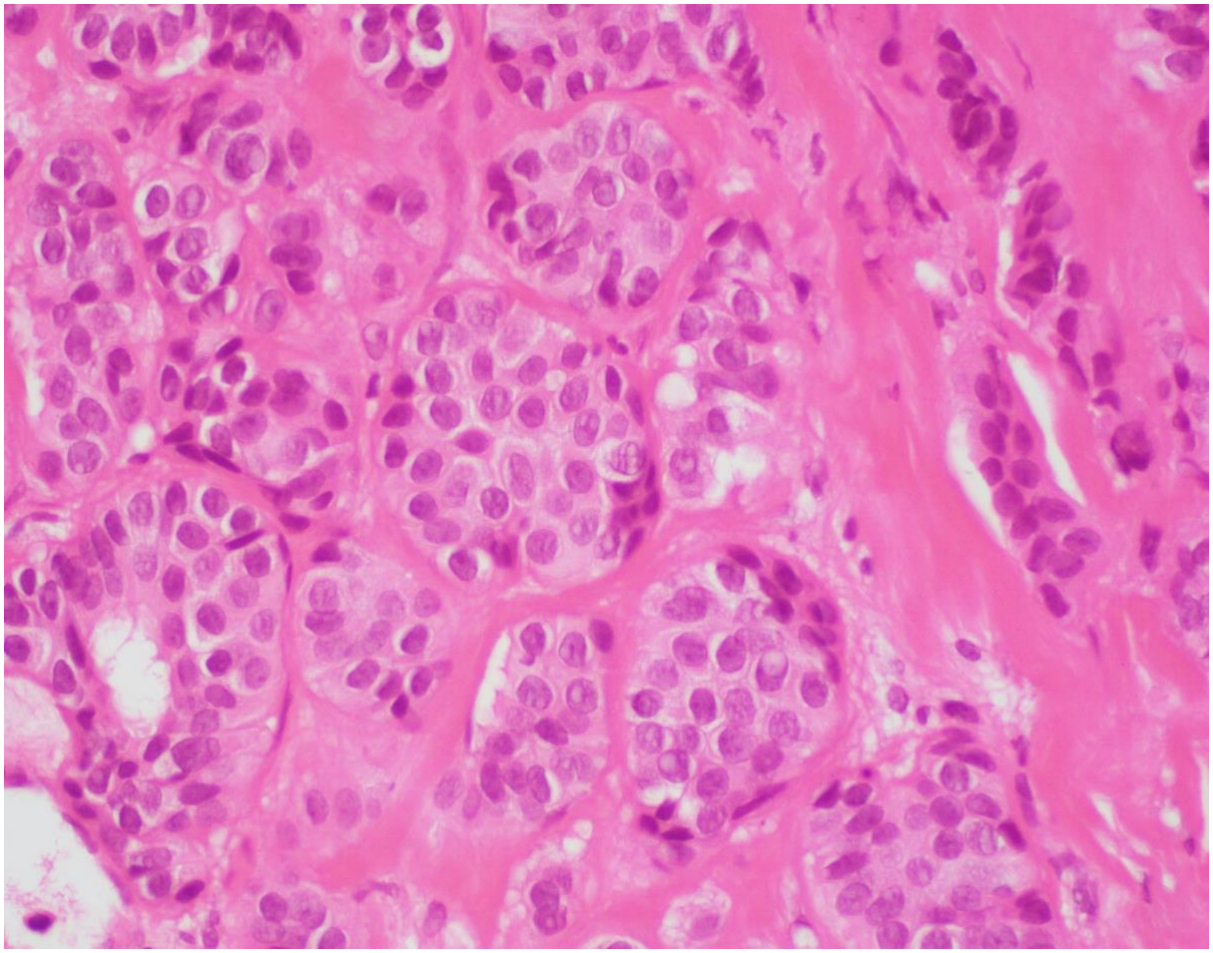

FIGURE 16. Compact nests and granular eosinophilic cytoplasm, suggesting neuroendocrine differentiation in IBCNED. Note the surrounding sclerotic stroma. (H&E, x400 magnification)

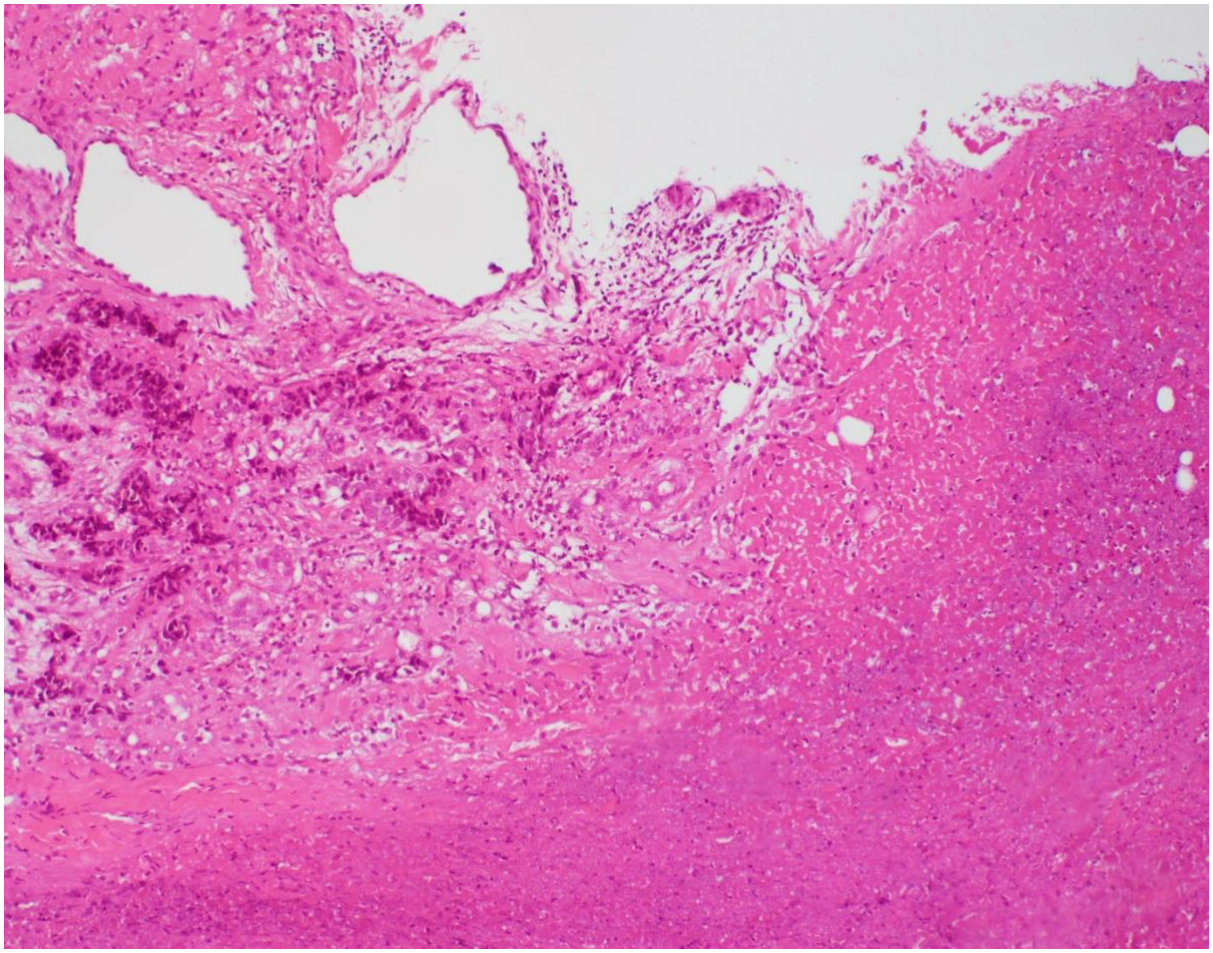

FIGURE 17. A case of IBCNED with coagulative necrosis. (H&E, x100 magnification)

4.4. Histologic grade

The distribution of histologic grade among the two subtypes were as shown in **TABLE 4.**

TABLE 5. Histologic grade of IBCNED and NST.

Histologic grade	**IBCNED**	**NST**
Grade 1	2 (6%)	2 (3%)
Grade 2	16 (46%)	30 (54%)
Grade 3	17 (48%)	24 (43%)

There was no statistically significant difference in histologic grade between IBCNED and NST (Fisher's exact; $p = 0.7$). The majority of both IBCNED and NST were moderately- to poorly-differentiated.

4.5 Molecular subtype

Invasive breast carcinomas with neuroendocrine differentiation showed a predominance of luminal B molecular subtype. Whilst the non-neuroendocrine tumours also favoured luminal B, the distribution of other subtypes was more widespread (**FIGURE 18)**:

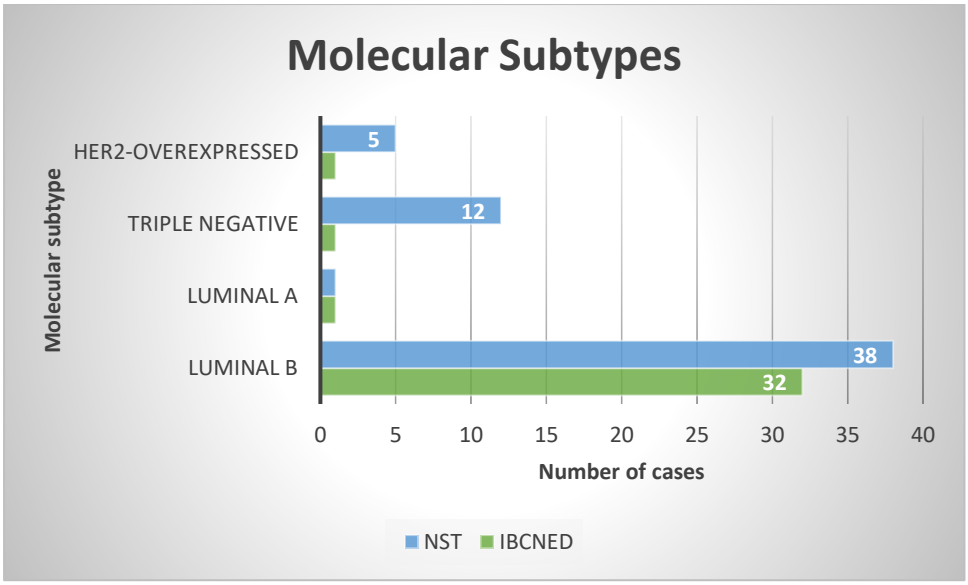

FIGURE 18. Molecular subtypes of IBCNED and NST.

Apart from one tumour with a HER2-enriched molecular subtype **(FIGURE 19)** and one with a triple negative profile, all the invasive beast carcinomas with neuroendocrine differentiation showed oestrogen receptor positivity **(FIGURE 20)**. Results for progesterone receptor were similar **(FIGURE 21)**, except that one tumour with a luminal B molecular subtype was negative for progesterone receptor. The Ki67 proliferation index **(FIGURE 22)** showed a mean value of 57% with a median of 70% and a range of 10-100%.

There was a statistically significant variation when comparing molecular subtypes between the two groups, in that IBCNED showed a strong predisposition for hormone receptor positivity (ER and PgR), particularly favouring luminal B (Fisher's exact; *p value = 0.01)*.

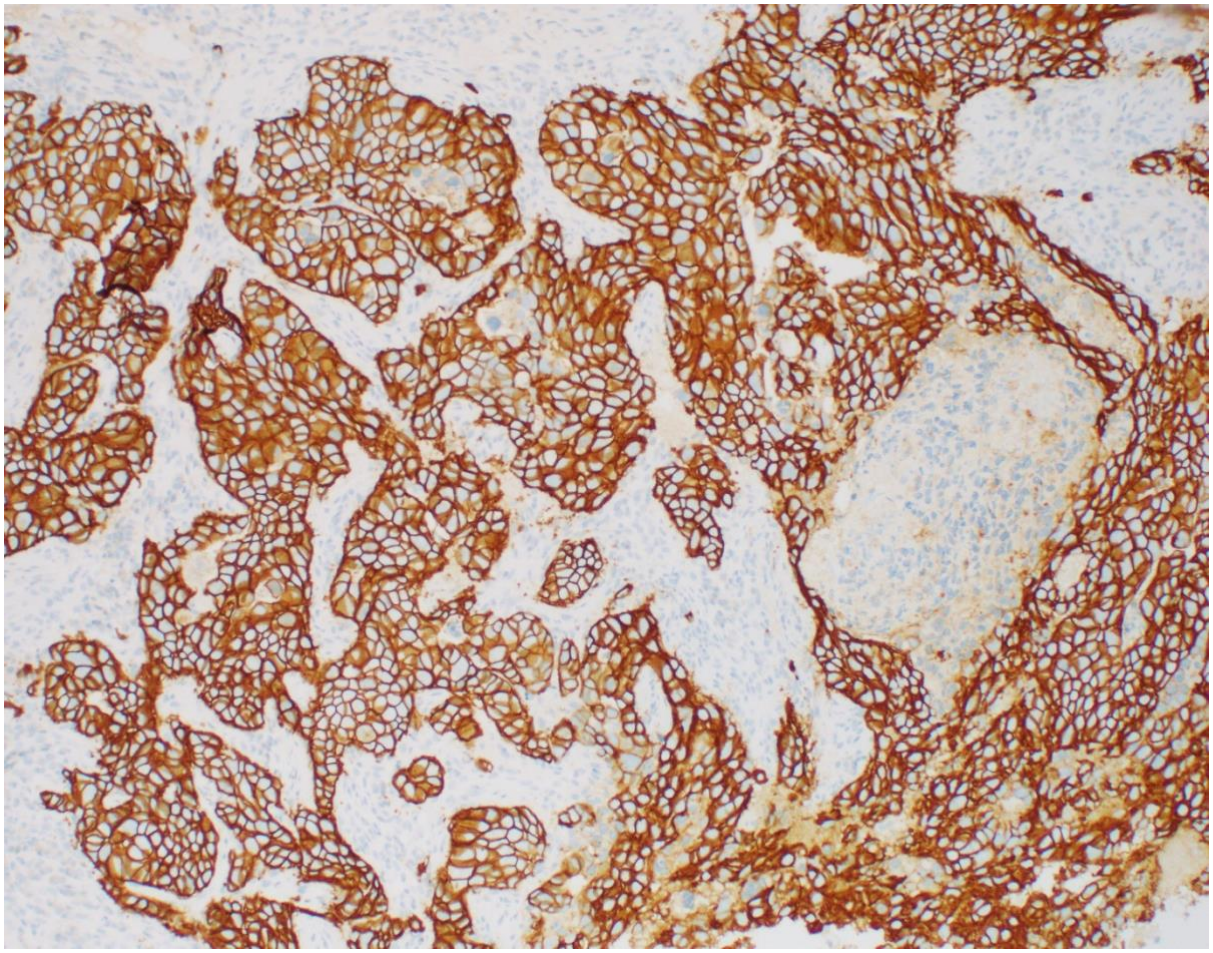

FIGURE 19. ASCO/CAP 3+ HER2 immunopositivity showing strong, continuous “rope-like” membranous staining in IBCNED. (x100 magnification)

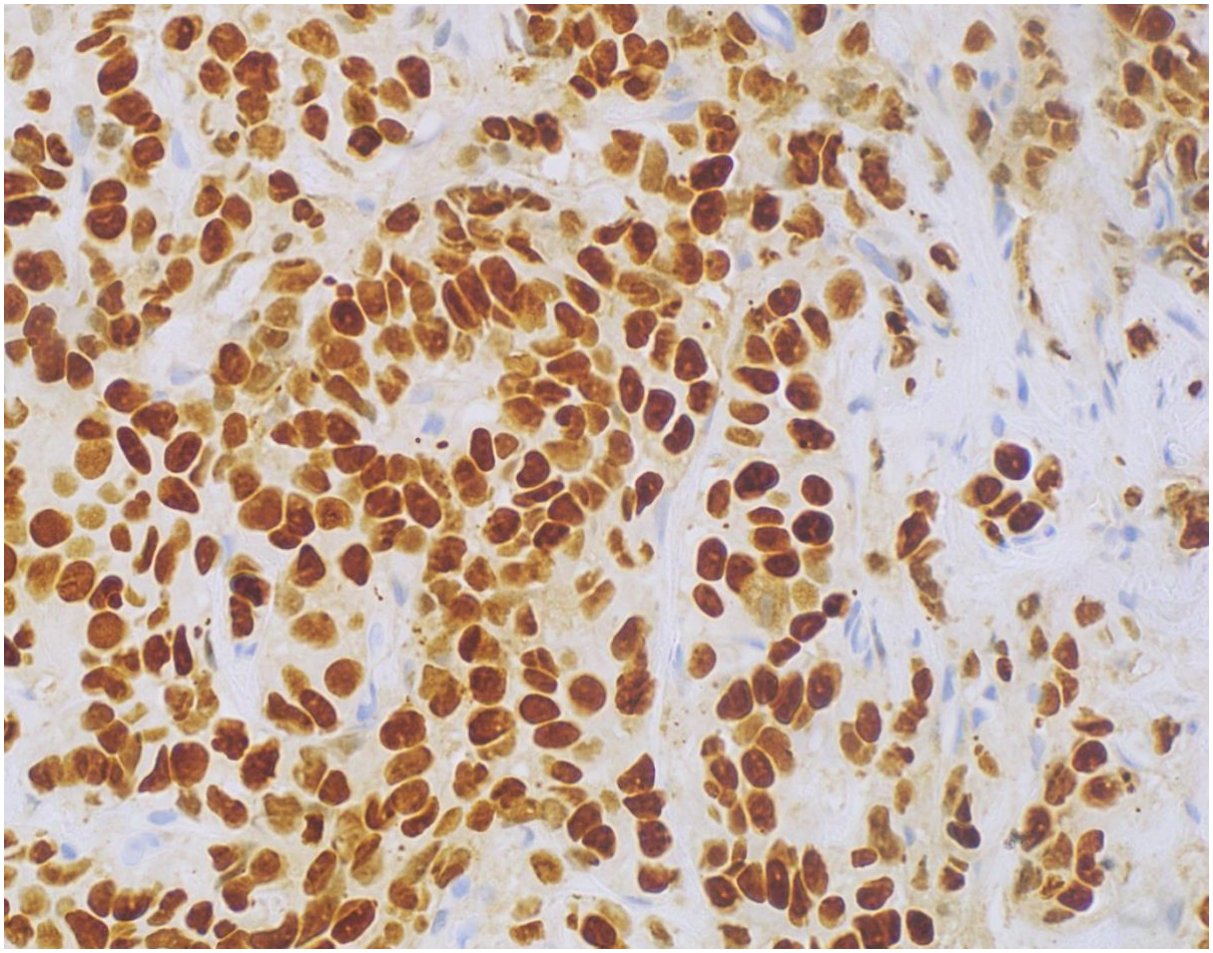

FIGURE 20. Strong oestrogen receptor nuclear positivity in IBCNED. (x400 magnification)

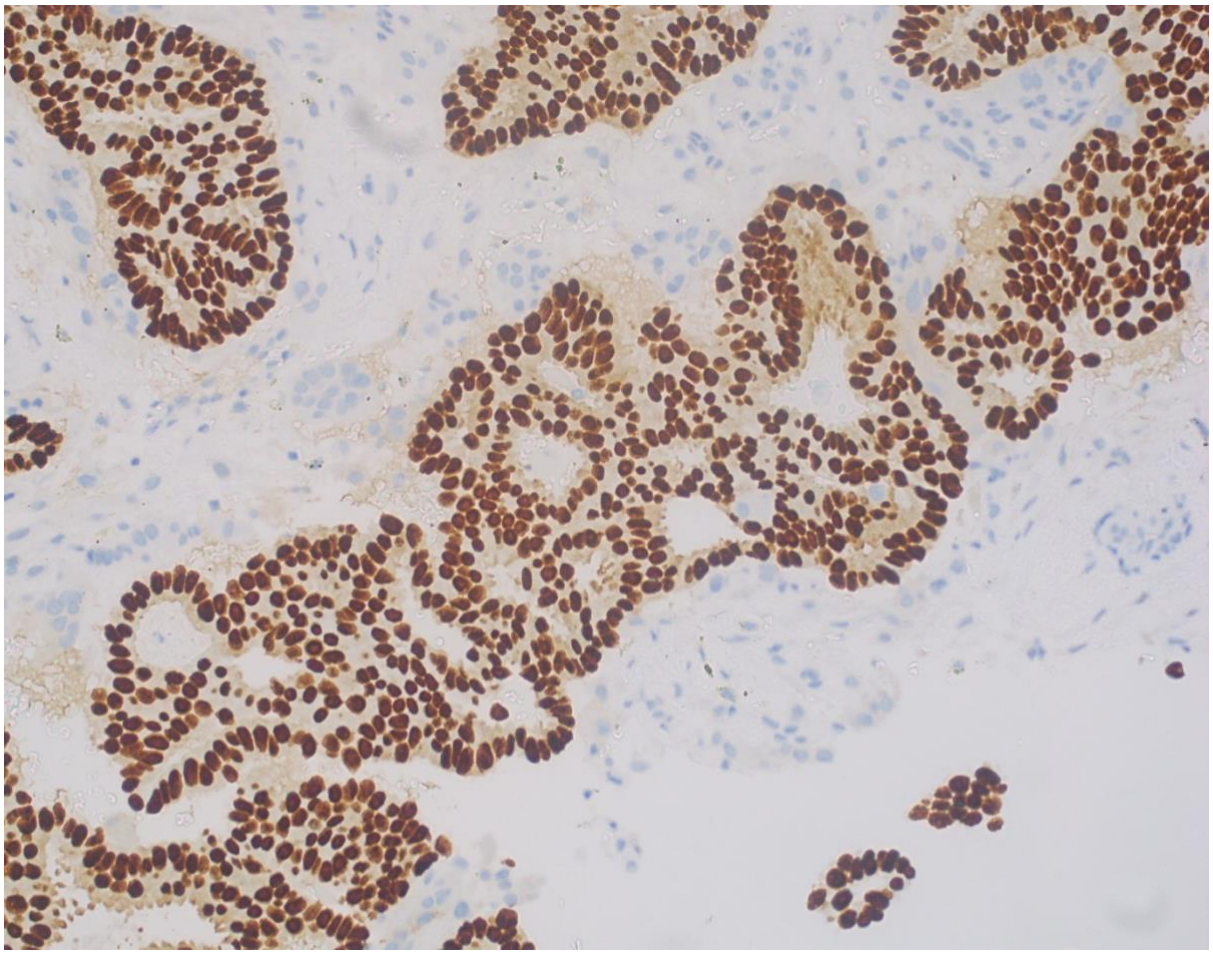

FIGURE 21. IBCNED with strong progesterone receptor positivity. (x400 magnification)

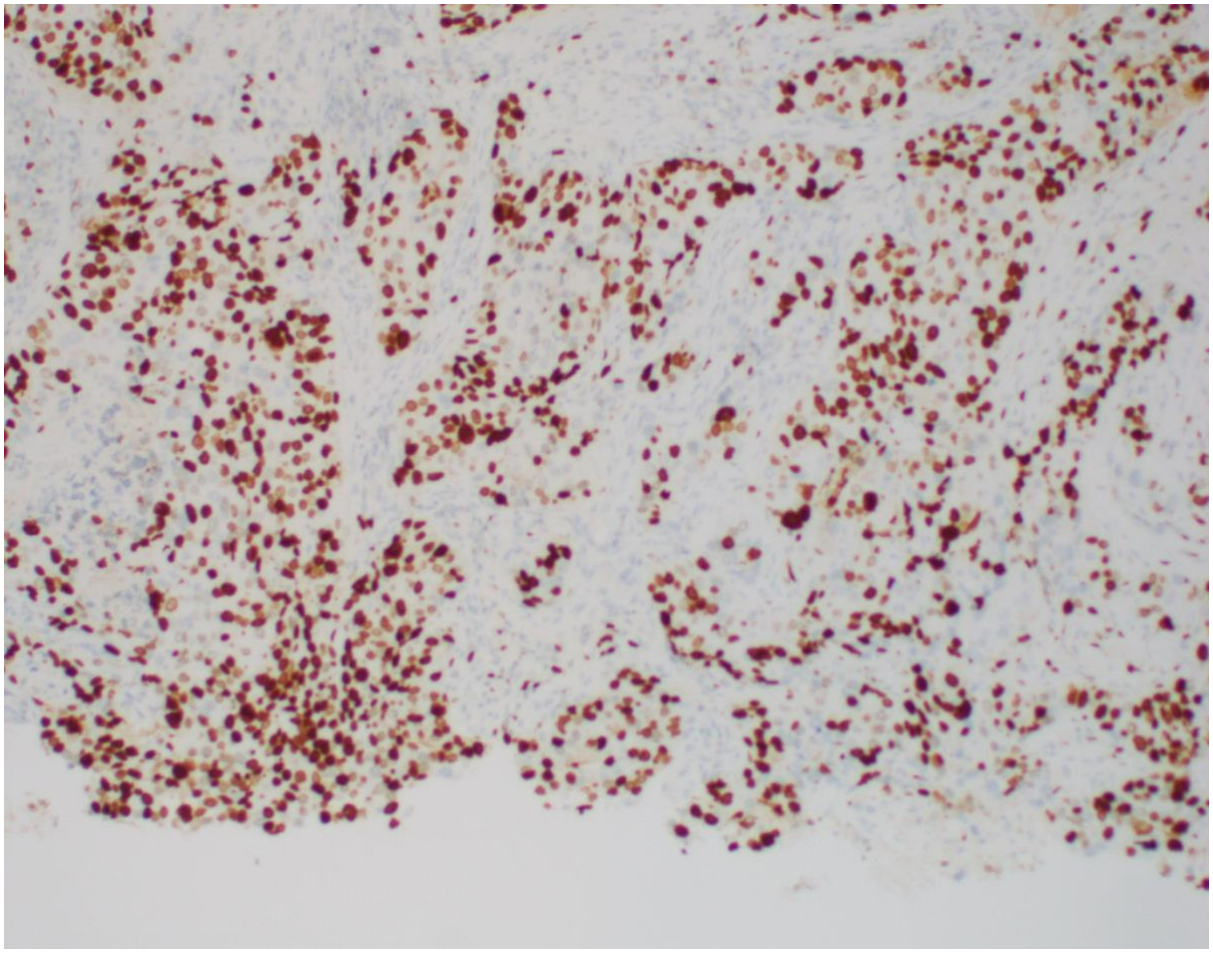

FIGURE 22. IBCNED with a high Ki67 proliferation index. (x100 magnification)

4.7 Electron microscopy

Dense core secretory granules were identified in the two cases of IBCNED (case 5 and case 91) in which ultrastructural studies were done. Case 5 showed numerous round cytoplasmic dense core granules ranging in size from 166nm to 944nm **(FIGURE 23)**. Case 91 displayed numerous round and oval dense core granules ranging in size from 171nm to 357nm **(FIGURE 24)**.

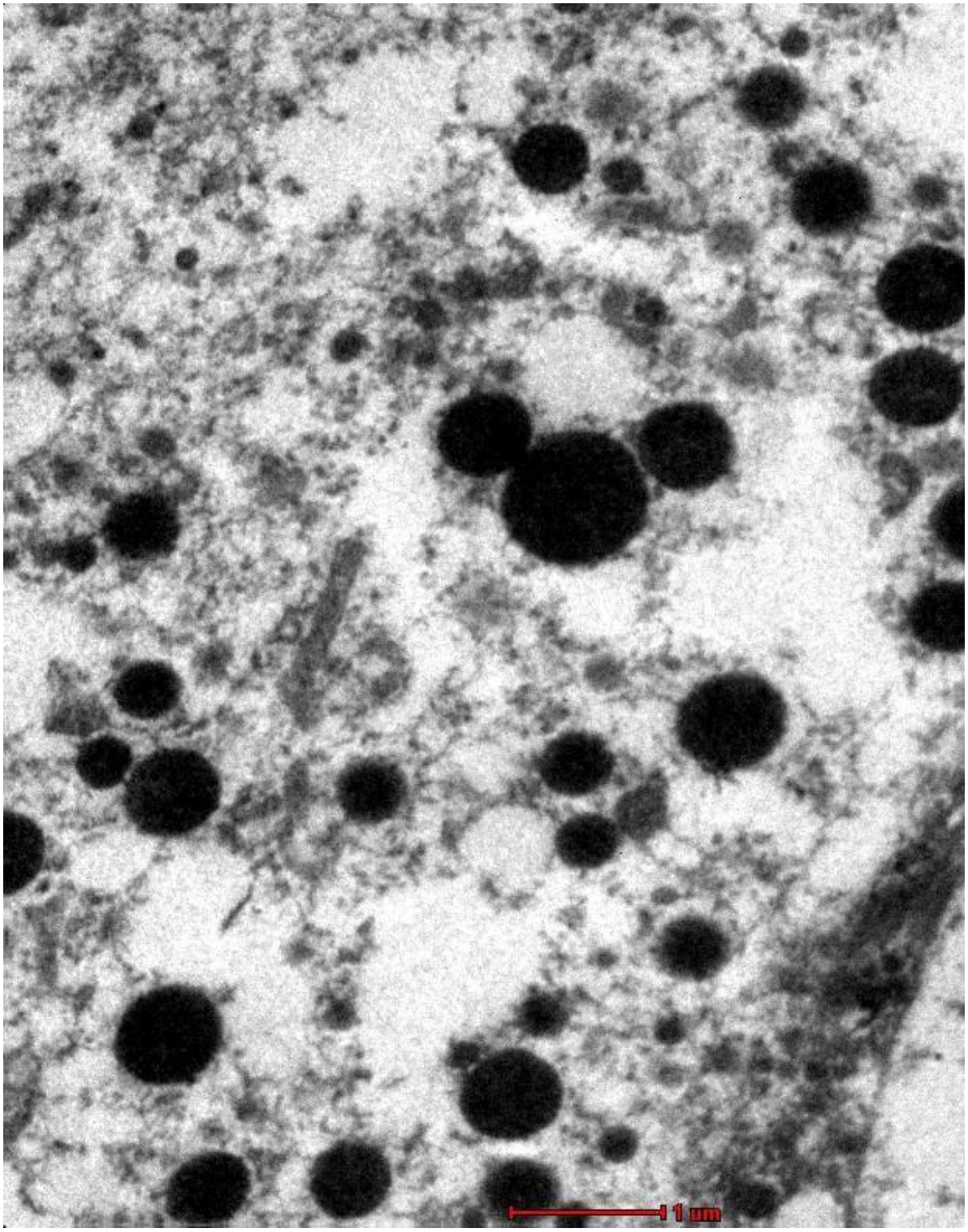

FIGURE 23. Numerous large dense core secretory granules showing a predominantly spherical shape. (x8200 magnification)

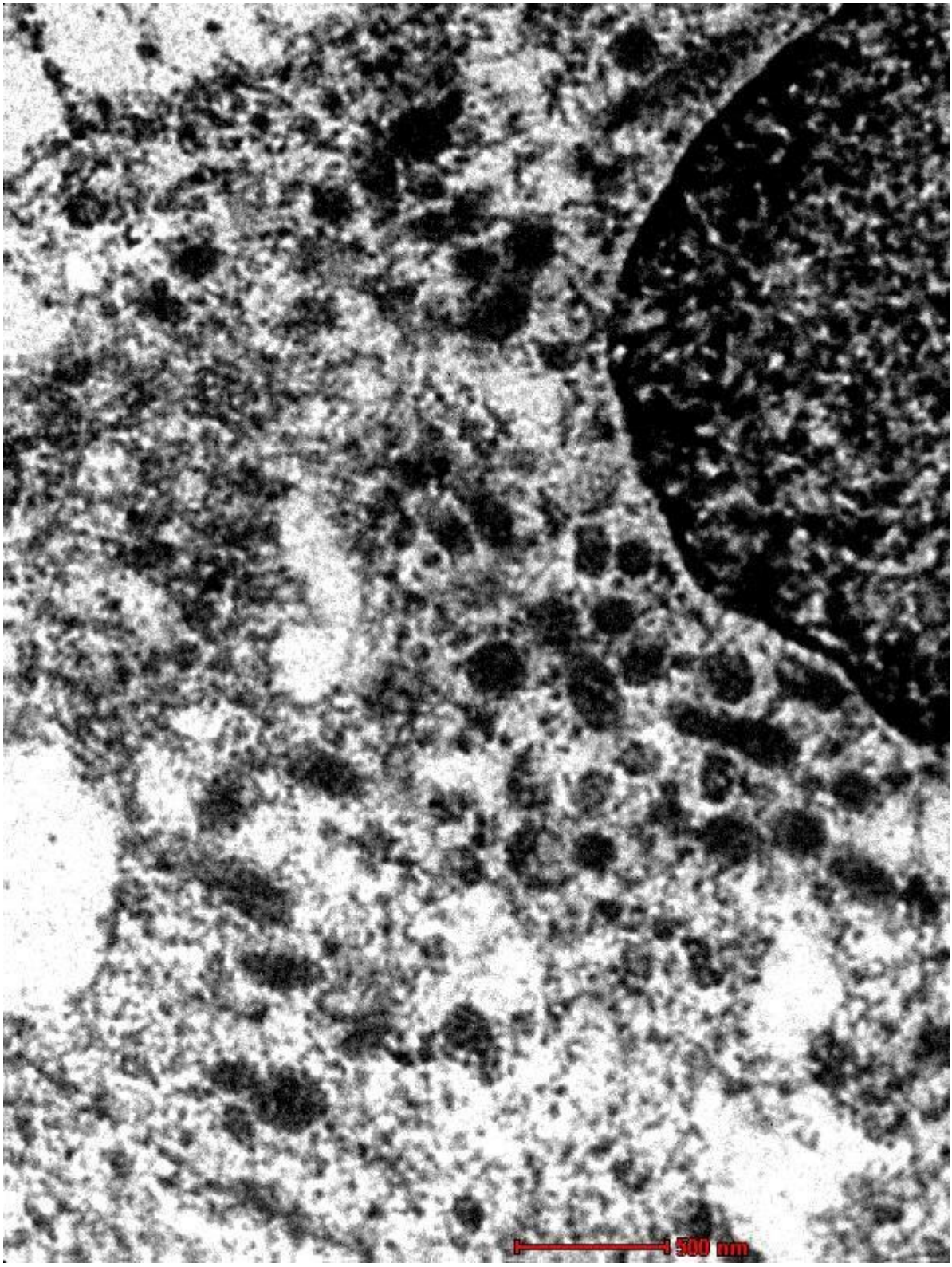

FIGURE 24. Multiple round and oval dense core secretory granules. (x16500 magnification)

CHAPTER 5

DISCUSSION

Invasive breast carcinomas are a heterogenous group of malignancies sharing origin from the mammary gland. The World Health Organization makes provision for numerous subtypes and variants, including those which demonstrate neuroendocrine differentiation(4). There have been many studies describing the different aspects of neuroendocrine breast carcinomas, however there is a paucity of literature which has analysed invasive breast carcinomas of no special type with neuroendocrine differentiation.

5.1 Incidence

The percentage of positive tumours in this study (38%) was significantly higher than the most current cited literature with the top end of the spectrum being 30%(7). One of the main reasons being adherence to the new WHO definition which places no cut-off value when assessing for neuroendocrine marker expression. Many other studies used a cut off value of 50% to categorise a tumour as having neuroendocrine differentiation(14, 15, 17, 47, 50). Because of the difference in criteria, it is expected that these cohorts would display a lower incidence of IBCNED. Numerous studies used a much lower criteria, ranging from a minimum of 1% to a minimum of 10%, but still only showed a maximum prevalence of 19.5%(19, 21-23, 51). Therefore, despite differences in criteria to define neuroendocrine differentiation, the study population in question appears to have a higher incidence of this tumour subtype. Additionally, it must be taken into consideration, that this study has a relatively small sample size which impacts the overall percentage of tumours showing neuroendocrine differentiation.

5.2 Neuroendocrine markers

Synaptophysin expression was higher than chromogranin A expression (100% vs 17%). Bogina et al(19) and Roininen et al(50) both showed 100% expression of synaptophysin with lower incidence of chromogranin A expression (59.3% and 69.8%), despite using different cut-off values for significant positivity (10% and 50%). The results in these studies are comparable to the ones from the current study. Bogina et al used different antibody clones for synaptophysin and chromogranin A, than used in this study. Information for the immunohistochemistry was not available for the study conducted by Roinenen et al. Three other studies showed variable expression of synaptophysin and chromogranin A, in that some cases showed only synaptophysin expression, only chromogranin A expression, or expression of both markers(21, 22, 46). This underscores the importance of utilising both synaptophysin and chromogranin A when assessing a tumour for neuroendocrine differentiation.

5.3 Gender

All patients with invasive breast carcinomas showing neuroendocrine differentiation were female, consistent with the international literature, which showed an overwhelming female predilection(14-16, 19, 52-54). Despite this, a larger sample size would infer a more accurate representation.

5.4 Age

The age range was 32-83 years with a mean age of 55 years and a median age of 54 years. There was no difference in age range between those tumours with and without

neuroendocrine differentiation (Pearson Chi²; $p = 0.5$). One study showed a similar age range (27-95 years) with a mean of 59 years(51). Another study showed a younger age range of 22-85 years with a median of 47(21). Other studies showed a slightly higher age range including 29-83 years with a median of 63(15);17-82 years with a mean of 59.2 and median of 61 years(47); 40-88 years with a median of 69.5 and a mean of 67.4 years(19). Other studies also showed a predilection of neuroendocrine differentiation to occur in the older age groups(22, 50, 53, 54). Overall, most of the research demonstrates that IBCNED tend to have a predilection for older patients compared to NST. The reason for the disparity of these findings with the reported cohort is unknown. Speculatively, the influence of HIV infection may be a factor. It has been shown in previous reports, that HIV positive patients tend to develop invasive breast carcinomas at a younger age(55). Additionally, general population characteristics such as mean/median age of the general population should be considered, as the NST group showed a similar age profile (Pearson chi²; $p = 0.5$).

5.5 Race/ethnicity

The Asian subpopulation showed a higher percentage of cases with neuroendocrine differentiation; however, this was not statistically significant due to the small sample size (Fisher's exact; $p = 0.11$). Most of the other cited literature did not specifically comment on race predilection. Wei et al(53) described a cohort which showed 80% of cases to be Caucasian and only 1% Asian. Wang et al(54) showed a similar ethnicity profile.

5.6 HIV infection

When comparing IBCNED to invasive breast carcinoma, NST, patients with IBCNED were much less likely to be HIV positive (Fisher's exact; $p = 0.006$). The one confounding factor

was the large number of cases in which the HIV status was unknown. To our knowledge, there have been no reports describing the association of HIV and IBCNED.

5.7 Histologic subtype

It has been shown that certain special histologic types of invasive breast carcinoma have a proclivity for neuroendocrine differentiation. The two commonest are type B invasive mucinous carcinoma (hypercellular variant) and solid papillary carcinoma. This cohort did not recapitulate these findings. Of five cases showing a mucinous component (2 focal, and 2 diffuse), only 2 showed neuroendocrine marker expression. There was no case of solid papillary carcinoma reported during the study period. Due to the few number of cases of these special subtypes, a definite conclusion cannot be made. A large study(19) showed similar findings in that the predominant histologic subtype is that of no special type. However, they did report a larger percentage of special types including lobular (4%), mucinous (6%) and solid papillary (16%). Other studies confirm the majority of cases to be of no special type(14, 15, 17, 21, 22).

5.8 Histologic grade

Most cases were of intermediate or high grade (moderate to poorly differentiated). Wang et al also showed that breast carcinomas with neuroendocrine differentiation tend to be grade 3(54).One study reported on the histologic grade(19) and showed a similar distribution, however this current cohort did show a higher percentage of grade 3 tumours than reported in that aforementioned study. Other studies showed similar findings with regards to histologic grade, in that breast carcinomas with neuroendocrine differentiation tend to be grade 2 or 3, the majority reporting a predominance of grade 2 tumours(21, 22, 53). Most invasive breast carcinomas are also classified as grade 2 tumours(14, 17, 19). In this studied population, both

IBCNED and NST showed a preponderance for grade 2/3 histologic grade with no difference between the two (Fisher's exact; $p = 0.7$).

5.9 Histomorphology

IBCNED tended to show large cohesive groups of tumour cells more commonly than NST (Fisher's exact; $p = 0.03$). Additionally, granular eosinophilic cytoplasm was more common in IBCNED whilst vacuolated cytoplasm was more common in NST (Pearson chi^2; $p<0.001$). These findings suggest that IBCNED do show some morphologic differences compared with invasive breast carcinomas of no special type. One study described that invasive breast carcinomas with neuroendocrine differentiation often lack cytomorphological evidence of the latter(56). However, to our knowledge, no other studies have performed a direct comparison between invasive breast carcinomas with neuroendocrine differentiation and those without. Additionally, the sample size in this study was relatively small and a larger cohort would be necessary to confirm these findings.

5.10 Molecular subtype

Within the confirmed cases of invasive breast carcinoma with neuroendocrine differentiation, an overwhelming majority showed a luminal B molecular subtype (91.4%). There was only one of each of the other major molecular subtypes present, comparatively less than what was seen in the group without neuroendocrine differentiation (Fisher's exact; $p = 0.01$). Other studies confirmed a predilection for luminal molecular subtype(14, 17, 19, 21, 47, 50, 53). One study showed a predominance of luminal A molecular subtype (91%)(14) whilst another showed a predominance of luminal B(19). Although the latter study showed a preponderance of luminal B molecular subtype, the percentage was much lower than the current cohort (51% vs 91%).

5.11 Electron microscopy

The electron microscopic features of IBCNED are similar to those of other tumours showing neuroendocrine differentiation, with the defining element for neuroendocrine differentiation being the identification of electron dense secretory granules(30-32). Cost-effectiveness evaluation studies have shown that electron microscopy is a less expensive diagnostic modality if more than three antibodies are necessary to arrive at the correct pathological diagnosis(32). It is important to note, however, that ultrastructural studies are no longer considered part of routine workup for neuroendocrine tumours.

5.12 Treatment

In view of the predominance of luminal B molecular subtype in IBCNED, treatment recommendations for invasive breast carcinomas were reviewed. Current recommendations(57) suggest that certain breast carcinoma molecular subtypes are more chemo-sensitive (HER2-enriched and triple negative). Chemotherapy is also indicated for luminal B-like tumours; however, the absolute benefit of chemotherapy is more pronounced in ER-negative tumours. The benefit of neoadjuvant therapy is multiple and includes less extensive surgical treatment (wide local excision rather than mastectomy) with better cosmetic outcome and prognostication. Additionally, chemotherapy is utilised in locally advanced and metastatic breast carcinomas. It seems then, as detailed above, that although chemotherapy is a mainstay treatment for luminal B-like carcinomas, it tends to respond less effectively than HER2-enriched and triple negative subtypes. In this report, the study population shows a high incidence of invasive breast carcinoma with neuroendocrine differentiation, the vast majority of which display a luminal B molecular subtype. Hence, it follows to ask the question, in view of these unique attributes affecting this specific tumour

subtype and the inherent biological characteristics (presence of neuroendocrine differentiation), whether other treatment modalities may be potentially feasible. This notion is submitted with the following taken into consideration: the current standard chemotherapy for breast carcinomas utilize anthracycline and/or taxane based regimens(57). In selected patients, cyclophosphamide/methotrexate/5-fluorouracil (CMF) may be used. However, 4 cycles of doxorubicin and cyclophosphamide show equal efficacy to 6 cycles of CMF. Additionally, sequential used of anthracyclines and taxanes is more efficacious and less toxic than concomitant use. The standard chemotherapy regimens for neuroendocrine carcinomas, whether extrapulmonary or of lung origin, tends to be platinum-based and includes cisplatin and etoposide(58-60). Ergo, the consideration of adding to current treatment regimens for invasive breast carcinomas, by including chemotherapeutic agents effective against neuroendocrine carcinomas, should be duly considered. A search of the current literature yielded no results regarding the use of neuroendocrine-specific chemotherapeutic agents in IBCNED. Therefore, the findings of this study, apart from the initial objectives, may provide a steppingstone for a randomized clinical trial to assess the potential for novel treatment regimens for invasive breast carcinomas with neuroendocrine differentiation.

CHAPTER 6

CONCLUSION & RECOMMENDATIONS

With regards to our initial objectives, it has been shown that the study population in question has a higher incidence of invasive breast carcinomas with neuroendocrine differentiation than that which is cited in the international literature. IBCNED show a heterogenous histomorphology comparable to that seen with invasive breast carcinomas of no special type, however they do have distinct cytomorphological features. These tumours have a predominantly moderate to high histologic grade, with a preponderance of luminal B molecular subtype and high Ki67 proliferation indices. Despite these compelling findings, a larger cohort would certainly improve the validity of these results.

Further recommendations include a larger clinicopathological study to assess the association of HIV infection and to determine any potential race predilection. Additionally, evaluation of treatment-related objectives could potentially provide new treatment regimens for this poorly recognised, yet evidently increasingly prevalent, category of invasive breast carcinomas.

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APPENDIX 1: DATA CAPTURE TOOL

CASE NO.

DATA SHEET

Age:

Sex:

Ethnicity:

HIV status:

Immunohistochemical profile

ER	PR	HER2	HER2 FISH	Ki67	Synaptophysin	Chromogranin A

SUBCLASSIFICATION

Molecular subtype	Histologic grade	Histologic subtype

OTHER FINDINGS

Ductal carcinoma in situ	Lymphovascular invasion	Perineural invasion	Necrosis

APPENDIX 1: DATA CAPTURE TOOL (Continued)

Architecture

Large / solid + cohesive	Small/medium/ribbons/glands	Mixed

Cell shape

Oval-polygonal	Plasmacytoid	Spindled

Cytoplasm

Eosinophilic/granular	Vacuolated

Chromatin

Even / stippled	Prominent nucleoli	Mixed

Stroma

Desmoplastic	Sclerotic	Fibroelastotic	Mixed

APPENDIX 2: ETHICS APPROVAL

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 October 2020

Dr Nimallen Naicker (207502003)
School of Lab Med & Medical Sc
Medical School

Dear Dr Naicker,

Protocol reference number: BREC/00001851/2020
Project title: A Morphologic and Immunohistochemical Appraisal of Invasive Breast Carcinomas with Neuroendocrine Differentiation
Degree: MMed

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 11 October 2020. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations dated 26th August 2020, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_2_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 11 October 2020. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 10 November 2020.

Yours sincerely,

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

APPENDIX 3: GATEKEEPER APPROVAL

UNIVERSITY OF KWAZULU-NATAL INYUVESI YAKWAZULU-NATALI COLLEGE OF HEALTH SCIENCES

National Health Laboratory Service
Department of Anatomical Pathology
Laboratory Building, Level 3
Inkosi Albert Luthuli Central Hospital
800 Vusi Mzimela (Bellair) Road, Mayville, 4091
Private Bag X03, Mayville, 4058
Tel: +27 (0)31 240 2693 Fax: +27 (0)31 240 2610

NATIONAL HEALTH LABORATORY SERVICE
Practice No. 5200296

30/09/2020

To whom it may concern,

Request for permission to conduct a study:

Title of research project: A Morphologic and Immunohistochemical Appraisal of Invasive Breast Carcinomas with Neuroendocrine Differentiation.

I, Dr Nimallen Naicker, am employed by NHLS KZN Academic Complex and The University of Kwazulu-Natal in the department of Anatomical Pathology, as a registrar. I would like to apply for permission to conduct a retrospective study pertaining to the entity described in the title above. As part of routine immunohistochemical staining protocols, I have noticed that numerous invasive breast carcinomas show neuroendocrine marker expression. There has not been a study conducted in South Africa to determine the incidence and further categorization of such tumours. This study is a starting point that can serve as launch pad for additional studies to evaluate this subset of cancer. I have received provisional approval from BREC.

The supervisor for this study is Dr GB Nhlonzi, Department of Anatomical Pathology, NHLS at IALCH and member of staff at UKZN.

[redacted]

Dr N Naicker (Principle Investigator)

[redacted]

Dr GB Nhlonzi (Supervisor)

Supported / Not supported

[redacted]

Dr MZ Msimang (Acting HOD - Anatomical Pathology)
30/09/2020

APPENDIX 4: CLINICOPATHOLOGICAL FEATURES

Case Number	DEMOGRAPHICS	HIV Status	ER	PgR	HER2 IHC	HER2 FISH	Ki67 (%)	SYNAPTOPHYSIN	CHROMOGRANIN A	MOLECULAR SUBTYPE	HISTOLOGIC SUBTYPE	HISTOLOGIC GRADE
1	F, A, 47	Negative	-	-	+	N/A	30%	-	-	HER2 overexpression	NST	2
2	F, C, 32	Negative	+	+	-	N/A	80%	-	-	Luminal B	NST, extracellular mucinous component	3
3	F, A, 52	Unknown	-	-	-	N/A	90%	-	-	Triple - (basal-like)	NST	3
4	F, A, 43	Negative	+	+	+	N/A	90%	50%	-	Luminal B	NST	3
5	F, A, 86	Unknown	+	+	2+	Negative	80%	90%	15%	Luminal B	NST	3
6	F, A, 83	Unknown	+	+	1+	N/A	80%	40%	-	Luminal B	NST	3
7	F, I, 60	Unknown	+	+	2+	N/A	70%	20%	-	Luminal B	NST	3
8	F, I, 58	Negative	+	+	2+	Negative	30%	100%	-	Luminal B	NST	2
9	F, I , 46	Negative	+	+	1+	N/A	20%	-	-	Luminal B	NST	2
10	F, A, 36	Negative	+	+	2+	Negative	80%	15%	-	Luminal B	NST	3
11	F, A, 43	Positive	-	-	1+	N/A	70%	-	-	Triple - (basal-like)	NST	2
12	F, I, 73	Negative	-	+	0+	N/A	90%	-	-	Luminal B	NST	2
13	F, A, 67	Unknown	+	-	0+	N/A	60%	-	-	Luminal B	NST	2
14	F, A, 57	Negative	+	+	1+	N/A	60%	-	-	Luminal B	NST	2
15	F, A, 83	Unknown	+	+	0+	N/A	70%	-	-	Luminal B	NST	2
16	F, A, 53	Unknown	-	-	0+	N/A	100%	-	-	Triple - (basal-like)	NST	2
17	F, A, 39	Unknown	-	-	0+	N/A	90%	-	-	Triple - (basal-like)	NST	3
18	F, A, 81	Negative	-	-	0+	N/A	100%	-	-	Triple - (basal-like)	NST	3
19	F, A, 71	Negative	-	-	0+	N/A	60%	-	-	Luminal B	NST	2
20	F, I, 52	Negative	+	+	1+	N/A	60%	95%	-	Luminal B	NST	3
21	F, A, 44	Unknown	+	+	1+	N/A	60%	10%	-	Luminal B	NST	3
22	F, A, 47	Negative	+	+	2+	Negative	100%	-	-	Luminal B	NST	3
23	F, I 51	Negative	+	+	1+	N/A	95%	10%	-	Luminal B	NST	3
24	F, A, 32	Unknown	+	+	1+	N/A	10%	10%	-	Luminal A	NST	1
25	F, A, 62	Unknown	+	+	3+	N/A	60%	15%	-	Luminal B	NST	3
26	F, A, 50	Positive	+	+	0+	N/A	10%	-	-	LuminalA	Type B invasive mucinous carcinoma	1
27	F, A, 76	Unknown	+	+	1+	N/A	70%	-	-	Luminal B	NST	2
28	F, A, 82	Negative	-	-	0+	N/A	75%	70%	-	Triple - (basal-like)	NST	3
29	F, I, 58	Negative	+	+	1+	N/A	30%	10%	-	Luminal B	NST	2
30	F, A, 66	Positive	+	+	0+	N/A	50%	-	-	Luminal B	NST	2
31	F, A, 82	Negative	+	+	0+	N/A	100%	2%	10%	Luminal B	NST	3
32	F, A, 41	Unknown	-	-	0+	N/A	90%	-	-	Triple - (basal-like)	NST	3
33	F, C , 37	Negative	-	-	0+	N/A	80%	-	-	Triple - (basal-like)	NST	3
34	F, A, 56	Positive	+	+	1+	N/A	50%	30%	-	Luminal B	NST	2
35	F, I, 58	Negative	-	-	0+	N/A	80%	-	-	Triple - (basal-like)	NST	3
36	F, I, 83	Negative	+	+	0+	N/A	20%	100%	-	Luminal B	NST	2
37	F, A, 95	Negative	+	+	1+	N/A	60%	40%	-	Luminal B	NST, focal mucinous	2
38	F, A, 69	Negative	+	+	1+	N/A	60%	10%	-	Luminal B	NST	2
39	F, A, 41	Positive	+	+	0+	N/A	50%	-	-	Luminal B	NST	2
40	F, C, 63	Negative	+	+	1+	N/A	25%	10%	-	Luminal B	NST	2
41	F, A, 78	Negative	-	-	1+	N/A	90%	-	-	Triple - (basal-like)	NST	3
42	F, A, 50	Unknown	+	+	1+	N/A	100%	-	-	Luminal B	NST	3
43	F, I, 62	Negative	+	+	0+	N/A	30%	40%	-	Luminal B	NST	2
44	F, A, 24	Negative	+	+	1+	N/A	80%	70%	90%	Luminal B	NST	2
45	F, A, 53	Negative	+	+	0+	N/A	90%	15%	-	Luminal B	NST	3
46	F, A, 37	Negative	+	+	1+	N/A	50%	10%	-	Luminal B	NST	2
47	F, A, 74	Negative	+	+	1+	N/A	20%	5%	5%	Luminal B	NST	2
48	F, I, 62	Negative	+	+	0+	N/A	30%	40%	-	Luminal B	NST	2
49	F, A, 43	Negative	+	+	3+	N/A	70%	-	-	Luminal B	NST	3
50	F, A, 72	Unknown	+	+	0+	N/A	40%	10%	-	Luminal B	NST	3
51	F, A, 38	Negative	+	+	1+	N/A	90%	90%	-	Luminal B	NST	3
52	F, A, 78	Negative	+	+	0+	N/A	25%	-	-	Luminal B	NST	2
53	F, A, 35	Negative	+	+	0+	N/A	15%	-	-	Luminal B	NST	2
54	F, A, 72	Positive	-	-	3+	N/A	40%	-	-	HER2 overexpression	NST	3
55	F, A, 43	Negative	+	+	1+	N/A	90%	-	-	Luminal B	NST	3
56	F, A, 54	Positive	+	+	0+	N/A	50%	-	-	Luminal B	NST	2
57	F, A, 32	Positive	+	+	0+	N/A	90%	-	-	Luminal B	NST	2
58	F, A, 38	Negative	-	-	0+	N/A	100%	-	-	Triple - (basal-like)	NST	2
59	F, A, 56	Negative	-	-	3+	N/A	50%	10%	-	HER2 overexpression	NST	2
60	F, A, 66	Negative	+	+	1+	N/A	80%	5%	-	Luminal B	NST	2
61	F, A, 32	Negative	-	-	0+	N/A	100%	-	-	Triple - (basal-like)	NST	3
62	F, A, 51	Positive	+	+	1+	N/A	60%	-	-	Luminal B	NST	2
63	F, A, 72	Unknown	+	+	0+	N/A	30%	-	-	Luminal B	NST	2
64	F, I, 56	Negative	-	-	3+	N/A	80%	-	-	HER2 overexpression	NST	3
65	F, A, 62	Unknown	+	+	1+	N/A	20%	-	-	Luminal B	NST	2
66	F, I, 45	Negative	+	+	0+	N/A	70%	-	-	Luminal B	NST	2
67	F, A, 54	Positive	-	+	0+	N/A	100%	-	-	Luminal B	NST	3
68	F, I, 59	Negative	-	-	3+	N/A	90%	-	-	HER2 overexpression	NST	3
69	F, A, 59	Negative	+	+	2+	Negative	60%	-	-	Luminal B	NST	2
70	F, A, 38	Positive	+	+	1+	N/A	60%	-	-	Luminal B	NST	2
71	F, A, 51	Unknown	-	+	3+	N/A	50%	-	-	Luminal B	NST	2
72	F, A, 36		+	+	1+	N/A	30%	-	-	Luminal B	Type B invasive mucinous carcinoma	2
73	F, A, 48	Negative	-	-	3+	N/A	40%	-	-	HER2 overexpression	NST	2
74	F, A, 75	Positive	-	+	1+	N/A	90%	-	-	Luminal B	NST	3
75	F, C, 59	Negative	-	+	1+	N/A	90%	-	-	Luminal B	NST	2
76	F, A, 70	Unknown	-	+	0+	N/A	80%	-	-	Luminal B	NST	3
77	F, A, 58	Unknown	+	+	0+	N/A	50%	-	-	Luminal B	NST	2
78	F, A, 58	Unknown	+	+	0+	N/A	80%	-	-	Luminal B	NST	2
79	F, A, 50	Positive	+	+	0+	N/A	40%	-	-	Luminal B	NST	2
80	F, A, 50	Positive	+	+	0+	N/A	90%	-	-	Luminal B	NST	3
81	F, C, 78	Negative	+	-	0+	N/A	30%	-	-	Luminal B	NST	2
82	F, A, 49	Positive	+	-	1+	N/A	90%	-	-	Luminal B	NST	3
83	F, A, 35	Negative	+	+	1+	N/A	90%	100%	100%	Luminal B	NST	3
84	F, A, 33	Negative	+	+	1+	N/A	20%	15%	-	Luminal B	NST	2
85	F, A, 72	Unknown	+	+	1+	N/A	30%	15%	-	Luminal B	NST	3
86	F, I, 71	Negative	+	-	0+	N/A	80%	5%	-	Luminal B	NST	3
87	F, A, 32	Negative	+	+	1+	N/A	80%	-	-	Luminal B	NST	3
88	F, A, 40	Positive	-	-	0+	N/A	70%	-	-	Triple - (basal-like)	NST	3
89	F, A, 34	Negative	+	-	0+	N/A	90%	-	-	Luminal B	NST	3
90	F, A, 60	Negative	+	+	1+	N/A	60%	20%	-	Luminal B	NST	2
91	F, A, 53	Negative	+	+	2+	Negative	20%	90%	20%	Luminal B	NST	1

F = Female	NST = No special type
A = African	IHC = Immunohistochemistry
I - Indian/Asian	FISH = Fluorescence in situ hybridization
C = Caucasian	N/A = Not applicable