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Breast Cancer: The relationship between histopathological features, immunohistochemical profile, and associated genetic mutations of breast cancer in Black women seen in breast oncology clinic at Inkosi Albert Luthuli Central Hospital, 2021.

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Research protocol submitted in partial fulfilment of the requirements for the degree of Master of Medicine, Department of Anatomical Pathology

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DECLARATION

I, Dr Blessing Bakhusele Bungane, 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 conceptualization of the study, 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 as a Supervisor and Dr Mpoi Makhetha as a Co-supervisor

Dedication

I am dedicating this work to my later mother, Mrs Busisiwe Bungane who died from oesophageal carcinoma and my aunt, Mrs D Base, who is a breast carcinoma survivor. To all the women who are currently battling breast carcinoma; to all the women who have lost the battle to breast carcinoma and to all the family members of these strong women. One day we will win this war!

Acknowledgement

I would like to thank my Supervisor Dr Gamalenkosi Nhlonzi and my co-Supervisor Dr Mpoi Makhetha for their enormous support throughout this long and challenging journey. You taught me what is truly means to be a Scholar. Thank you very much!

I would like to thank the laboratory staff members at the Department of Anatomical Pathology, Inkosi Albert Luthuli Central Hospital, National Health Laboratory Services, University of KwaZulu-Natal, Durban for their support.

To my family member, Cyprian, Bukiswa and Mangaliso Bungane, I will forever be grateful for the support you have given me throughout this journey.

Last but not least, I would like to thank God and my Ancestors for the wisdom.

EXECUTIVE SUMMARY

This research is a sub-study of a Ph.D. research conducted by Dr Mpoi Makhetha titled "Exploring methylation patterns in the promoter regions of selected breast cancer genes in South African female breast cancer patients presenting to the breast cancer clinic at the Inkosi Albert Luthuli Central Hospital," which was approved by the Biomedical Research Ethics Committee (BREC/00000613/2019). The current study was also approved by Biomedical Research Ethics Committee (BREC/00005294/2023)

Overview:

In 2022, breast cancer was reported as the most common female cancer and the second most prevalent cancer worldwide, with an estimated total of 2.3 million cases (11.6% of all cancers). It is the fourth leading cause of cancer related deaths, associated with 670000 deaths (6.9% of all cancer related deaths). The incidence of breast cancer is lower in African women compared to European women, but the mortality rate is higher in African women. In addition, there is an increase in the prevalence of breast cancer amongst African women globally. African women present with histological high grade breast cancer, triple-negative breast cancer, especially basal-like molecular subtype and have the worst prognosis. Additionally, the majority of African women with breast cancer are young and premenopausal which is the opposite of global trends. Normally, the basal-like molecular subtype breast cancers have breast cancer gene 1 (*BRCA1*) mutations, but in African women, that is not the case. Also, African women with luminal subtypes have the worst prognosis compared to European women (39). Regardless of these peculiar presentations of breast cancer in African women, there are limited studies on the pathological features of tumours in African patients, especially those that focus on the histomorphology, the immunohistochemical profile and the genetic mutations. The absence of literature does emphasize on the urgent need of studying African women with breast cancer.

The purpose of this observational study was to determine the relationship between histomorphological features, immunohistochemical profiles, and genetic mutations of breast cancer in Black South African women consulting at the breast cancer clinic at Inkosi Albert Luthuli Central Hospital in 2021. The objectives were to define the histomorphological features of breast

cancer in Black South African patients and to assess the association between the histological variants and histological grade of the breast cancers. In this study, we found that invasive breast carcinomas of no special type was the most common morphological subtype. Although most tumours (63.0%) showed histological grade 2, there was a significant number (29.6%) of histological grade 3 tumours. Luminal B-like molecular was the most common (70.4%) molecular subtype. Triple-negative breast cancers were the second most common (18.5%) molecular subtype, and BRCA mutations are rare because they were found in a small proportion of young/TNBC patients. Most cases showed poor treatment response.

LIST OF ABBREVIATIONS

Abbreviation	Meaning
ASCO	American Society of Clinical Oncologists
CAP	College of American Pathologists
DCIS	Ductal carcinoma in situ
IBC, NST	Invasive breast carcinoma, no special type
BC	Breast carcinoma
SPC	Solid papillary carcinoma
IHC	Immunohistochemistry
ER	Oestrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor receptor 2
Ki-67	Antigen Kiel 67
CK	Cytokeratin
SOX10	SRY-box transcription factor 10
AR	Androgen receptor
CD4	Cluster of differentiation 4
HIV	Human immunodeficiency virus
WHO	World Health Organisation
BRCA1/2	Breast cancer gene 1 and/or 2
PALB2	Partner and Localiser of *BRCA2*
CDH1	Cadherin 1 gene
TP53	Tumour Protein 53
BMI	Body mass index
H&E	Haematoxylin and eosin
PASD	Periodic acidic Schiff with diastase
BREC	Biomedical Research Ethics Committee
SA	South Africa
SPSS	Statistical Package for the Social Sciences
KZN	KwaZulu-Natal

IALCH	Inkosi Albert Luthuli Central Hospital
TNBC	Triple-negative breast carcinoma

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CHAPTER 1

INTRODUCTION

AND

LITERATURE REVIEW

1.1 Breast Cancer

Normal breast tissue consists of ducts and lobules, which are lined by outer myoepithelial cells and inner epithelial cells, and surrounded by fibrous stroma and adipose tissue (1). Breast cancer arises from the epithelial lining of the breast ducts. It can be invasive, in-situ/carcinoma in-situ, or may have both invasive and in-situ components. Invasive breast cancer is a large and heterogeneous group of malignant epithelial neoplasms of the glandular elements of the breast, where malignant glandular epithelial cells infiltrate the surrounding fibrofatty connective tissue (2). Carcinoma in-situ consists of malignant glandular epithelial cells that remain confined within the glandular elements, without infiltrating the surrounding fibrofatty connective tissue (2).

Female breast cancer has become the second most prevalent cancer worldwide, with an estimated 2.3 million new cases (11.6% of all cancers) (3). Breast cancer is the most common cancer in females and the fourth leading cause of cancer-related deaths (3). The mortality rate accounts for 6.9% of all cancer-related deaths, with 670,000 deaths recorded in 2022 alone (3, 4). It is more common in high-income countries than in low-income countries; however, there has been a significant rise in incidence in low-income countries (4). For example, in the United States, breast cancer accounts for 30% of all female cancers, while in Africa, the breast cancer incidence is estimated at 8.6% (5, 6).

1.2 Risk Factors for Breast Cancer Development

Breast cancer is classified into sporadic and hereditary forms. The risk factors for sporadic breast cancer include obesity, gender, aging, alcohol consumption, physical inactivity, nulliparity, not breastfeeding, use of hormonal contraceptives, hormone replacement therapy, breast implants, radiation exposure, early menarche, late menopause, and advanced maternal age (2, 7, 8). Obesity, defined as a body mass index (BMI) greater than 30 kg/m^2, is a serious biological risk factor for sporadic breast cancer (9). It is associated with tumor size, lymph node metastasis, poor prognosis and survival, and recurrence, with its impact being more pronounced among postmenopausal women (7, 9, 10, 11).

The risk factors for hereditary breast cancer include a family history of cancer and genetic mutations in high- and medium-penetrance genes such as *BRCA1*, *BRCA2*, Partner and Localizer of BRCA2 (*PALB2*), and Tumor Protein p53 (*TP53*) (2, 7). BC's associated with BRCA1 germline mutations usually display a medullary pattern, are often high-grade, and show a triple-negative molecular profile, while BC's with *BRCA2* germline mutations are generally invasive and of a lower grade (12, 13, 14).

1.3 Morphology, Grading, and Molecular Classification

BC is categorized as an epithelial tumor of the breast (2). Within this category, BC is further subdivided into papillary neoplasms, non-invasive lobular neoplasia, DCIS, and invasive BC (2). Invasive BC also has subcategories. First, IBC-NST refers to tumors with non-special type morphology, or a combination of non-special and special type morphology, where the special type is less than 10% of the tumor (2). IBC-NST is the most common histologic subtype, accounting for 60-75% of all primary BC's (15, 16, 17). Second, mixed invasive BC includes both non-special type morphology and special type morphology ranging from 10-90%. Third, special type tumors, where more than 90% of the tumor consists of special type morphology (2). All special subtypes of breast cancer account for at least 25% of all primary BC's (15).

There are at least 18 described morphological subtypes of BC, and this classification is crucial for disease diagnosis and prognosis (2, 15, 16). The classification includes the histologic subtype and tumor histologic grade, with grading done using the Nottingham grading system/modified Bloom and Richardson grading system (2, 18). Histologic grading is a valuable prognostic indicator and is currently used in combination with other factors, such as clinical stage and tumor histologic subtype (2, 15, 16, 18, 19). For example, tubular carcinoma is a grade 1 tumor, comprising more than 90% tubular/glandular morphology, and is associated with an excellent prognosis, while invasive micropapillary carcinoma subtypes are linked with poor prognosis, low survival rates, and high recurrence (2, 15, 16, 18, 19).

BC is also classified based on molecular profile/signature, which serves as a predictive, prognostic, and diagnostic tool. IHC stains used for molecular classification of BC include ER, PR, HER2,

and Ki-67. Additionally, AR, CK5/6, CK14, and SOX10 are used for TNBC as basal markers. Tumors are then classified into the following molecular subtypes: luminal A-like (50-60% of cases), luminal B-like (15-20%), HER2-enriched (10-15%), and triple-negative (10-20%) (2, 18, 20). Luminal A-like BC is associated with the best prognosis, luminal B-like BC with a high recurrence rate and poor prognosis, HER2-enriched BC is aggressive but has better survival with HER2-targeted therapy, and TNBC can have both good and poor outcomes (2, 20). Some histopathological subtypes are associated with specific molecular subtypes (2, 21).

DCIS is graded using the Royal College of Pathologists grading system and is classified into low grade, intermediate grade, and high grade (2, 22). Lobular carcinoma in-situ is divided into classic, florid, and pleomorphic types (2, 22). Carcinoma in-situ typically causes no symptoms and rarely metastasizes. Over time, carcinoma in-situ invades the surrounding stroma, becomes invasive carcinoma, metastasizes to the lymph nodes and other organs, and eventually causes death if not completely treated (2, 22).

1.4 Racial Disparities in Breast Cancer

There are distinct differences in breast cancer development, progression, and mortality between women of African and European origin. Studies report a lower breast cancer incidence among women of African descent; however, they are more likely to present with breast cancer at a younger age, often in the premenopausal period, and to die from smaller-sized tumors (23, 24, 25). In the United States, studies show the median age of diagnosis for Black women is slightly younger (around 60 years) compared to White women (around 64 years) (5, 23, 26). Additionally, Black women are more likely to present with TNBC, have an increased mortality rate, and are more likely to present with advanced disease compared to women of other races (5). Bowen *et al*. (2008) found that Black patients in the UK were diagnosed earlier than White patients, with a median age of 46 compared to 67, respectively (26). They also reported higher incidences of triple-negative cases, higher tumor grade, advanced disease, and increased death rates among African American patients (26). These trends are also observed in the UK (23).

Further disparities are reported in histopathological and molecular subtypes. IBC-NST, which is usually common in older women, is disproportionately frequent in younger African women (26).

Studies also report high incidences of triple-negative, grade 3 aggressive tumors with associated high mortality rates in younger Africans compared to other races (5, 23, 26). Some studies show that even with low-grade tumors, the mortality rate remains high among African women (23). The reasons for these trends among African women are still not well understood.

1.5 BC in SA

In SA, racial classification was first introduced during colonial times, and the categories were: Black/African, White/European, Asian/Indian, and Coloured/Mixed race (27). This classification system still exists today. Breast cancer is the most common cancer among SA women, accounting for 23.22% of all female cancers (28, 29). The incidence of breast cancer-related deaths is also high in SA, accounting for 16% of cancer deaths (4, 28, 29). There has been a significant increase in breast cancer incidence in SA from 2002 to 2022, with rates of 7.6 cases per 100,000 women in 2002 to 190.4 cases per 100,000 women in 2022 (29, 30, 31). In 2002, the incidence of breast cancer was lower among Black women (11.3 cases per 100,000), compared to Coloured (14.7 cases per 100,000) and White women (40.9 cases per 100,000) (32). Despite having a lower incidence rate, Black women have a higher mortality rate compared to women of other racial groups (33). Dlamini *et al*. (2023) found that Black women in SA tend to be diagnosed with more aggressive forms of breast cancer and have a poorer prognosis compared to women from other racial groups (34). To the best of our knowledge, there is currently no literature describing the morphology and molecular profile of breast cancer in Black SA women.

1.6 Study Setting

The study took place at IALCH in KZN province. Approximately 8 million people (12.7% of SA population) are living with HIV in SA (35). KZN province has the second-highest prevalence of HIV in SA (36). Although HIV status is not associated with breast cancer development, it is linked to therapy-related toxicity, worse outcomes, lower survival rates, and higher mortality (37, 38, 39, 40). Calabresi *et al.* (2013) found that patients living with HIV develop breast cancer much earlier (~42 years) than HIV-negative patients (~65-69 years) (41). While the underlying cause for worse prognosis and increased mortality in patients living with HIV and concurrent breast cancer has not

been isolated, the role of HIV infection-related deaths presents a confounding factor in evaluating survival outcomes among patients living with HIV (36).

Patients with multiple comorbidities are at an increased risk of metastatic disease and have a higher mortality rate (28). The high prevalence of HIV infections and obesity in the KZN population makes it more vulnerable than the average population. Therefore, it is essential to prioritize the treatment of breast cancer patients in this population.

CHAPTER 2

AIMS

AND

OBJECTIVES

Aim of this study was to determine the relationship between histopathological features, IHC profile, and genetic mutations of breast cancer in Black women seen in breast cancer clinic at IALCH, 2021.

Objectives of this study were to define the histopathological features of breast cancer in Black SA women, and to assess the association between the histopathological variants and histopathological grade of the breast cancers.

CHAPTER 3

MATERIALS

AND

METHODS

3.1 Study design

This observational study was conducted at the Department of Anatomical Pathology, National Health Laboratory Services (NHLS), IALCH, Durban, KZN, SA, between 2023 and 2024. We used archived tissues from an umbrella study for which participants gave informed consent. The principal investigator, Dr Makhetha, had recruited Black SA patients above 18 years with clinically and histopathologically confirmed breast cancer diagnosis consulting at IALCH. The study included patients diagnosed with stage I to stage III disease, and who required surgery as part of their disease management. All stage IV patients, or patients whose cancer was not of breast origin were excluded from the study. The study was approved by BREC with registration of BREC/00000613/2019. The patients' clinical data was obtained from the clinical files and is presented in Table 1.

This sub-study was also approved by the BREC (BREC/00005294/2023). Our aim was to describe the histopathological variants and associated histopathological grades of breast cancer in African women by comparing morphological features with the IHC profile and associated genetic mutations. We included tumours obtained from 27 patients who participated in the main study, which is the total number of patients who participated in the PhD study. Our study variables and confounders were histopathological variants, histopathological grade and IHC profile/molecular classification. To maintain confidentiality, new identities were generated for each patient and all documents containing identifiable data and images captured for research were stored in a password protected computer which was only accessible to the principal investigator.

3.2 Tissue collection and clinical data collection

Our study utilized these archived H&E slides and immunoperoxidase stained slides from the Anatomical Pathology department's archives for analysis. The cases were independently and jointly appraised by at least two pathologists using the Olympus BX43 microscopes and the findings were recorded on a data collection sheet Appendix 1. All participants had been diagnosed with breast cancer initially from a core needle biopsy. The excision specimens had titanium wire in-situ which was used as a guide to the exact tumour location. Both normal breast tissue and carcinoma in-situ or invasive carcinoma or both were assessed.

Grossly, invasive tumour was solid, tan-white in colour with irregular margins invading into the normal fibrofatty connective tissue of the breast. Carcinoma in-situ was seen as irregular calcifications. Three to 5mm thick by 1cm wide tissue sections were taken and placed in small cassettes from all the areas of interest (excision margins, tumour, areas of calcification, normal areas of fibrosis and the nipple areolar complex or lymph nodes if present) for histopathological appraisal. These sections were fixed overnight in Sakura Tissue-Tek VIP machine. After fixation, the tissue sections in cassettes were embedded in wax.

After embedding, the tissues were cut using Leica RM2235 microtome at 4 microns (2 microns for lymph nodes and >5 microns for fat). The cut tissue sections were placed in hot waterbath with a temperature of 45 ºC, this removed small folds/wrinkling of the tissue. The best tissue sections were picked up from the waterbath onto labelled (with patient laboratory number) glass slides and the glass slides with the tissue sections are placed in Leica HHP6 hot plate for at least 5 minutes at 60 ºC to fix the tissue onto the glass slide. The glass slides were then stained with H&E stain. Reagent preparation for the H&E stain was as follows: Commercially available Mayer's Haematoxylin (Cell Path Services, SA), 0.5% alcoholic eosin (20g eosin Y to 4000ml of absolute alcohol) (Eosin is from Merck & Co, Inc, United States of America/Germany and alcohol is from Purple Moss, SA), acetic acid (Merck & Co, Inc, United States of America/Germany) was added prior to staining (2.5ml of concentrated acetic acid to 500ml of filtered eosin-alcohol solution), and lithium carbonate solution (0.5ml of saturated lithium carbonate to 100ml tap water) (SRL Chem, India).

The following clinical information was collected from patient's files: patient age, age of diagnosis with BC, menstrual history, BMI, family history of BC, HIV status, contraceptives used, Social habits, genetic results and TNM stage.

3.3 Methods of staining

The slides with tissue sections were heat-fixed and the tissue sections were dewaxed on the glass slide in Xylene (Cell Path Services, SA). The sections were then rehydrated through descending grades of alcohol to water. The glass slides were stained in Mayer's haematoxylin solution for 5

minutes, followed by a rinse in running tap water. The nuclei of the sections were blued in lithium carbonate solution for 1 minute until they appeared blue macroscopically. The slides were rinsed in running tap water for 2 minutes. They were then quickly dipped in SVR (96% alcohol) and counterstained in 0.5% alcoholic eosin for 5 minutes. After rinsing the slides in running tap water, the tissue sections were dehydrated in ascending grades of alcohol, cleared in xylene, and mounted in DPX with a glass coverslip. Excess xylene was dried with soft tissue paper.

3.4 Analysis of H&E glass slides

Analysis included a confirmation by the pathologists for the presence of tumour, the tumour subtype, the histopathological grade of the tumour, the presence/absence of DCIS, lymphovascular invasion, perineural invasion, Paget disease of the nipple or lymph nodal metastasis. For the histopathological grading of the tumour, modified Bloom and Richardson/Nottingham grading system was used. When the invasive tumour was confirmed, a panel of IHC stains was done. The standard IHC stain panel for invasive BC includes ER, PR, HER2 and Ki-67 stains. After the interpretation of IHC stains, tumours were given a molecular class and tumour subtype.

For TNBC, basal markers (CK14, CK5/6 and SOX10) and AR were done. IHC stain GATA3 was used to confirm breast origin of the tumour in TNBC and in metastatic BC E-cadherin was used in cases where the tumour morphology was suspicious for an invasive lobular carcinoma. E-cadherin protein is an adhesion molecule that is encoded by the cadherin 1 (*CDH1)* gene (4). When there is *CDH1* gene mutation, there is loss of E-cadherin adhesion molecule. Aberrant E-cadherin IHC patterns of expression (associated with *CDH1* gene mutation): a. circumferential, reduced intensity staining pattern; b. partial, fragmented, bead-like staining pattern; c. cytoplasmic staining pattern; and d. dot-like, perinuclear Golgi-type staining pattern are associated with invasive lobular carcinoma. Strong and diffuse membranous staining pattern of E-cadherin IHC stain is associated with IBC-NST and other special type other than invasive lobular carcinoma. For IBC-NST with mucinous components, histochemical stains mucicarmine, periodic acid-Schiff and periodic acid-Schiff with diastase were done to highlight the extracellular and intracytoplasmic mucin.

3.5 Statistical analysis

We described our data using histopathological grade and IHC profiles which were recorded as frequencies, means and proportions. These data points were analysed using Statistical Package for Social Sciences (SPSS) software.

3.6 Study Funding and Progress:

Funding was not required to conduct this study as all laboratory testing had been conducted for diagnostic purposes.

3.7 Study team, contributors and authorship

Role	Names	Speciality	Institution
Principal investigator	Blessing Bakhusele Bungane	Registrar: Anatomical Pathology	National Health Laboratory Services University of KwaZulu-Natal
Supervisor	Gamalenkosi B Nhlonzi	Pathologist: Anatomical Pathology	National Health Laboratory Services AMPATH Laboratory University of the Kwa-Zulu Natal
Co-Supervisor	Mpoi Makhetha	Geneticist	University of the Kwa-Zulu Natal

3.8 Publication of research findings

The data was presented as a full report to the School of Laboratory Medicine and Basic Medical Science, University of Kwazulu-Natal and KZN Department of Health. Publication of the findings in a peer reviewed journal will be undertaken.

CHAPTER 4

RESULTS

4.1 Clinical findings

A total of twenty-seven cases used in the Ph.D. research conducted by Dr Mpoi Makhetha titled "Exploring methylation patterns in the promoter regions of selected breast cancer genes in South African female breast cancer patients presenting to the breast cancer clinic at the Inkosi Albert Luthuli Central Hospital," which was approved by the BREC (BREC/00000613/2019), were retrieved from the archives of the Department of Anatomical Pathology, National Health Laboratory Services, University of KwaZulu-Natal, Durban between the years, 2023 and 2024.

Majority, 55.6%, of patients were above age 50 years and pre-menopausal, 51.9%. 44.4% and 48.1% of patients were below age 50 years and post-menopausal, respectively. The mean age at diagnosis with breast cancer was 48.52 years. A vast majority, 81.5%, of patients fell in the overweight/obese BMI category. The number of patients who tested positive and negative for HIV was almost similar, 14 and 13, respectively. 18.5% of patients reported having a positive family history of breast cancer. 81.5% of patients did not have a positive family history of breast cancer.

At least 59.3% of patients reported using/having used hormonal contraceptives in the past 5 years. Of the patients who reported using hormonal contraceptives, 51.9% reported using medroxyprogesterone acetate. 92.6% of patients reported to have never smoked nor drank alcohol in their lifetime and only 7,4% of patients admitted to be drinking alcohol. Young patients, below age 50 at the time of diagnosis with breast cancer and patients who had triple-negative molecular profile were tested for *BRCA1*, *BRCA2* and *RAD54L* gene mutations. 37% of patients had negative mutation of the 3 above mentioned genes, 11.1% had *BRCA2* mutation, 3.7% had *BRCA1* mutation and 3.7% had *RAD54L* mutation. One patient was erroneously not tested for genetic mutations.

48.1% of patient had tumour stage two (T2) disease at presentation and significant number of patients, 33.3% had T4 disease at presentation. About 48.1% of patients had local lymph node metastasis (N1) at presentation. None of the patients in our cohort had distant metastatic disease at presentation. The characteristics of patients have been listed in detail in Table 1.

Table 1. The number and clinical characteristics of breast cancer in Black female patients seen at the breast cancer clinic at IALCH, 2021.

Characteristic		**Number**	**Percentage**
Age	Below 50 years	12	44.4%
	Above 50 years	15	55.6%
Age of diagnosis	Minimum	23	
	Mean	48.52	
	Maximum	75	
Menstrual history	Pre-menopausal	14	51.9%
	Post-menopausal	13	48.1%
BMI	Normal	5	18.5%
	Overweight	6	22.2%
	Obese	16	59.3%
Family history of breast cancer	Yes	5	18.5%
	No	22	81.5%
HIV status	Positive	14	51.9%
	Negative	13	48.1%
Contraceptives used	Medroxyprogesterone acetate	14	51.9%
	Oral contraceptives	2	7.4%
	None	11	40.7%
Social habits	Smoke/drink alcohol	2	7.4%
	Sober habits	25	92.6%
Genetic results	*BRCA1* positive	1	3.7%
	BRCA2 positive	3	11.1%
	RAD54L positive	1	3.7%
	Negative	10	37%
	Not done	1	3.7%
Tumour	T1	0	0%
	T2	13	48.1%
	T3	4	14.8%

	T4	9	33.3%
	N/A	1	3.7%
Lymph node	N0	10	37.0%
	N1	13	48.1%
	N2	1	3.7%
	N3	2	7.4%
	N/A	1	3.7%
Metastasis	0	0	0%

4.2 Morphological findings

Table 2. The number and clinico-morphological characteristics of breast cancer in Black female patients seen at the breast clinic at IALCH, 2021.

Characteristic		Number	Percentage
Histologic subtypes	IBC, NST	26	96.3%
	SPC	1	3.7%
Histopathological grade	Grade 1	1	3.7%
	Grade 2	17	63%
	Grade 3	8	29.6%
	N/A	1	3.7%
DCIS	Low grade	0	0%
	Intermediate grade	5	18.5%
	High grade	10	37%
	Absent	12	44.5%
Age and DCIS	Total patients with DCIS	15	
	Below age 50	9	60%
	Above age 50	6	40%
	High grade DCIS		
	Below age 50	7	46.7%
	Above age 50	3	20%
	Intermediate grade DCIS		
	Below age 50	2	13.3%
	Above age 50	3	20%
Molecular classification	Luminal A	2	7.4%
	HER2 positive luminal B	5	18.5%
	HER2 negative luminal B	14	51.9%
	Triple negative	5	18.5%
	Not applicable (N/A)	1	3.7%

	HER2 Enriched	0	0%
Age and Molecular class	Luminal A Below age 50 Above age 50 HER2 positive luminal B Below age 50 Above age 50 HER2 negative luminal B Below age 50 Above age 50 Triple negative Below age 50 Above age 50	 1 1 4 1 7 7 4 1	
Genetic results and molecular classification	Luminal A *BRCA1* positive *BRCA2* positive *RAD54L* positive Negative HER2 positive luminal B *BRCA1* positive *BRCA2* positive *RAD54L* positive Negative HER2 negative luminal B *BRCA1* positive *BRCA2* positive *RAD54L* positive Negative Triple negative	 0 0 0 1 0 1 0 2 0 2 0 5	

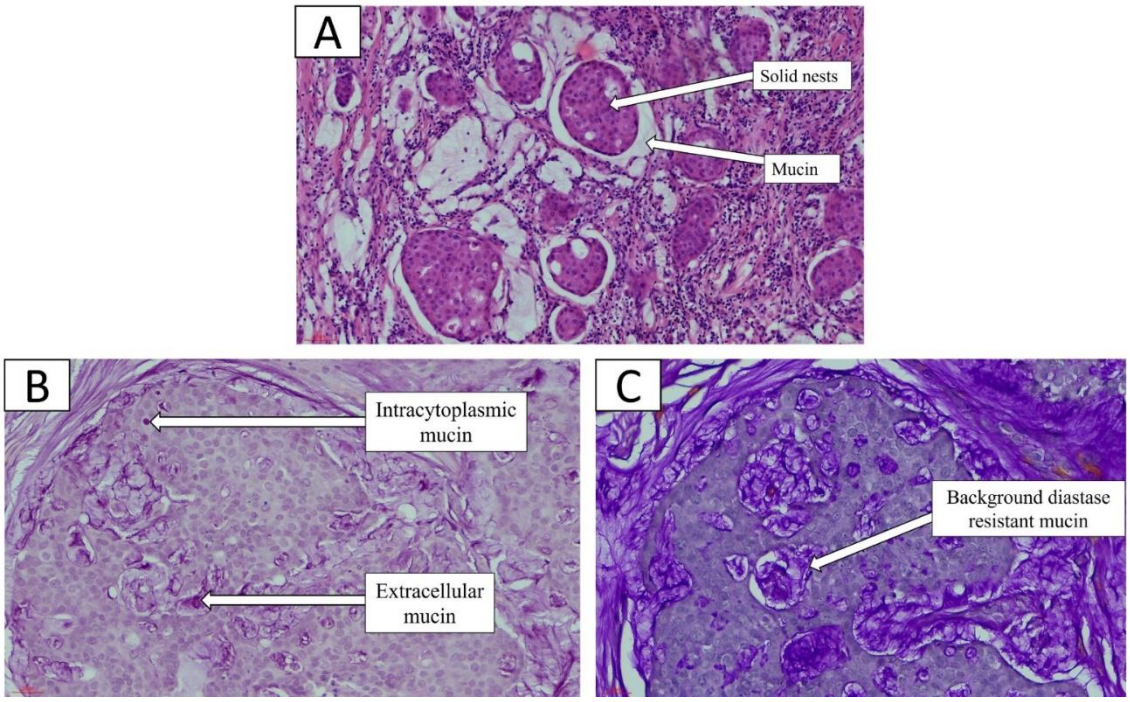
	BRCA1 positive *BRCA2* positive *RAD54L* positive Negative	1 0 1 2	
Treatment response Sataloff method	T-A T-B T-C T-D N/A	4 8 13 0 2	14.8% 29.6% 48.1% 0% 7.4%
Treatment response and molecular class	Luminal A T-A T-B T-C T-D HER2 positive luminal B T-A T-B T-C T-D HER2 negative luminal B T-A T-B T-C T-D N/A Triple negative T-A T-B T-C	 1 0 1 0 1 2 2 0 2 4 6 0 2 0 2 3	

	T-D	0	

4.3 Histopathologic subtypes

Twenty-six cases showed IBC-NST and one case showed solid papillary carcinoma, without invasion. Figures 1-4, show some of the different morphological features of IBC-NST observed in this study.

Figure 1



The patient with the tumour represented in Figure 1. represent a tumour that was obtained from a 47-year-old female who tested positive for HIV. In this image, **A** shows an H&E image showing IBC-NST with a mucinous component. The tumour is arranged as solid nests within a mucinous background (x200 magnification). **B** shows Mucicarmine histochemical stain highlighting background/extracellular and intracytoplasmic mucin, staining the mucin magenta (x200 magnification). **C** shows PASD histochemical stain highlighting background and intracytoplasmic diastase resistant mucin (x400 magnification).

The patient with the tumour represented in Figure 1 was pre-menopausal; had a positive family history of breast cancer; had normal BMI of (23.65 kg/m2); and had never used hormonal contraceptive therapy. She was clinically stage T4BN1M0. Histopathologically, she was diagnosed with IBC, NST with a mucinous component and associated high nuclear grade DCIS, modified Bloom and Richardson grade 2, and triple negative molecular subtype. Her genetic studies showed *RAD54L* mutation but negative for *BRCA1* and *BRCA2* mutations. After treatment, she had a T-C treatment response (Sataloff method).

Figure 2

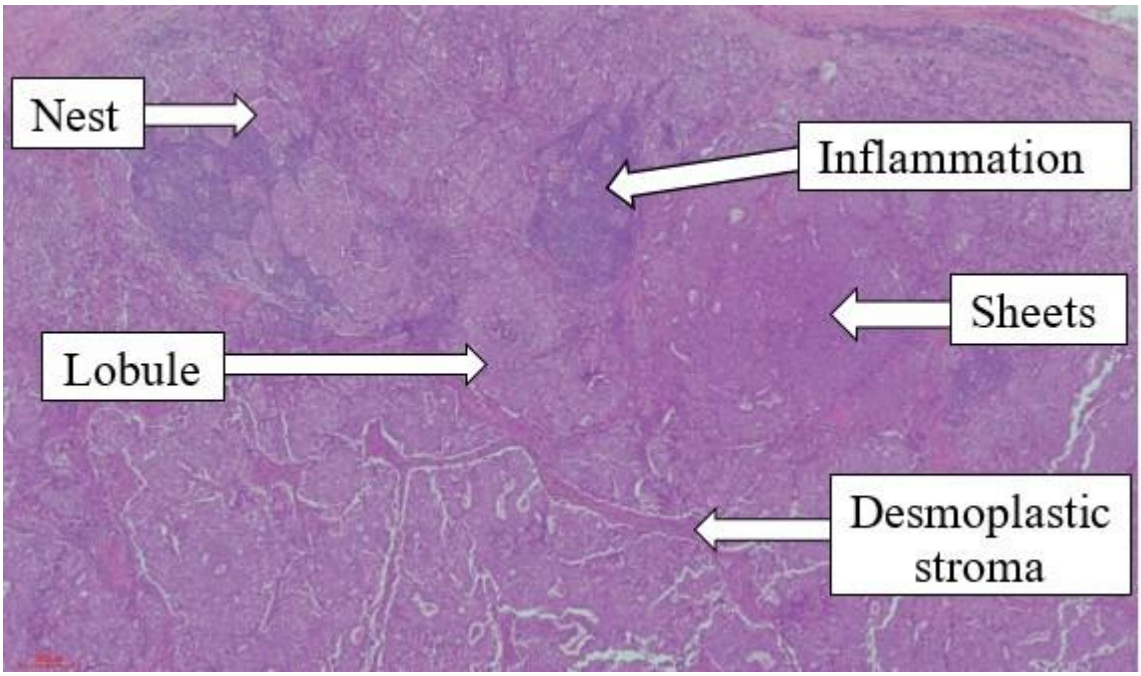


Figure 2 is an H&E image showing IBC-NST. The tumour is arranged as nests, sheets and lobules within a desmoplastic and inflamed stroma (x100 magnification).

The patient with the tumour represented in Figure 2 was a 48-year-old pre-menopausal female who tested positive for HIV. She had a positive family history of breast cancer; she was obese with BMI of (31.8 kg/m2); and at the time of diagnosis, was using medroxyprogesterone acetate hormonal contraceptive therapy. She was clinically stage T2N1M0. Histopathologically, she was diagnosed with IBC, NST, modified Bloom and Richardson

grade 3 and triple negative molecular subtype. Her genetic studies showed negative *BRCA1*, *BRCA2* and *RAD54L* gene mutations. No other genetic studies were conducted. After treatment, she had a T-C treatment response (Sataloff method).

Figure 3

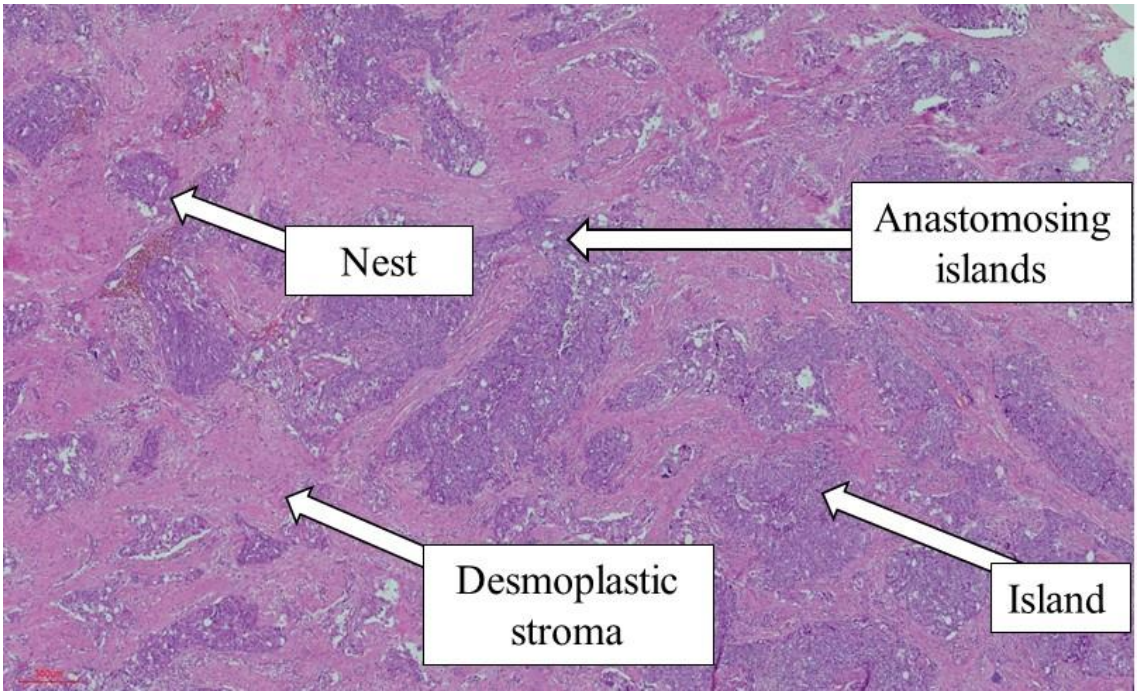


Figure 3 is an H&E image showing IBC-NST. The tumour is arranged as nests, islands and anastomosing islands within a desmoplastic and collagenous stroma (x100 magnification).

The patient with the tumour represented in Figure 3 was a 58-year-old post-menopausal female who tested positive for HIV. She had a positive family history of breast cancer; obese BMI (31.3 kg/m2); and reported using medroxyprogesterone acetate hormonal contraceptive therapy in the last 10 years. She was clinically stage T2N1M0. Histopathologically, she was diagnosed with IBC-NST, modified Bloom and Richardson grade 3 and HER2 positive luminal B-like molecular subtype. After treatment, she had a T-C/N-A treatment response (Sataloff method).

Figure 4

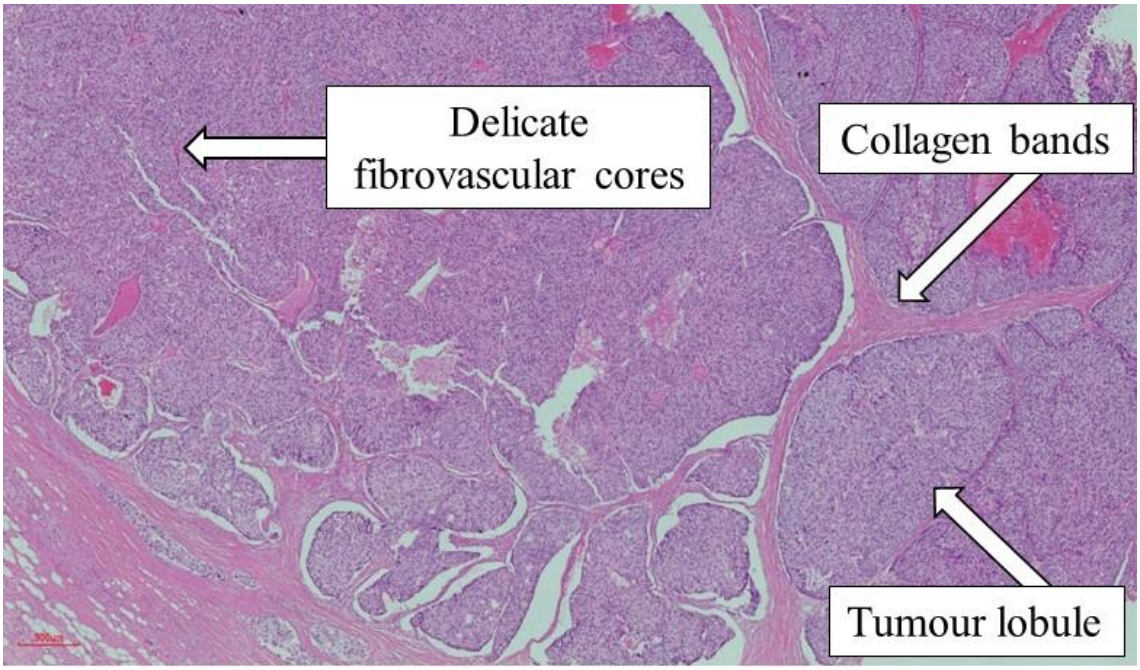


Figure 4 is an H&E image showing solid papillary carcinoma, without invasion. The tumour is arranged as lobules with pushing borders, separated by collagen bands. Delicate fibrovascular cores are present within the tumour lobules (x100 magnification).

The patient with the tumour represented in Figure 4 was a 54-year-old post-menopausal female who tested positive for HIV. She had no positive family history of breast cancer; had a normal BMI (22.38 kg/m2); and was not using hormonal contraceptive therapy. She was diagnosed with breast cancer at the age of 51. She was clinically stage T2N1M0. Histopathologically, she was diagnosed solid papillary carcinoma without invasion. The tumour was excised with clear margins.

4.4 Histopathological grade

The tumours in this study were graded using the modified Bloom and Richardson/Nottingham grading system. Majority of cases, 63%, were grade 2, followed by 29.6% cases which were grade 3 and lastly 3.7% showed grade 1 features. Not applicable (N/A) represents the case of solid papillary carcinoma without invasion. According to WHO, solid papillary carcinomas without invasion should not be graded using the modified Bloom and Richardson/Nottingham

grading system nor should we appraise the receptor status of these tumours (3). Figures 5-7 show different grades of IBC-NST.

Figure 5

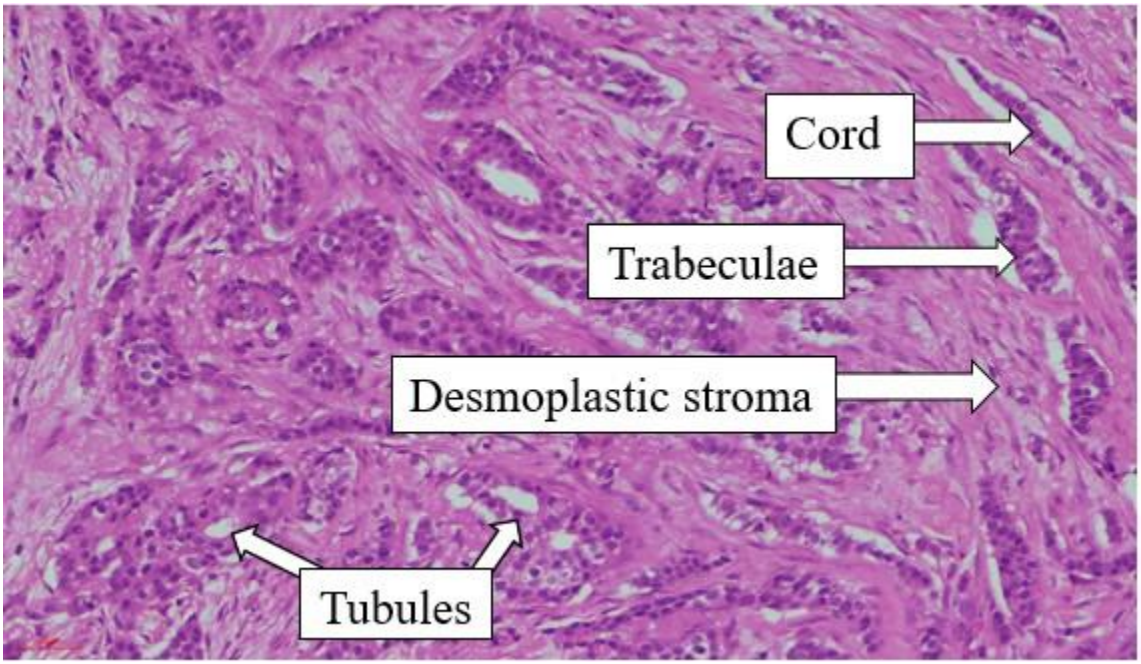


Figure 5 is an H&E image showing grade 1 tumour. Note tubular formation and mild nuclear atypia (x200 magnification).

The patient with the tumour represented in Figure 5 is a pre-menopausal female who tested positive for HIV. She had no positive family history of breast cancer; normal BMI (22.7 kg/m2); and was using medroxyprogesterone acetate hormonal contraceptive therapy. She was clinically stage T2N0M0. Histopathologically, she was diagnosed with IBC-NST, modified Bloom and Richardson grade 1 and luminal A-like molecular subtype. After treatment, she had a T-C (Sataloff method). The modified Bloom and Richardson was as follows: 2 points for tubule formation (there were more than 10% tubules with clear lumen, but less than 75%), 1 point for nuclear pleomorphism (the tumour cells displayed mild nuclear pleomorphism) and 1 point for mitotic figures (there were 2 mitotic figures in 10 high power fields). The total score was 4/9, in keeping with grade 1 (which ranges from 3-5/9 points).

Figure 6

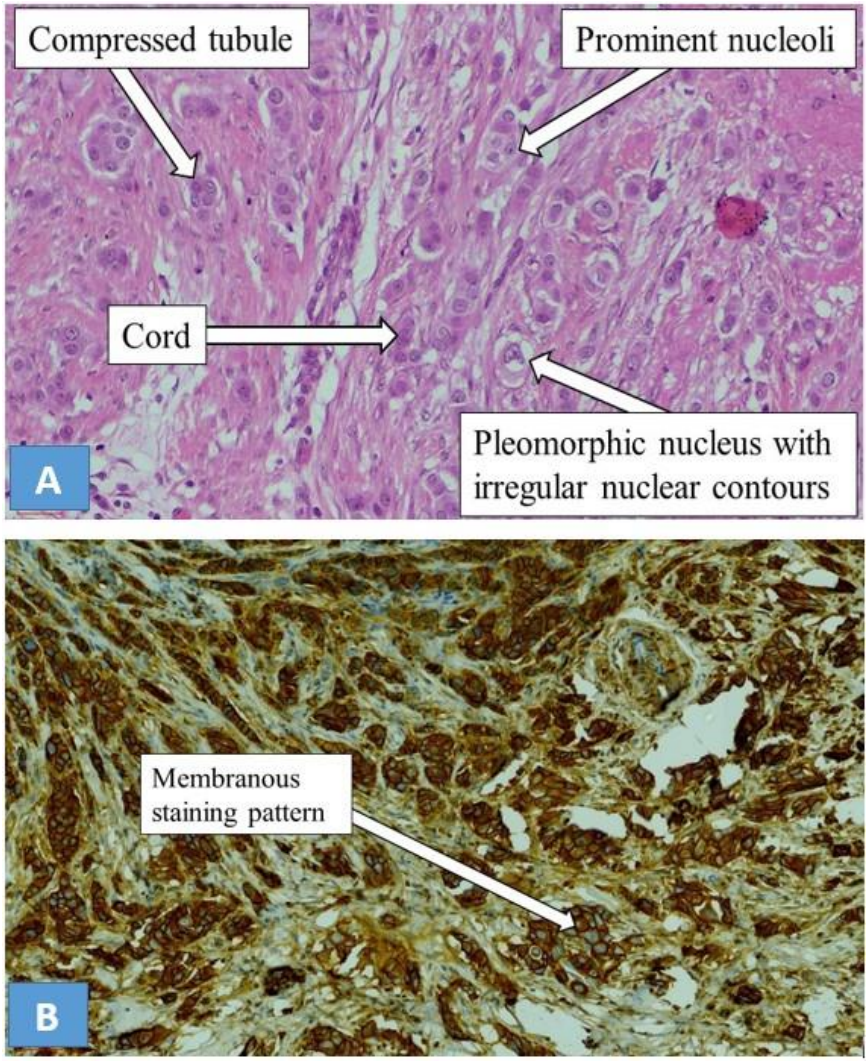


Figure 6. **A** is an H&E image showing IBC-NST, grade 2. Note nuclear pleomorphism. The tumour is growing as compressed ducts, cords, single cells and nests within a desmoplastic and collagenous stroma (x400 magnification). **B** is the tumour seen in Figure 6, **A**, showing retained E-cadherin characterised by complete, strong and diffuse membranous immunostaining pattern; ruling out invasive lobular carcinoma (x400 magnification).

The patient with the tumour represented in Figure 6 A-B is a post-menopausal female female who tested negative for HIV. She had no positive family history of breast cancer; obese BMI (37.9 kg/m2); and never used hormonal contraceptive/hormone replacement therapy. She was

clinically stage T4BN1M0. Histopathologically, she was diagnosed with IBC-NST, modified Bloom and Richardson grade 2 and triple negative molecular subtype. After treatment, she had a T-C (Sataloff method). She had negative genetic test for *BRCA1, BRCA2* and *RAD54L* gene mutations.

The modified Bloom and Richardson was as follows: 3 points for tubule formation (there were less than 10% tubules with clear lumen), 2 point for nuclear pleomorphism (the tumour cells displayed moderate nuclear pleomorphism) and 2 point for mitotic figures (there were 10 mitotic figures in 10 high power fields). The total score was 7/9, in keeping with grade 2 (which ranges from 6-7/9 points).

Figure 7

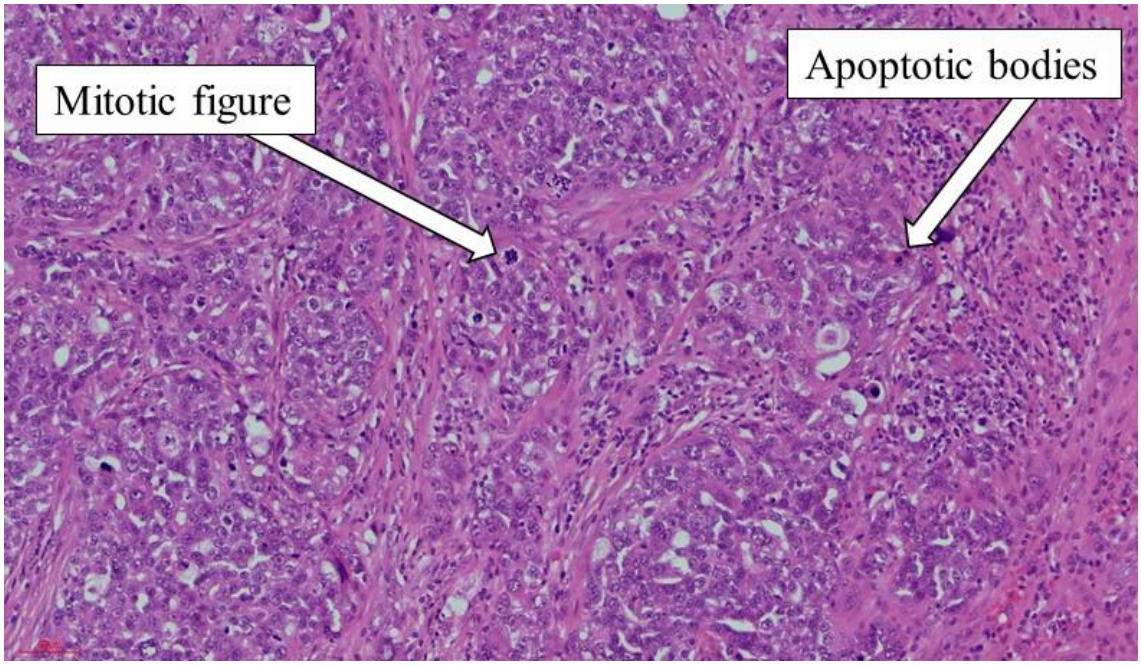


Figure 7. H&E image showing IBC-NST, grade 3. Note nuclear pleomorphism, brisk mitotic figures and numerous apoptotic bodies. The tumour is growing as solid nests and islands within a desmoplastic and inflamed stroma (x200 magnification).

The patient with the tumour represented in Figure 7 is a pre-menopausal female who tested positive for HIV. She had positive family history of breast cancer; obese BMI (52.87 kg/m2);

and was using medroxyprogesterone acetate hormonal contraceptive therapy. She was clinically stage T2N1M0. Histopathologically, she was diagnosed with IBC-NST, modified Bloom and Richardson grade 3 and triple negative molecular subtype. After treatment, she had a T-C (Sataloff method). She had negative genetic test for *BRCA1, BRCA2* and *RAD54L* mutations.

The modified Bloom and Richardson was as follows: 3 points for tubule formation (there were less than 10% tubules with clear lumen), 2 point for nuclear pleomorphism (the tumour cells displayed moderate nuclear pleomorphism) and 3 point for mitotic figures (there were 30 mitotic figures in 10 high power fields). The total score was 8/9, in keeping with grade 3 (which ranges from 8-9/9 points).

4.5 DCIS

A total of 15 patients of the cases with invasive BC had associated DCIS; of which five were intermediate nuclear grade DCIS and 10 were high nuclear grade DCIS. There was no case with low nuclear grade DCIS. Of the 15 patients with DCIS, nine (60%), were below age 50 and six (40%), were above age 50. Majority, 46.7%, of patients with DCIS who were below age 50, they demonstrated high nuclear grade DCIS. In patients above age 50, there was similar prevalence of intermediate nuclear grade DCIS and high nuclear grade DCIS, both 20%. Figures 8-11 show different grades of DCIS seen in the study and the IHC stains used.

Figure 8

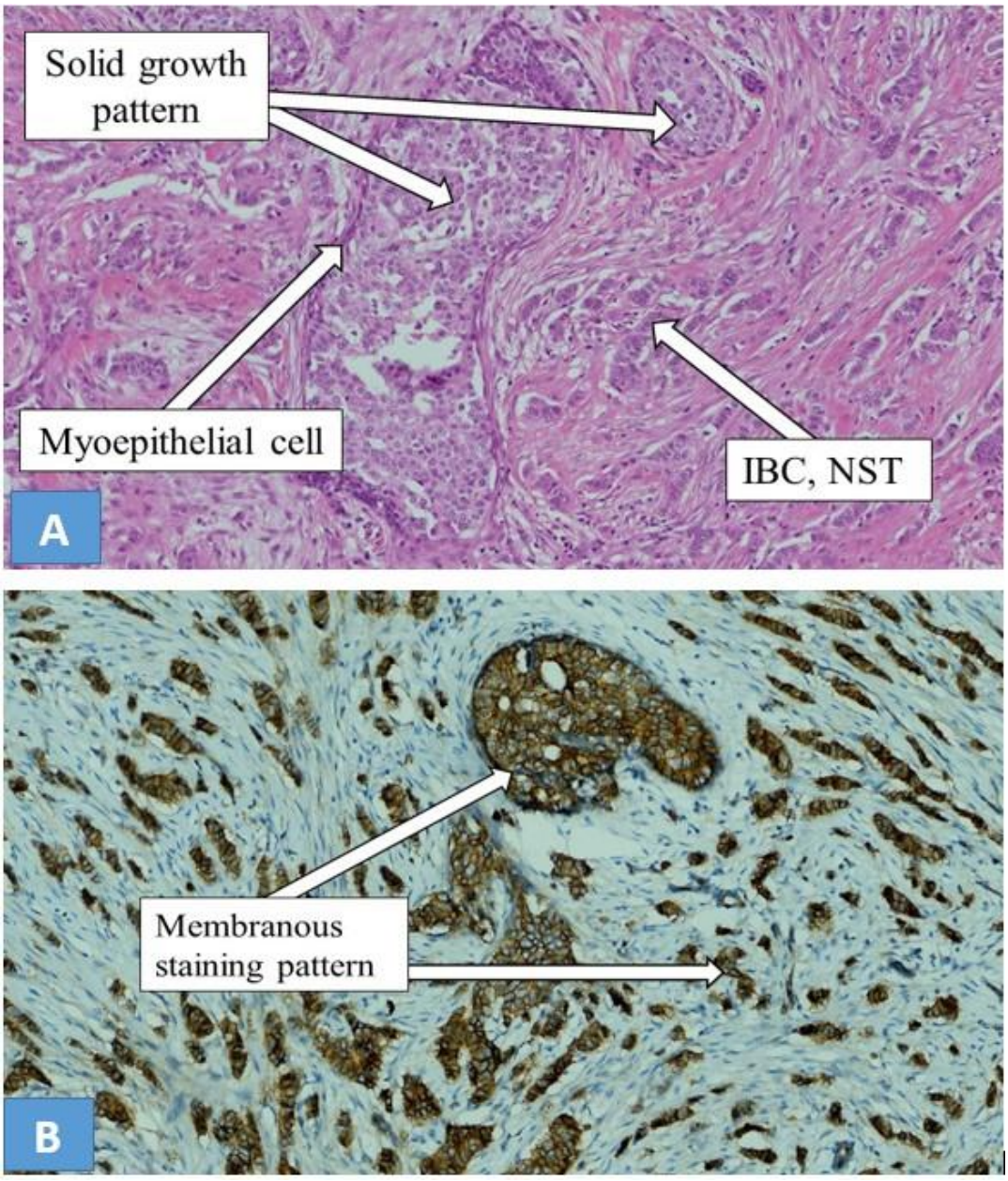


Figure 8. **A** is an H&E image of a tumour seen in Figure 6, showing intermediate nuclear grade DCIS with solid growth pattern. Note retained myoepithelial cells in the DCIS component (x200 magnification). **B** is the tumour seen in Figures 6, showing retained complete, strong and diffuse membranous immunostaining pattern for p120 immunostain in both the invasive tumour component and the carcinoma in-situ component (x200 magnification).

Figure 9

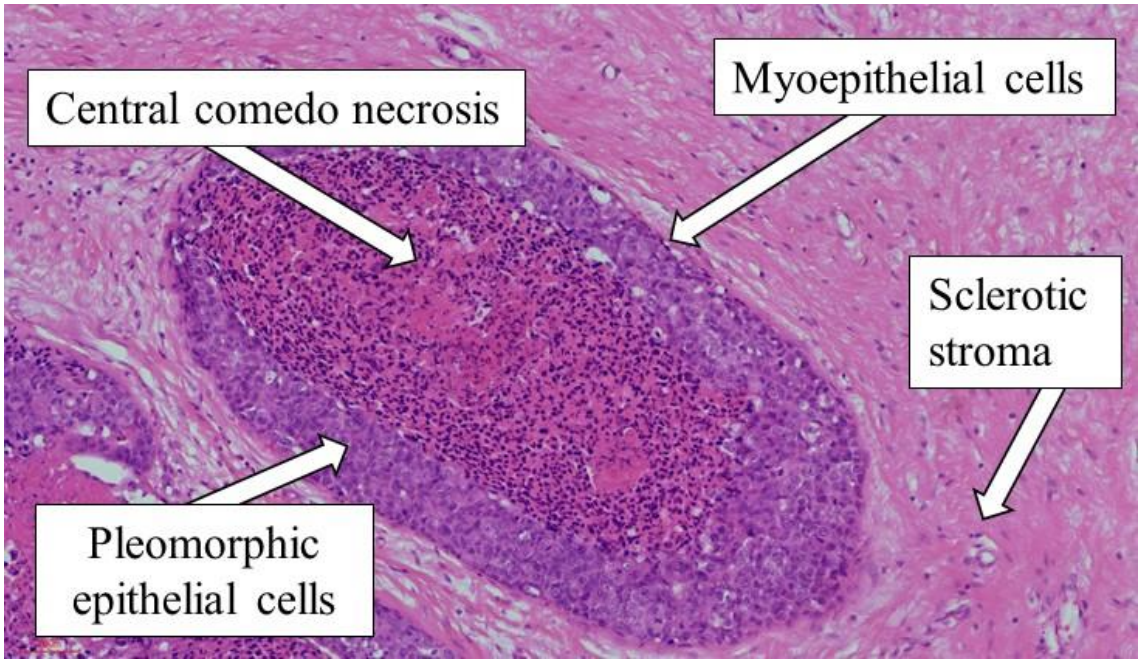


Figure 9. H&E image showing high nuclear grade DCIS with comedo necrosis. Note nuclear pleomorphism, brisk mitotic figures, retained myoepithelial cells and surrounding sclerotic stroma (x200 magnification).

Figure 10

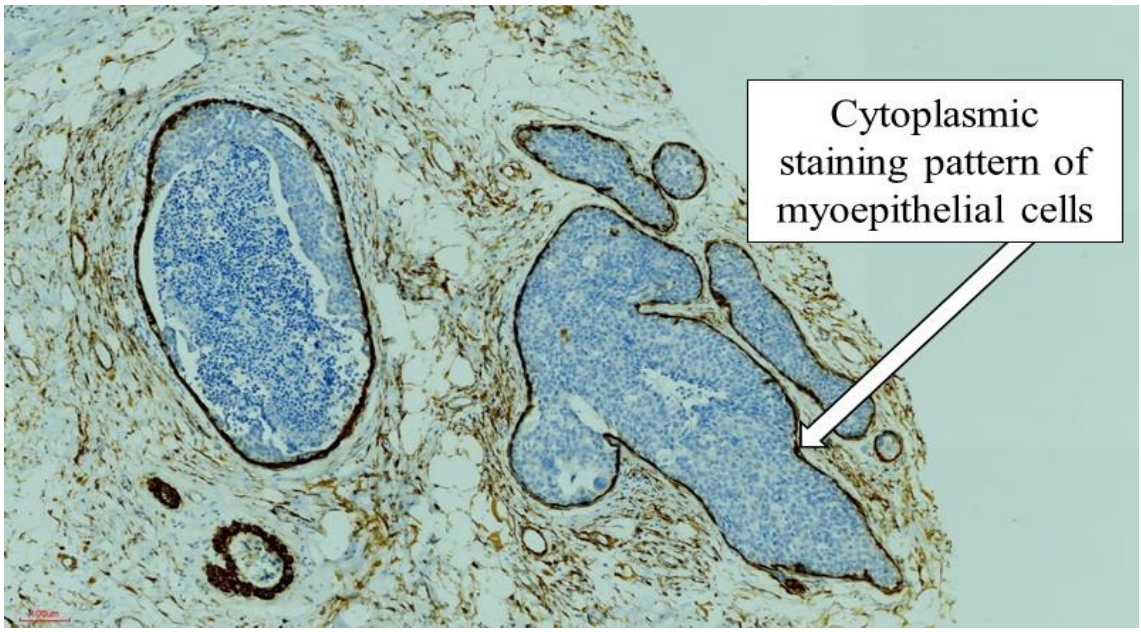


Figure 10. Calponin IHC stain highlighting retained myoepithelial cells, cytoplasmic staining pattern, in high nuclear grade DCIS (x100 magnification).

Figure 11

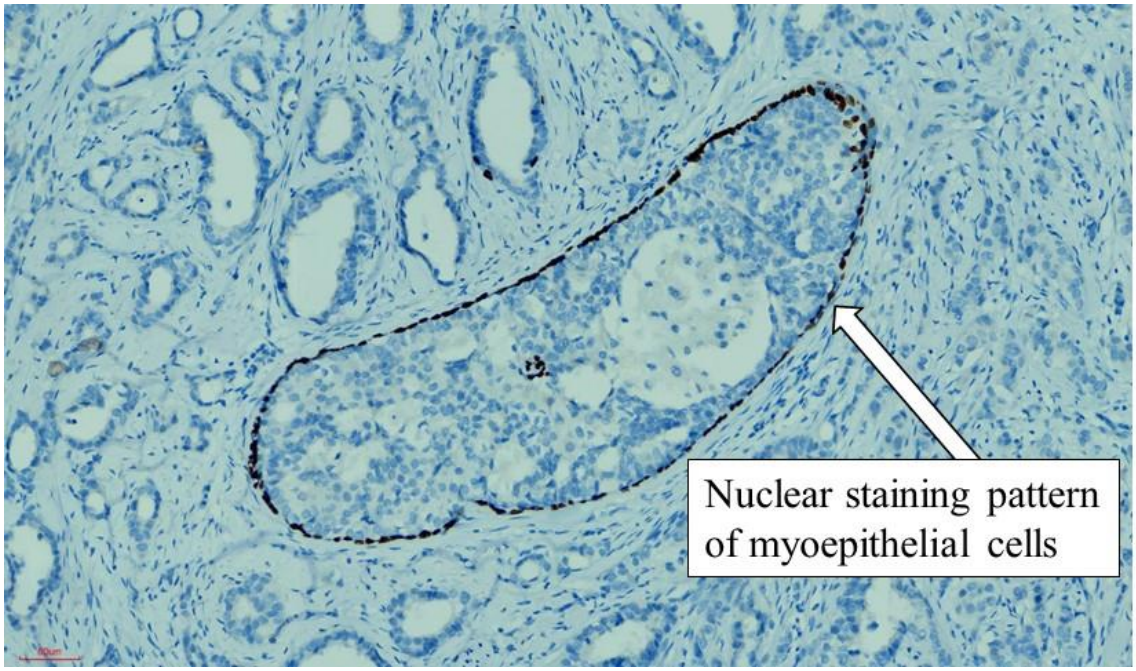


Figure 11. p63 IHC stain highlighting retained myoepithelial cells, nuclear staining pattern, in intermediate nuclear grade DCIS (x200 magnification).

4.6 Molecular classification

A total of 19 (70.4%) cases were luminal B-like molecular subtype, five (18.5%) cases were triple negative molecular subtype, with four patients having basal-like immunoprofile, and two (7.4%) cases were luminal A-like molecular subtype. Majority of patients with HER2 positive luminal B-like and triple negative immunoprofile were below age 50. Only one patient from each of these molecular classes was above age 50. There were equal numbers of patient age groups in cases of luminal A-like and HER2 negative luminal B-like molecular subtypes. Of the patients with triple negative immunoprofile, one patient had *BRCA1* gene mutation, one patient had *RAD54L* gene mutation and two patients had negative *BCRA1*, *BRCA2* and *RAD54L* gene mutations. The patient who was not tested for genetic mutations had a triple negative immunoprofile. Figures 12 to18 show IHC stains used for the molecular classification of IBC.

Figure 12

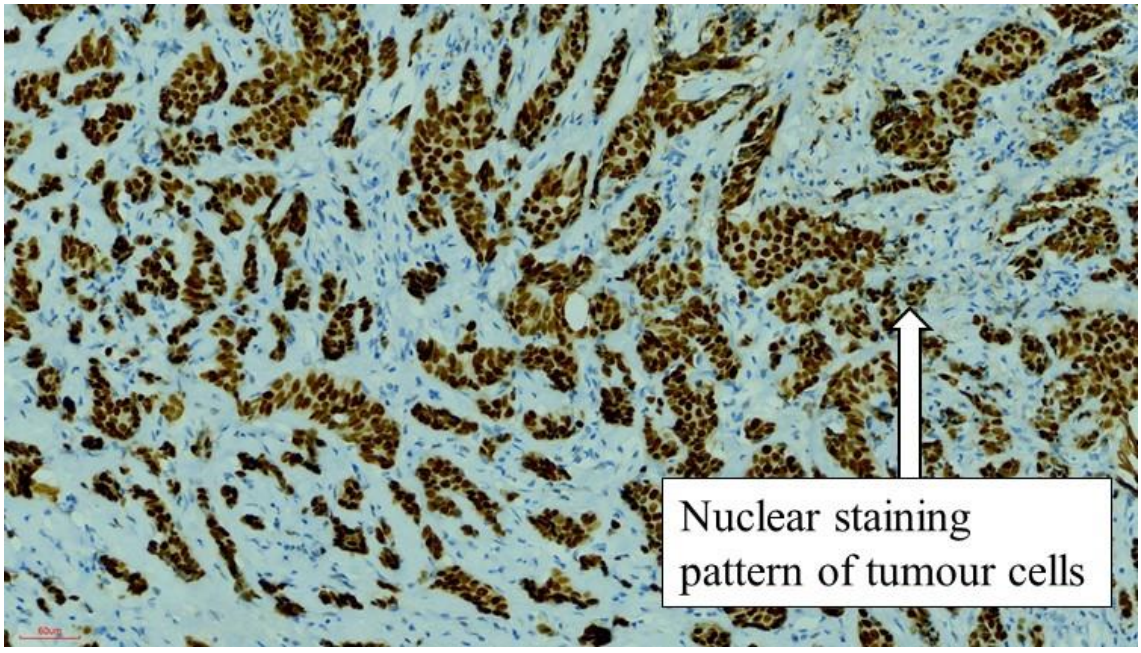


Figure 12. ER IHC stain showing strong and diffuse nuclear staining pattern in IBC-NST (x200 magnification).

Figure 13

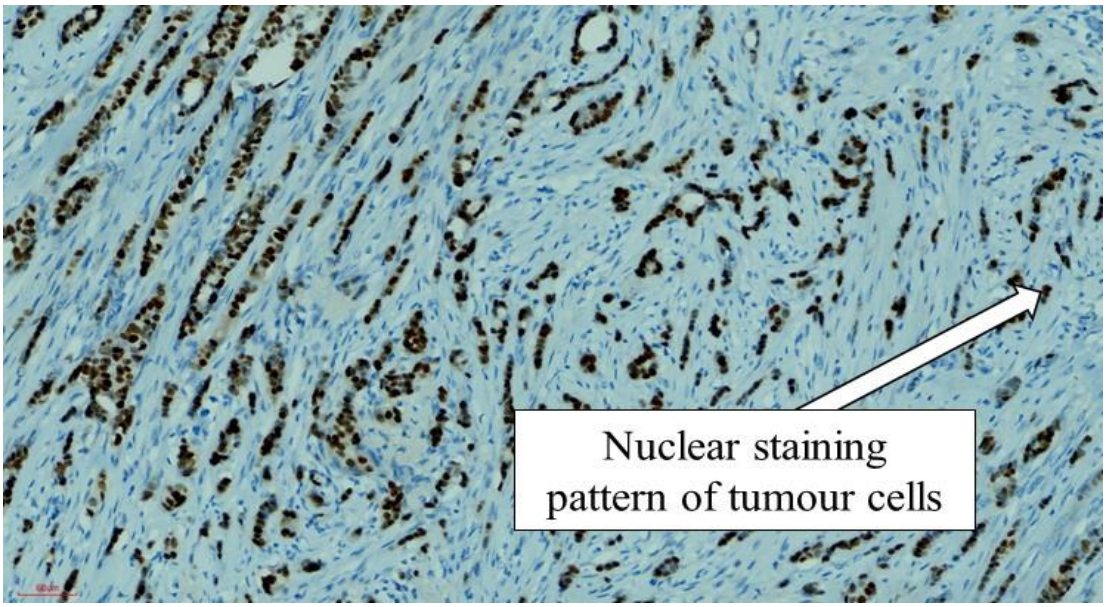


Figure 13. PR IHC stain showing strong and diffuse nuclear staining pattern in an IBC-NST seen in Figure 8 (x200 magnification).

Figure 14

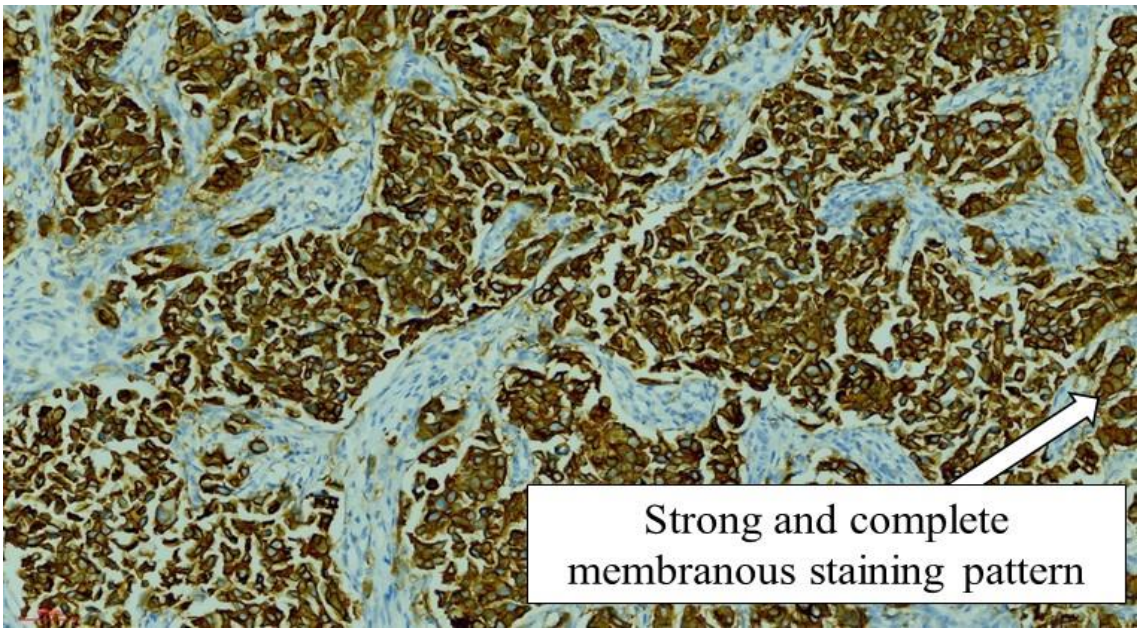


Figure 14. HER2 IHC stain showing strong and complete membranous staining pattern in more than 10% of the tumour cells; IBC-NST (x200 magnification).

Figure 15

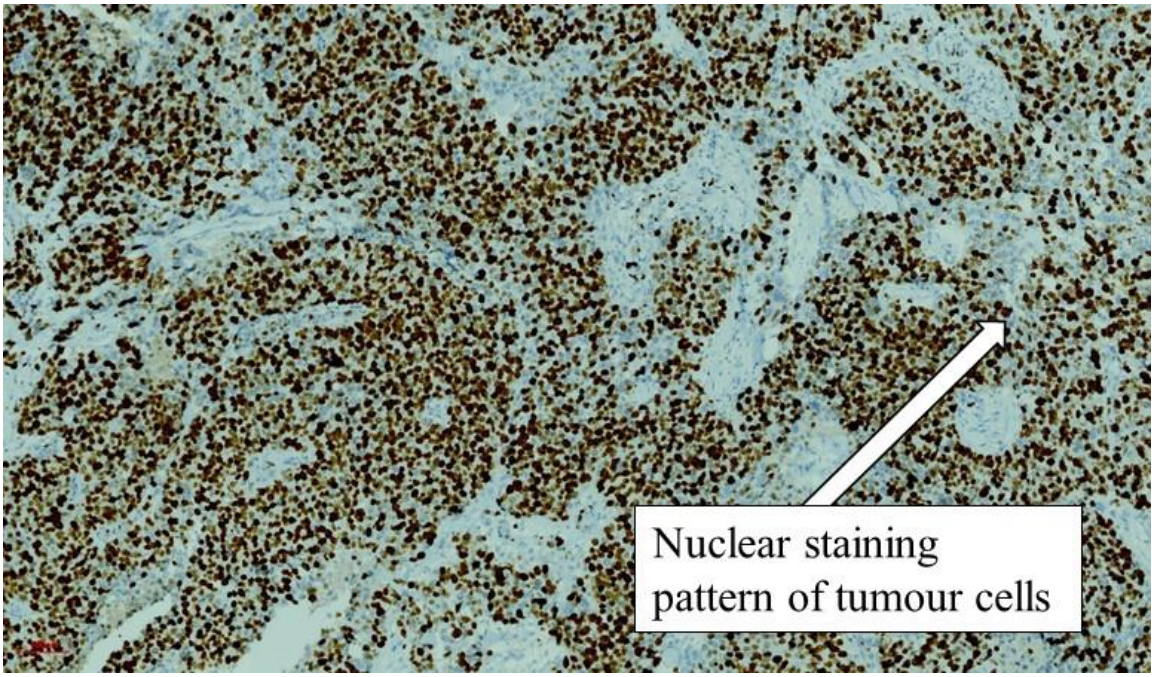


Figure 15. Ki-67 IHC stain showing high proliferative index characterised by nuclear staining pattern of tumour cells; IBC-NST (x200 magnification).

Figure 16

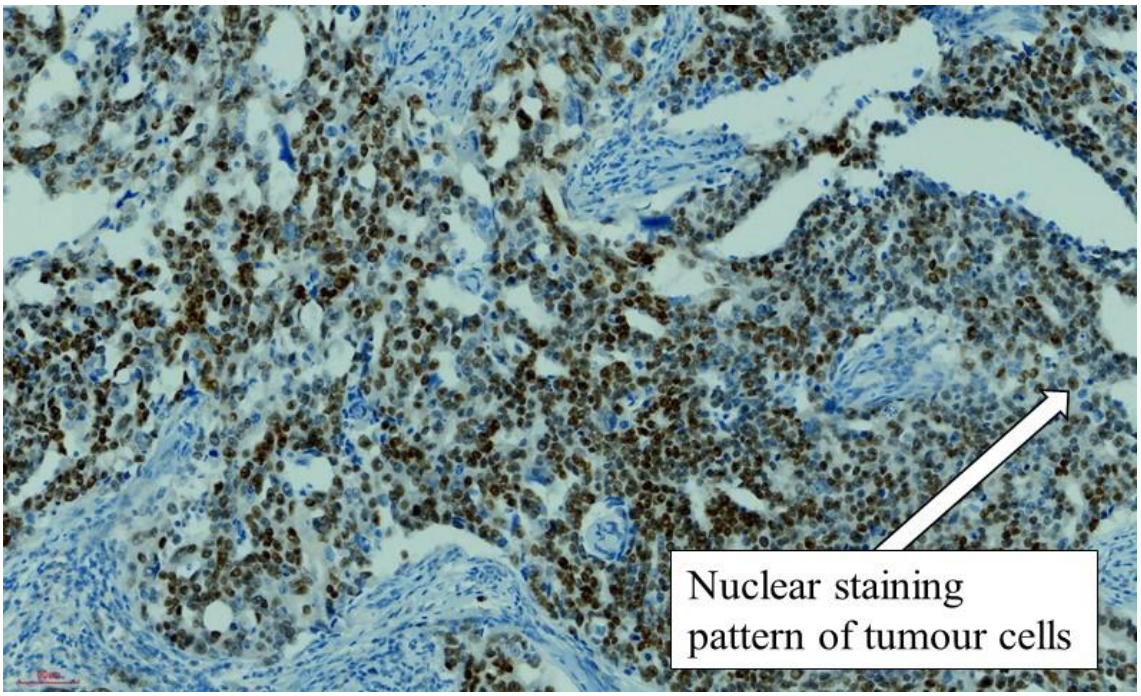


Figure 16. SOX10 IHC stain showing nuclear staining pattern; TNBC (x200 magnification).

Figure 17

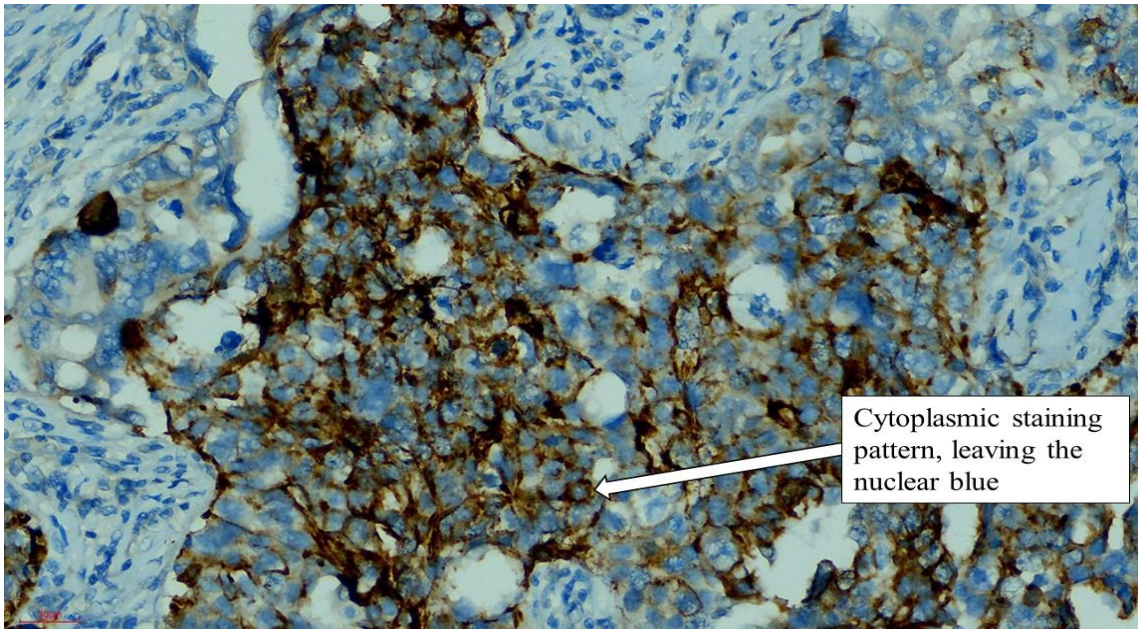


Figure 17. CK5/6 IHC stain showing cytoplasmic staining pattern; TNBC (x400 magnification)

Figure 18

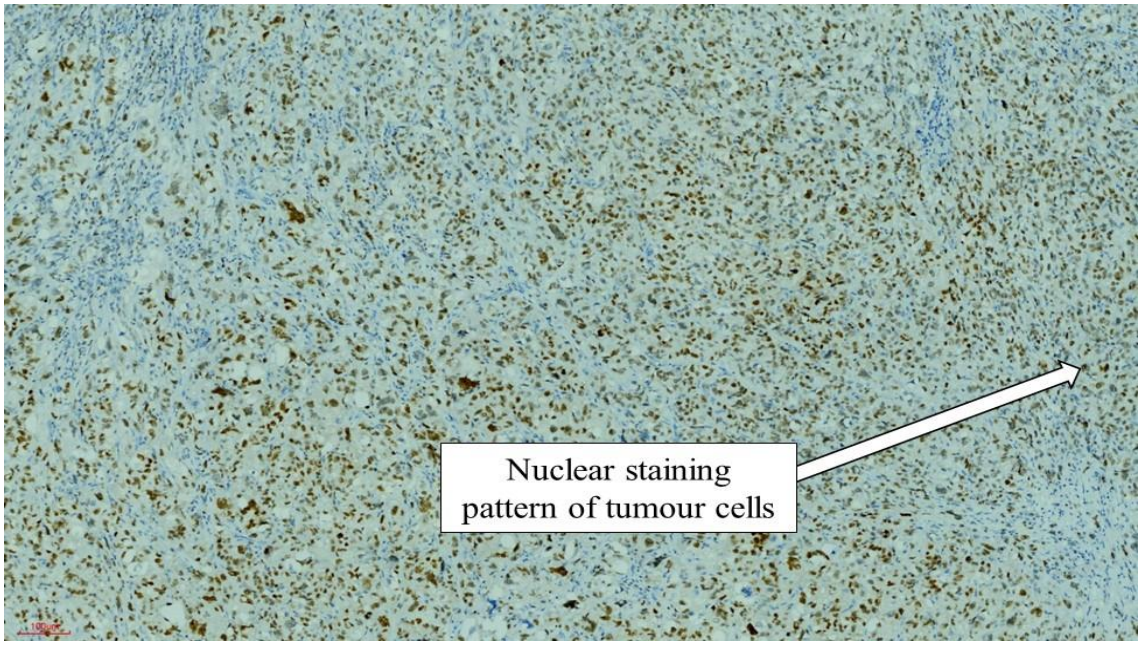


Figure 18. GATA3 IHC stain showing nuclear staining pattern; TNBC (x400 magnification).

4.6 Treatment response

Sataloff method was used for assessing treatment response. Majority of patients displayed poor treatment response, with 48.1% falling in the T-C category. Majority of patients who had triple negative immunoprofile, were in the T-C category and the rest of the patients were in the T-B category. None of the patients with triple negative molecular immunoprofile were in the T-A category. N/A represent patients who had surgical treatment upfront (without any chemoradiation therapy).

Figure 19

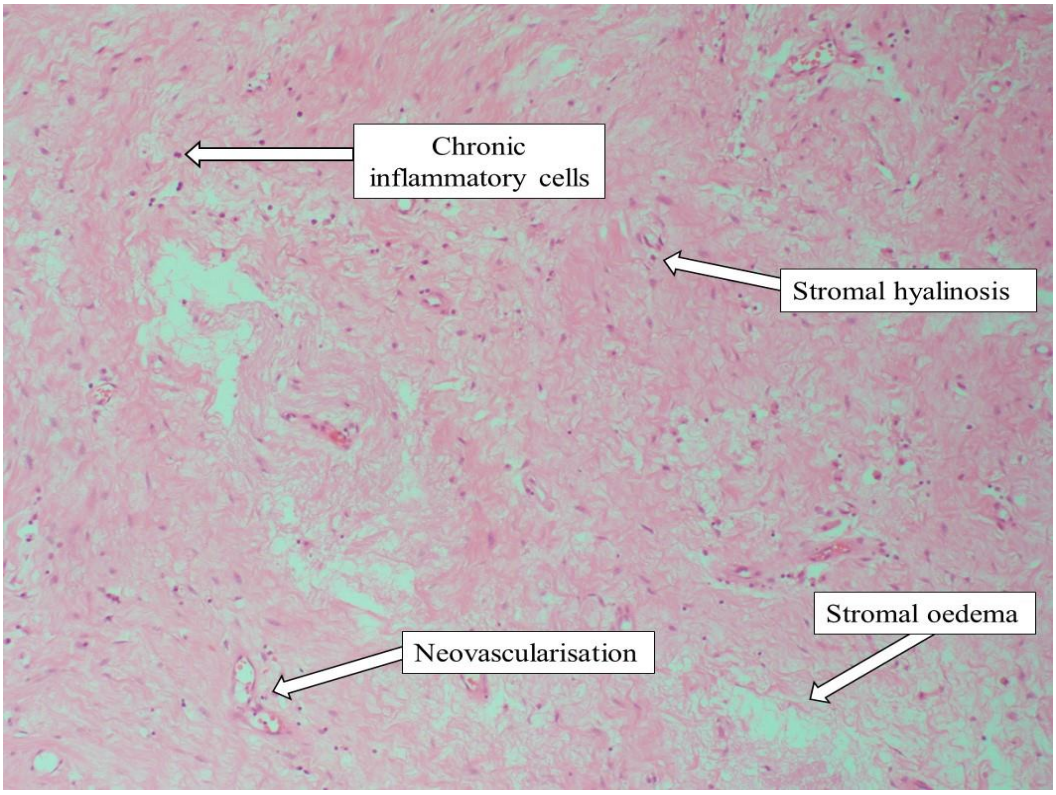


Figure 19. H&E image showing complete pathological treatment response. Treatment changes characterised by stromal hyalinosis, neovascularisation, scattered chronic inflammation and stromal oedema is evident (x100 magnification).

Figure 19 depicts complete treatment response (Sataloff method category T-A). The image was obtained from a 68-year-old female who tested negative for HIV, overweight BMI (26.54 kg/m2), and clinically T4BN0M0. Histopathological

IBC-NST, grade 2 and luminal A-like molecular subtype.

CHAPTER 5

DISCUSSION

5.1 Age

Most patients in our cohort were pre-menopausal with the mean age of diagnosis of 48.5 years which might suggest that breast cancer does indeed affect younger female Black patients when compare to female patients from other racial groups (26). However, more studies with bigger samples sizes are needed to draw a definitive conclusion.

5.2 BMI

In SA, approximately 50% of adults are either obese or overweight (42, 43, 44, 45). Obesity is a well-established risk factor for breast cancer, particularly in post-menopausal women because endogenous oestrogen in post-menopausal women is produced from conversion of adipose tissue (10, 51). Other studies have shown that being overweight or obese impacts overall survival in patients with BC, is associated with an increased risk of lymph node metastasis, and correlates with larger tumour size (7, 9). Van Baelen K *et al* (2023), found that higher BMI was linked to higher histopathological grade, larger tumour size, nodal metastasis and tumour multifocality in ER positive / HER2 negative tumours (52). Majority of patients in our cohort were overweight and/or obese, had T2 and above tumours at presentation, had nodal metastasis at presentation, displayed ER positive / HER2 negative tumours and higher histopathological grade. Although our sample size was small, our findings seem to correspond with findings from published data. However, there was no significant morphological difference in tumours between obese, overweight, and normal BMI women. Studies with bigger sample sizes and including women from other racial groups in SA are recommended.

5.3 Family History of Cancer

A positive family history of breast cancer or any cancer associated with *BRCA1* and *BRCA2* gene mutations (e.g., gastric carcinoma) is associated with increased risk of breast cancer (2, 5). We found that 81.5% of the patients in our study did not have a family history of cancer. Five patients with family history, either the mother or a maternal relative had breast cancer. None reported a family history of breast cancer / any other form of cancer from the paternal side. This raises the question of whether a positive family history of breast cancer is a significant risk factor for the development of invasive breast cancer in Black women in KZN.

5.4 HIV

Approximately 12.6% of people live with HIV in SA, and about 21.8% of people in KZN tested positive for HIV (35, 46). HIV status is not directly associated with breast cancer development, it is linked to therapy-related toxicity, worse outcomes, lower survival rates, and higher mortality (37, 38, 39, 40). Calabresi *et al*. (2013) found that patients living with HIV develop breast cancer much earlier (~42 years) compared to HIV-negative patients (~65–69 years) (41). More than 50% of patients in our cohort are living with HIV, which might indicate a significant overlap between HIV and BC in cohort. These findings underscore the need for special consideration and targeted interventions for patients with breast cancer who are living with HIV.

5.5 DCIS

DCIS is a precursor to invasive breast cancer, and high-grade DCIS can increase the risk of progression to invasive disease if left untreated (2). In our cohort we found that majority of patients had DCIS and most patients who had DCIS, had high grade DCIS. When breaking down the age distribution of DCIS, most high-grade DCIS cases were seen in younger women, below age 50. This indicates that younger patients in our cohort tend to present with more aggressive forms of DCIS. Nassar *et al.* (2009) found that DCIS was commonly diagnosed at a significantly older age in African-American women (67 years) compared to Caucasian women (56 years) (47). Similarly, Taj *et al.* (2011) found that DCIS was diagnosed at an older age in African-American women (48). These findings differ from our cohort, however, the smaller sample size in our cohort precludes accurate comparison. It remains uncertain whether factors, such as obesity or the use of hormonal contraceptives which were prominent in our cohort, contributed to the earlier diagnosis of DCIS in Black women in KZN. Further research with a larger sample size is needed to reach a definitive conclusion.

5.6 Morphological subtypes and histopathological grade

The majority of patients (96.3%) in our cohort had IBC-NST, which is consistent with findings in the global literature, where IBC-NST is the most common histologic subtype of breast cancer (2, 15, 16, 17). However, these findings still require validation with a bigger sample size.

Histopathological grading provides insight into the aggressiveness of the tumour (2, 15, 16, 18, 19). In our cohort, most cases were graded as Grade 2 (63%), indicating moderate differentiation. Grade 3 tumours, representing poorly differentiated cancers, accounted for 29.6% of the cases. This is concerning because poorly differentiated tumours are typically associated with a more aggressive disease course and poorer prognosis (2, 15, 16, 18, 19). Only a small proportion (3.7%) were graded as Grade 1, which usually suggests a better prognosis due to well-differentiated tumour cells (2, 15, 16, 18, 19). To our knowledge, no studies have provided exact figures for tumour grades in different population groups. While existing studies report a high incidence of Grade 3 tumours among Black / African women (5, 26), our cohort showed that nearly 30% of women had Grade 3 tumours. A larger sample size and inclusion of women from other racial / ethnic groups would provide more accurate results on whether Black women indeed have a higher incidence of Grade 3 tumours.

5.7 Molecular Classification

Molecular classification divides breast cancer into distinct subtypes, which help predict prognosis and guide treatment (2, 20). Most patients in our cohort had HER2-negative luminal B-like molecular subtype BC, which is associated with a higher risk of recurrence and poorer prognosis compared to the luminal A-like molecular subtype BC. Luminal A-like molecular subtype, typically associated with a better prognosis, comprised a smaller percentage of the cases (7.4%) (2, 20). These findings differ from the general population, where luminal A-like molecular subtype is the most common (2). Kerlikowske *et al.* (2011) found that increased BMI is associated with an increased risk of ER-positive breast cancer in post-menopausal women and an increased risk of ER-negative breast cancer in premenopausal women (49). Although most patients in our cohort were pre-menopausal, majority of our patients had increased BMI. It remains uncertain whether BMI had an influence in BC development in our patients.

A significant number of patients in our cohort had TNBC, which may be in line with the literature findings that Black women tend to have higher rates of TNBC compared to other racial groups (5, 23, 26). TNBC in our cohort was mostly seen in younger patients, below age 50, which is

consistent with current literature which states that TNBC is more common in younger Black women (5, 23, 25).

5.8 Molecular Classification and Genetic Results

The presence of *BRCA1* and *BRCA2* mutations is linked to a higher risk of BC development (2). BC's associated with *BRCA1* germline mutations typically display a medullary pattern, are often high-grade, and show a triple-negative molecular profile. In contrast, BC's with *BRCA2* mutations are generally invasive and of a lower grade (12, 13, 14). According to the WHO, majority of TNBC harbor *BRCA1* germline mutations (2). *BRCA1*, *BRCA2,* and *RAD54L* mutations were rare in our cohort as they were only found in a minute proportion of young / TNBC patients. These findings suggest that Black women in our cohort may have other genetic mutations responsible for breast cancer development. Further studies are needed to investigate the causative genetic mutations of breast cancer in Black women in KZN. It would also be interesting to include women from other racial groups and compare the findings.

5.9 Treatment Response

Almost all patients in our cohort received chemotherapy or endocrine therapy as part of their treatment. Majority of patients in our cohort had a poor treatment response. According to the literature, a treatment response of less than 50% (T-C) is associated with poor survival and prognosis (50). Since majority of patients in our cohort had luminal B-like molecular profile, which is associated with poor prognosis (2, 20), this may explain why most patients had poor treatment responses. However, these findings still require validation with a bigger sample size. If validated, these findings may call for more research on treatment approaches in KZN Black women.

Our study had several limitations. First, this was a retrospective study, therefore our findings need to be verified through prospective studies. Second, the principal investigator of this study had no role in selecting participants of the study. Third, our sample size was small, which precludes accurate comparison to other studies done in international cohorts. Fourth, to the best

of our knowledge, there are no studies in SA that have looked at BC morphology, molecular profile and genetic findings. Validation with different ethnicities is needed. The extent of BRCA1 and BRCA2 testing could not be determined from the patient records.

CHAPTER 6

CONCLUSION

AND

RECOMMENDATIONS

Study findings

In this study, IBC-NST was the most common morphologic subtype (96.3%) in Black women. While most tumours were classified as histopathological grade 2 (63.0%), a significant proportion were grade 3 tumours (29.6%). This aligns with the findings that Black women have a higher rate of grade 3 tumours. The Luminal B-like molecular subtype (70.4%) was the most common, contrasting with the WHO findings, where Luminal A-like molecular subtype (7.4%) is the most prevalent. Triple-negative molecular subtype (18.5%) was the second most common molecular subtype in Black women in our study. The majority of cases were negative for *BRCA1* and *BRCA2* gene mutations. Notably, most TNBC cases were negative for the *BRCA1* mutations. This suggests that other genetic variations may be responsible for the pathogenesis of BC in Black SA women.

Of note, overweight, obesity, and use of hormonal contraceptives were highly associated with breast cancer in Black SA women. There was high prevalence of HIV amongst patients, which was higher than that of the general population. BC in KZN Black women is more common in younger women (median age at diagnosis:48.52 years) compared to older women. The majority of cases showed poor treatment response, but the reasons for this remain unclear.

In conclusion: Black SA women based in KZN present with IBC-NST morphologic subtype of BC and higher histopathological grade tumours. Although patients in our cohort mostly have Luminal B-like molecular subtype, there is a significant rate of TNBC. *BRCA1* and *BRCA2* gene mutations are rare in young / TNBC, only found in small proportion.

Recommendations:

We encourage more studies with larger sample sizes to examine the morphology, tumour grade, molecular profile, and genetic profile of breast cancer in Black women in KwaZulu-Natal. A comparative study including women from other racial groups would be valuable. It would also be insightful to explore the role of risk factors such as obesity, HIV and the use of hormonal contraceptives in breast cancer development, treatment response, and overall long-term survival.

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Appendices

APPENDIX 1

Modified Bloom and Richardson/Nottingham grading system

1. **Tubular formation:**

- Low power (*4x objectives*) throughout the whole tumour.
- Tubules with clear central lumina and devoid of myoepithelial cells.

Scores:

- 1 point= >75% of the tumour growth pattern is tubular.
- 2 points= 10-75% of the tumour growth pattern is tubular.
- 3 points= <10% of the tumour growth pattern is tubular.

2. Mitotic count

- Started from more active area (hotspot).
- 10 high power fields.
- 1 HPF= 40X objective.
- Not necessarily contiguous- ONLY fields with representative tumour cell burden were assessed.

Scores:

- 1 point= 0-8 mitotic figures in 10 high power fields.
- 2 points= 9-18 mitotic figures in 10 high power fields.
- 3 points= >18 mitotic figures in 10 high power fields.

3. Nuclear pleomorphism

- Look in areas with the greatest atypia.
- Nuclear: size, shape and contours.
- Nucleoli: size and number.
- Compare to normal epithelial cells in adjacent breast tissue.

- The preferred magnification for nuclear scoring is 40× objective.

Scores:

1 point= minimal variation.

- Nuclei are very similar in size to the nuclei of benign pre-existing epithelial cells (< 1.5 times the size).
- They show minimal pleomorphism, an even chromatin pattern, and nucleoli that are either not visible or very inconspicuous.

2 points= moderate variation.

- Nuclei are larger (1.5–2 times the size of benign epithelial cell nuclei).
- Mild to moderate pleomorphism and visible but small and inconspicuous nucleoli.

3 points= marked variation.

- Nuclei are even larger (> 2 times the size of benign epithelial cell nuclei).
- Vesicular chromatin.
- They vary markedly in size and shape and often show prominent nucleoli.

Final scores:

- 3-5 points= Grade I (well differentiated)
- 6-7 points= Grade II (moderately differentiated)
- 8-9 points= Grade III (poorly differentiated)

APPENDIX 2

Table 3: Royal College of Pathologists DCIS grading.

DCIS Grade	**Growth patterns seen**	**Nuclear features**	**Other features**
DCIS of low nuclear grade	o Cribriform, micropapillary, papillary or (less often) solid patterns involving more than two complete spaces (or measuring > 2 mm). o Microrosettes with small luminal spaces.	o Uniform in size and shape, with regular chromatin and inconspicuous nucleoli. o The nuclei are 1.5–2 times the size of an erythrocyte.	o Mitotic figures are rare. o Microcalcification is commonly seen in secretions within the luminal spaces and may be psammomatous. o Necrosis is uncommonly present in low-grade DCIS, but it does not preclude the diagnosis. o No comedonecrosis.
DCIS OF intermediate nuclear grade	o Same as low grade.	o Cells show moderate variability in size, shape, and polarization. o The nuclei have variably coarse chromatin and sometimes have prominent nucleoli.	o Mitoses may be present. o Necrosis (either punctate or comedo) may be seen. o Microcalcifications may be present in secretions and/or in necrotic material.

DCIS of high nuclear grade	○ Predominantly solid growth pattern. ○ However, other growth patterns can be seen too. ○ Uncommonly, a single layer of large, highly atypical cells lines spaces in flat DCIS of high nuclear grade (previously sometimes called the pleomorphic subtype of clinging DCIS).	○ Large and typically pleomorphic, with irregular contours, coarse chromatin, and often prominent nucleoli. ○ The nuclei are > 2.5 times the size of an erythrocyte in diameter.	○ Mitoses are usually conspicuous. ○ Central comedonecrosis bearing microcalcification is often present but is not mandatory for the diagnosis. ○ DCIS of high nuclear grade may also present as Paget disease of the nipple.

APPENDIX 3

Molecular Classification of invasive breast carcinoma:

1. **Luminal A–like molecular subtype**

- ER: positive
- PR: positive
- HER2: negative
- Ki-67 proliferation index: low (<14%)

2. **Luminal B–like (HER2-negative) molecular subtype**

- ER: positive
- HER2: negative
- At least one of the following:
  - ✓ Ki-67 proliferation index: high (>14%)
  - ✓ PR: negative or low

3. **Luminal B–like (HER2-positive) molecular subtype**

- ER: positive
- HER2: overexpressed or amplified
- Ki-67 proliferation index: any
- PR: any

4. **HER2-positive (non-luminal) molecular subtype**

- HER2: overexpressed or amplified
- ER: absent
- PR: absent

5. **Triple negative molecular subtype**

- ER: absent
- PR: absent
- HER2: negative

✓ Types of triple negatives MS:

- Basal-like 1
- Basal-like 2
- Immunomodulatory
- Mesenchymal-like
- Mesenchymal stem-like
- Luminal androgen receptor

APPENDIX 4

Allred scoring system:

A. **Intensity**

- Score of 0= negative even at 40x objective.
- Score of 1= weak positive seen best on high power.
- Score of 2= moderate positive seen best on intermediate power.
- Score of 3= strong positive seen on low power.

B. **Proportion**

- Score of zero= 0% cells staining (negative).
- Score of 1= <1% cells staining, any intensity (negative).
- Score of 2= 1-10% cells staining, any intensity (low positive).
- Score of 3= 11-33% cells staining, any intensity (positive).
- Score of 4= 34-66% cells staining, any intensity (positive).
- Score of 5= >66% of cells staining, any intensity (positive).

1. **Oestrogen receptor:**

- Validated for:
  - Prediction of benefit from hormone therapies if positive.
- Other uses:
  - Categorisation for overall treatment pathways.
  - Characterisation as the IHC luminal group if positive.
  - Poor prognostic marker if negative.
- Positive:
  - >/=1% of invasive cancer has nuclear staining of any intensity.
- Negative:

- <1% or 0% of invasive cancer has nuclear staining (follow proper QA and most-recent guidelines to ensure not a false negative result).

2. Progesterone receptor:

- Validated for:
  - Primarily prognostic in ER-positive cancers (not well-validated for prediction of endocrine therapy benefit).
- Other uses:
  - Poor prognostic marker if negative (in ER-positive cancers).
  - Further characterisation of the IHC group subtype.
- Positive:
  - >/=1% of invasive cancer has nuclear staining of any intensity.
- Negative:
  - < 1% or 0% of invasive cancer has nuclear staining (follow proper QA and most-recent guidelines to ensure not a false negative result).

3. ERBB2 (HER2) (using American Society of Clinical Oncology/ College of American Pathologists (ASCO/CAP) guidelines)**:**

- Validated for the
  - Prediction of benefit from HER2-targeted therapy if positive (administered with chemotherapy).
- Other uses:
  - Categorisation for overall treatment pathways.
  - Characterisation as the HER2-enriched IHC subtype (if ER-negative) or luminal B (if ER-positive).
  - Marker of aggressive biology.

- IHC:
  - Positive= 3+**:** Circumferential membrane staining that is complete, intense, and in > 10% of tumour cells (at 2-5x objectives).
  - Equivocal= **2+:** Weak to moderate complete membrane staining observed in > 10% of tumour cells (at 10-20x objectives). ISH (dual-probe ISH or single probe ISH) is done for such cases.
  - Negative=
    - **1+:** Incomplete membrane staining that is faint / barely perceptible and in > 10% of tumour cells (at 40x objectives).
    - **0:** No staining is observed, or incomplete membrane staining that is faint / barely perceptible and in ≤ 10% of tumour cells (at 40x objectives).

4. Ki-67 proliferation index:

The interpretation of the Ki-67 proliferation index is important for assigning a hormone receptor (oestrogen receptor) positive tumour to luminal A-like molecular subtype or luminal B-like molecular subtype when using immunohistochemistry as a surrogate for gene expression profile. This is important for the choice of management and prognosis. There are many counting methods that are currently used for interpreting the Ki-67 proliferation index. The WHO method of Ki-67 interpretation was utilised in this study. The Ki-67 is calculated in 10 high power fields (with 1 high power field being x40 objectives/x400 objectives) (3). The WHO recommends spotting an area with the highest number of mitotic figures (hot-spot area) which is usually the peripheral leading edge of the tumour. Once the hot-spot area is picked, you start calculating from there and move randomly (only in areas with viable tumour cells) until you reach 10 high power fields (3).

APPENDIX 5

Sataloff Method

a. Tumour

- T-A Total or near total therapeutic effect (pCR)
- T-B 50% therapeutic effect, but less than total or near total (pPR)
- T-C 50% therapeutic effect, but effect evident (pPR)
- T-D No therapeutic effect (pNR)

b. Nodes

- N-A Evidence of therapeutic effect, no metastatic disease
- N-B No nodal metastasis or therapeutic effect
- N-C Evidence of therapeutic effect, but nodal metastasis present
- N-D Viable metastatic disease, no therapeutic effect

APPENDIX 6

Design of Data Collection Tool & Testing

Name:

Hospital number:

Age: DOB: Gender: Race: First visit at ADH:

PRESENTING COMPLAINT

- History of presenting complaint

RISK ASSESSMENT:

- Previous breast problems
- Family history breast cancer
- Other cancers Previous radiation Estrogen window:
  - Hormonal Therapy: o Age first pregnancy: o Age last pregnancy:
  - Menarche:
  - Menopause:

MEDICAL HISTORY

- Co-morbidities:
- Medications:
- Effort tolerance:
- Past surgery:
- Allergies: Social Habits:

FOCUSED BREAST EXAMINATION

Bra size: Height: Weight: BMI:

- Left breast and axilla
- Right breast and axilla

DIAGNOSIS

Clinical:

Mammogram:

- Breast density:
  - o Left breast and axilla o Right breast and axilla

Ultrasound:

- Left breast and axilla
- Right breast and axilla

COMMENT

Histopathology:

STAGING INVESTIGATIONS:

- CXR
- Bloods
- Ultrasound liver
- U+E/Cr:
- Bone scan
- FBC:
- MUGA scan
- Calcium:
- LFT:
- HIV:

SUMMARY

MANAGEMENT

- Primary systemic therapy
- Rationale
- Surgery
- Adjuvant therapy
- Radiotherapy
- Other

Genetic assessment:

APPENDIX 7

Table 4: Primary antibodies utilized in this study:

Antibody	Supplier	Clone	Concentration	Staining pattern	External control used
ER	Ventana	SP1	Predilute 1µg/mL	Nuclear	Breast carcinoma
PR	Ventana	1E2	Predilute 1µg/mL	Nuclear	Breast carcinoma
HER2/NEU	Ventana	4B5	Predilute 6µg/mL	Membranous	Breast carcinoma
Ki-67	Ventana	30-9	Predilute 2µg/mL	Nuclear	Lymph node
P63	Ventana	4A4	Predilute 0.14µg/mL	Nuclear	Normal prostate
Calponin	Roche-cell Marque	EP798Y	Predilute 0.17µg/ml	Cytoplasm	Normal breast
CK5/6	Ventana	D5/16 B4	Predilute 10.4µg/mL	Cytoplasm	Normal prostate
CK14	AB CAM	EPR17350 monoclonal Ab	0.5µL in 1000 µL. 2.289mg/ml	Cytoplasm and nuclear	Normal prostate

SOX10	Cell Marque	EP268	199µg/ml which is made at 1:100	Nuclear	Melanoma
GATA3	Cell Marque	L50-823	Ready to use 1.57µg/ml	Nuclear	Normal breast
E-cadherin	Cell Marque	EP700Y	Predilute 0.85µg/ml	Loss of membranous	Normal breast
P120	Ventana	J10318	Predilute 0.076µg/mL	Loss of membranous	Normal breast

For each antibody used, a positive external quality control was used for accurate interpretation. Oestrogen receptor (ER) and progesterone receptor (PR) are the hormone antibodies. Their positivity is important for management, especially using endocrine therapy. HER2/NEU positivity is also important for management because these tumours tend to respond well to Trastuzumab/Herceptin drug. Calponin and p63 antibodies are used to highlight the presence of myoepithelial cells, to diagnose carcinoma in-situ. E-cadherin and p120 antibodies are used to rule out invasive lobular carcinoma or lobular carcinoma in-situ. Loss of membranous staining pattern for E-cadherin and p120 supports the diagnosis of invasive lobular carcinoma and/or lobular carcinoma in-situ. For triple negative invasive carcinoma, CK5/6, CK14 and SOX10 are used as the basal markers. The positivity of these antibodies, support, basal-like immunophenotype. GATA3 is important, especially in triple negative and metastatic carcinoma (in addition to clinical history and radiology findings) to support primary breast origin.

Appendix 8

Clinicopathological features:

Patient/Parameters	Age	Menarche	Menopause
KZN1	43	12	N/A
KZN2	52	13	45
KZN3	48	13	N/A
KZN4	59	14	40
KZN5	48	14	N/A
KZN6	44	18	N/A
KZN7	27	13	N/A
KZN8	65	12	43
KZN9	68	17	55
KZN10	47	16	N/A
KZN11	51	15	47
KZN12	64	15	49
KZN13	44	14	N/A
KZN14	38	14	N/A
KZN15	85	17	47
KZN16	54	15	46
KZN17	79	17	50
KZN18	58	16	53
KZN19	35	12	N/A
KZN20	59	12	54
KZN21	47	14	N/A
KZN22	35	16	N/A
KZN23	50	12	N/A
KZN24	52	14	N/A
KZN25	47	13	N/A
KZN26	61	11	50
KZN27	61	15	49

Hormonal therapy	Family history of breast cancer	HIV status
None	YES	Positive
Medroxyprogesterone acetate (Depo-Provera)	NO	Positive
Medroxyprogesterone acetate (Depo-Provera)	NO	Positive
None	NO	Positive
None	NO	Positive
Medroxyprogesterone acetate (Depo-Provera)	NO	Positive
None	NO	Negative
Oral contraceptive use	NO	Negative
None	NO	Negative
Medroxyprogesterone acetate (Depo-Provera)	NO	Positive
None	NO	Negative
Medroxyprogesterone acetate (Depo-Provera)	NO	Negative
Medroxyprogesterone acetate (Depo-Provera)	NO	Negative
None	NO	Positive
None	NO	Negative
None	NO	Positive
Oral contraceptive use	NO	Negative
Medroxyprogesterone acetate (Depo-Provera)	YES	Negative
Medroxyprogesterone acetate (Depo-Provera)	NO	Negative
Medroxyprogesterone acetate (Depo-Provera)	NO	Positive
None	YES	Positive
Medroxyprogesterone acetate (Depo-Provera)	YES	Positive
Medroxyprogesterone acetate (Depo-Provera)	NO	Negative
Medroxyprogesterone acetate (Depo-Provera)	YES	Positive
Medroxyprogesterone acetate (Depo-Provera)	NO	Negative
None	NO	Positive
Medroxyprogesterone acetate (Depo-Provera)	NO	Negative

BMI	Age at diagnosis of breast cancer	Genetic results
41,38	40	BRCA1
28,2	48	
31,8	44	Negative
34,9	55	
28,9	44	BRCA2
35,9	40	BRCA2
31,2	23	Negative
40,7	62	
26,54	64	
22,7	44	Negative
37,9	47	Negative
28,14	60	
26,75	40	BRCA2
21,67	34	Negative
55	72	N/A
22,38	50	
39,86	75	
31,3	54	
30,8	29	Negative
37,48	55	
26,8	43	RAD54L
30,53	32	Negative
23,65	47	Negative
52,87	49	Negative
39,6	43	Negative
24	58	
36,2	58	

Patient/Parameters	Histological subtype	Histological grade
KZN1	Invasive breast carcinoma, no special type	Grade 3
KZN2	Invasive breast carcinoma, no special type	Grade 2
KZN3	Invasive breast carcinoma, no special type	Grade 2
KZN4	Invasive breast carcinoma, no special type	Grade 2
KZN5	Invasive breast carcinoma, no special type	Grade 2
KZN6	Invasive breast carcinoma, no special type	Grade 2
KZN7	Invasive breast carcinoma, no special type	Grade 2
KZN8	Invasive breast carcinoma, no special type	Grade 3
KZN9	Invasive breast carcinoma, no special type	Grade 2
KZN10	Invasive breast carcinoma, no special type	Grade 1
KZN11	Invasive breast carcinoma, no special type	Grade 2
KZN12	Invasive breast carcinoma, no special type	Grade 2
KZN13	Invasive breast carcinoma, no special type	Grade 3
KZN14	Invasive breast carcinoma, no special type	Grade 3
KZN15	Invasive breast carcinoma, no special type	Grade 2
KZN16	Solid papillary carcinoma	N/A
KZN17	Invasive breast carcinoma, no special type	Grade 2
KZN18	Invasive breast carcinoma, no special type	Grade 2
KZN19	Invasive breast carcinoma, no special type	Grade 3
KZN20	Invasive breast carcinoma, no special type	Grade 3
KZN21	Invasive breast carcinoma, no special type	Grade 2
KZN22	Invasive breast carcinoma, no special type	Grade 3
KZN23	Invasive breast carcinoma, no special type	Grade 2
KZN24	Invasive breast carcinoma, no special type	Grade 3
KZN25	Invasive breast carcinoma, no special type	Grade 2
KZN26	Invasive breast carcinoma, no special type	Grade 2
KZN27	Invasive breast carcinoma, no special type	Grade 2

Molecular classification	Carcinoma in-situ	Treatment response
Triple negative, basal-like	Absent	T-B
HER2 positive luminal B	High grade ductal carcinoma in-situ	T-A
HER2 positive luminal B	Intermediate grade ducal carcinoma in-situ	T-B
HER2 negative luminal B	Absent	N/A
HER2 negative luminal B	Absent	T-B
HER2 negative luminal B	Absent	N/A
HER2 positive luminal B	High grade ductal carcinoma in-situ	T-B
Triple negative, unspecified	High grade ductal carcinoma in-situ	T-B
Luminal A	High grade ductal carcinoma in-situ	T-A
Luminal A	High grade ductal carcinoma in-situ	T-C
Triple negative, basal-like	Absent	T-C
HER2 negative luminal B	Intermediate grade ducal carcinoma in-situ	T-C
HER2 negative luminal B	High grade ductal carcinoma in-situ	T-C
HER2 negative luminal B	Absent	T-A
HER2 negative luminal B	Absent	T-B
N/A	Absent	T-C
HER2 negative luminal B	Absent	T-B
HER2 negative luminal B	Intermediate grade ductal carcinoma in-situ	T-C
HER2 positive luminal B	Absent	T-C
HER2 positive luminal B	Absent	T-C
Triple negative, basal-like	High grade ductal carcinoma in-situ	T-C
HER2 negative luminal B	High grade ductal carcinoma in-situ	T-B
HER2 negative luminal B	Intermediate grade ducal carcinoma in-situ	T-C
Triple negative, basal-like	Absent	T-C
HER2 negative luminal B	High grade ductal carcinoma in-situ	T-A
HER2 negative luminal B	High grade ductal carcinoma in-situ	T-C
HER2 negative luminal B	Intermediate grade ducal carcinoma in-situ	T-C

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

03 April 2023

Mr Bakhusele Blessing Bungane (212508546)
School of Lab Med & Medical Sc
Medical School

Dear Mr Bungane,

Protocol reference number: BREC/00005294/2023
Project title: The relationship between histopathological features, immuhistochemical profile and associated genetic mutations of breast cancer in Black South African women attending at Inkosi Albert Luthuli Central Hospital, 2021
Degree Purposes: 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 03 April 2023. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 03 April 2023. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG 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 (2020) (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 the full Committee at its next meeting on 09 May 2023.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

STUDY TEAM, CONTRIBUTORS AND AUTHORSHIP

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- National Health Laboratory Services and University of KwaZulu-Natal.

Co-investigators:

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- Pathologist, Anatomical Pathology.
- National Health Laboratory Services and later AMPATH Laboratory, KwaZulu-Natal.
- University of the Kwa-Zulu Natal Supervisor

- Mpoi Makhetha
- Geneticist
- University of the Kwa-Zulu Natal
- University of the Kwa-Zulu Natal Co-Supervisor