MOLECULAR SUBTYPE CHARACTERIZATION OF PRIMARY BREAST CANCER AT THE UNIVERSITY TEACHING HOSPITAL, LUSKA ZAMBIA

BY

ALLEN CHOMBA CHAMA

A dissertation submitted to the University of Zambia in partial fulfilment of the requirement for the award of the degree of Master of Medicine in Pathology

UNIVERSITY OF ZAMBIA

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DECLARATION

I, **Allen Chomba Chama** this 10th day of June 2019, declare that this dissertation represents my own work. This work has not been done in Zambia before and neither has it been published for any qualification at the University of Zambia or any other University. Various sources to which I am indebted are clearly indicated in the text and in the references.

Signed: …… Date……

APPROVAL

This dissertation for Dr. **Allen Chomba Chama** has been approved as partial fulfillment of the requirements for the award of the Master of Medicine in Anatomical Pathology at the University of Zambia.

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Internal Examiner 1	Signature	Date
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Internal Examiner 2	Signature	Date
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External Examiner	Signature	Date
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Chairperson/Board of Examiners	Signature	Date
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Supervisor	Signature	Date

ABSTRACT

Breast cancer is the second most common cancer in women in Zambia. It has been established that Oestrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) are important markers for treatment and prognosis of breast cancer**.** Treatment has now become targeted and depends on the molecular subtype. However, at University Teaching Hospitals (UTHs) routine testing for ER, PR and HER2 is not done resulting in patients receiving suboptimal treatment. The aims of this study were to determine the age distribution of breast cancer patients, to classify these cancers according to their histologic type and grade and to molecular classify them as Luminal A, Luminal B, HER2 type and TNBC using ER, PR and HER2 immunohistochemistry (IHC).

A total of 68 paraffin embedded breast biopsies from female adults with a histologic diagnosis of breast cancer between 2016 and 2018 were evaluated using standard IHC in this laboratory based cross sectional study. Allred scoring and CAP/ASCO guidelines were used to determine ER/PR and HER2 status, which were considered valid only in the presence of positive controls. Luminal A (Lum A) cancers were defined as ER+/PR+/HER- with a Nottingham mitotic score of 1 while mitotic scores of 2 or 3 were classified as Luminal B (Lum B). HER2 type was defined as any HER2+ (>10%, 3+), and triple negative as ER-/PR-/HER2- (TNBC). FISH was not available and so equivocal HER2 were considered negative.

Data was collected for 68 patients, ranging in age from 29-93 years (average and Median 51.3 and 51years respectively). More than half of the cases were ER and/or PR positive (61.8%). However only 13.2% of the tumors examined were the least aggressive Luminal A subtype. 48.5% were of the Luminal B subtype followed by TNBC (29.4%) and the aggressive HER2 type was only seen in 8.8% of the cases. Invasive ductal carcinoma (NOS) was the most common histologic type and majority of tumors were grade 2. Data was analyzed using SPSS version 22 and Chi-square test was done for association between age and dependent variables, all analyzed at 95% confidence interval and p-values <0.05 were considered significant.

The study demonstrated breast cancer heterogeneity at UTHs. We therefore recommend a routine ER, PR and HER2 IHC panel for breast cancer in selection of patients for targeted therapy.

Keywords: Breast cancer, molecular subtype

DEDICATION

To my parents, William Felisho (late) and Idah Chitalu Chama for being my inspiration

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ACRONYMS / ABBREVIATIONS

ASCO	American Society of Clinical Oncology
CAP	College of American Pathologists
CI	Confidence Interval
DNA	Deoxyribonucleic acid
DPX	Distyrene Plasticizer Xylene
ER	Oestrogen receptor
ERES	Excellence in Research Ethics and Science
FISH	Fluorescence in Situ Hybridization
HER2	Human epidermal growth factor receptor 2
H&E	Hematoxylin and Eosin
HRP	Horseradish Peroxidase
IDC	Invasive Ductal Carcinoma
IHC	Immunohistochemistry
IS	Intensity Score
IT	Information Technology
LIMS	Laboratory Information Management System
LM	Light Microscopy
mRNA	Messenger Ribonucleic acid
NOS	Not Otherwise Specified

NR3C3	Nuclear Receptor subfamily 3 member 3
P	Prevalence
PAS	Periodic Acid Schiff
PR	Progesterone Receptor
PS	Proportion Score
QA	Quality Assurance
SD	Standard Deviation
SEER	Surveillance Epidemiology and End Results
SOP	Standard Operating Procedures
SPSS	Statistical Package for Social Sciences
TNBC	Triple Negative Breast Cancer
TS	Total Score
USA	United States of America
UTHs	University Teaching Hospitals
Z	Percentage of confidence level

DEFINITIONS

Breast cancer: Any type of malignant neoplasm arising in the breast.

Gene: A functional unit of hereditary that occupies a specific place (locus) on a chromosome that is capable of reproducing itself exactly at each cell division and directs the formation of an enzyme or other protein.

Gene amplification: A process for producing an increase in pertinent genetic material. It also includes the production of extra chromosomal copies of the genes for RNA.

Heterodimerization: Formation of biologically active dimers derived from two or more different monomers.

Human epidermal growth factor receptor 2: This is a type of receptor tyrosine kinase, a protein involved in normal cell growth.

Immunohistochemistry: Demonstration of specific antigens in tissues by use of markers that are either fluorescent dyes or enzymes.

Luminal: Luminal type breast cancer cells resemble the epithelial cuboidal cells that form the innermost (Luminal) layer of bilayered ductulobular system of the breast.

Messenger RNA (mRNA): The RNA reflecting the exact nucleoside sequence of the genetically active DNA and carrying the "message" of the latter coded in its sequence, to the ribosomes where protein is made in amino acid sequences specified by the mRNA.

Mitotic score: The number of cells dividing in a certain amount of tumor tissue**.**

Oestrogen receptors: A structural protein molecule on the cell surface that binds to oestrogen.

Progesterone receptors: Also known as NR3c3 or nuclear receptor subfamily 3, group c, number 3, is a protein found inside cells. It is activated by the steroid hormone progesterone.

Protein overexpression: Formation of too many copies of a particular protein.

CHAPTER ONE

INTRODUCTION

1.1 Background

Breast cancer is amongst the most common cancers and an important cause of cancer related deaths among women in the world(Parkin, Bray et al. 2001). It is the second most common cancer in women in Zambia(Zyaambo, Nzala et al. 2013). In the recent past, it has been established that Oestrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) are important markers for treatment and prognosis of breast cancer(Ludwig and Weinstein 2005). Treatment has now become targeted and depends on the St Gallen molecular subtype classification which defines Luminal A breast cancers as ER/PR positive, HER2 negative with a Nottingham mitotic score of 1, while those with mitotic score of 2 or 3 are classified as Luminal B. HER2 type defined as HER2 positive >10% or 3+ score with negative ER/PR and Triple Negative Breast Cancer (TNBC) as ER/PR and HER2 negative(Goldhirsch, Winer et al. 2013). One of the major risk factors to developing breast cancer is related to oestrogen hormone exposure. Oestrogen and related hormone progesterone are important in stimulation of breast growth during puberty and pregnancy in women thereby increasing the number of cells that can potentially give rise to cancer. The actions of oestrogen and progesterone are mediated by Oestrogen receptors (ERs) and Progesterone receptors (PRs) respectively, found in the Luminal cells of the mammary ducts. These two receptors are also found in some breast cancer cells. These breast cancers are termed “Luminal” because they most closely resemble normal breast Luminal cells in terms of their mRNA expression pattern (Elledge, Green et al. 2000; Lange and Yee 2008). The HER2 protein is a member of transmembrane tyrosine kinase growth factor receptors. A subset of invasive breast cancers shows HER2 gene amplification and protein overexpression. These cancers are associated with aggressive phenotypes and have poor prognosis(Barron, Cziraky et al. 2009).

Therefore ER/PR and HER2 testing are critical in selection of patients for ER blockade treatment and HER2 targeted therapy respectively. Luminal A and TNBC have been shown to carry the best and worst prognosis respectively (Sorlie 2004; Brenton, Carey et al. 2005; Goldhirsch, Winer et al. 2013).

Several studies around the world have shown that the Luminal type breast cancers are the most common molecular subtype in women of non-African ancestry(Ihemelandu, Leffall et al. 2007; Del Casar, Martin et al. 2008; Chuthapisith, Permsapaya et al. 2012; Verma, Bal et al. 2012; Howlader, Altekruse et al. 2014; Pervaiz, Rehmani et al. 2015). However, the molecular subtype characterization in African women remains controversial as some studies report a high incidence of triple negative disease (ER-/PR-/HER2-) (Huo, Ikpatt et al. 2009; Seshie, Adu-Aryee et al. 2015) while other studies have concluded that the prevalence of receptor status (ER/PR) and HER2 is comparable with that in non-African ancestry women(Adebamowo CA 2008; Sayed, Moloo et al. 2014).

Despite the high prevalence of breast cancer in Zambia, the molecular subtype characterization critical for treatment and prognosis in not known. Therefore this study aims at characterizing the breast cancers according to the molecular subtype classification using ER, PR and HER2 immunohistochemistry.

1.2 Statement of the problem

ER, PR and HER2 testing in breast cancer help to guide treatment. ER positive cancers respond to hormonal therapy (Tamoxifen) and carry a better prognosis, whereas HER2 positive tumors have a predictive response to anti-HER2 (Herceptin) therapy (Goldhirsch, Winer et al. 2013). However, at UTHs routine testing for ER/PR and HER2 is not done for breast cancer resulting in patients receiving suboptimal treatment. Furthermore, it is difficult to predict prognosis in patients with unknown molecular subtype.

1.3 Study justification

Studies from different parts of Africa have yielded different breast cancer molecular subtype results, with studies in Nigeria and Ghana suggesting that majority of the cases are triple negative breast cancer (TNBC) (Huo, Ikpatt et al. 2009; Seshie, Adu-Aryee et al. 2015). Other studies conducted in Nigeria and Kenya suggests that the breast cancer molecular subtype in African women is similar to women in other populations around the world (Adebamowo CA 2008; Sayed, Moloo et al. 2014). Therefore, more studies need to be conducted in women of

African ancestry with breast cancer. In addition, the study will help improve breast cancer patients' care in Zambia.

1.4 Study question

What is the molecular subtype characterization of primary breast cancer at the University Teaching Hospital?

1.5 Study objectives

1.5.1 General Objective

To explore the molecular subtype characterization of primary breast cancer at UTHs.

1.5.2 Specific Objectives

1. To determine the age distribution of breast cancer patients
2. To classify the breast cancers according to their histological type and grade.
3. To classify the breast cancers as Luminal A, Luminal B, HER2 type and Triple negative breast cancer using ER, PR and HER2 immunohistochemistry.

1.6 Organization of dissertation

The dissertation is organized in six chapters. Chapter one is composed of the: background to the study, statement of the problem, justification of the study, study question, study objectives and organization of the study. Chapter two presents reviewed literature relevant to the study while Chapter three contains the methodology. Research findings are presented in chapter four. Chapter five discusses the findings and limitations of the study and chapter six contains the conclusion and recommendations.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Breast cancer is a heterogeneous disease, comprising multiple entities associated with distinctive histological and biological features, clinical presentations and responses to therapy (Weigelt, Geyer et al. 2010). Most breast malignancies arise from epithelial elements and are categorized as breast carcinomas. These can either be *in situ* or invasive carcinomas. The *in situ* carcinomas of the breast are either ductal (also known as intraductal carcinoma) or lobular. This distinction is primarily based upon the growth pattern (invasive vs non-invasive) and cytologic features of the lesion, rather than their anatomic location within the mammary ductal-lobular system. The invasive breast carcinomas consist of several histologic subtypes based upon the architectural pattern exhibited by the tumour under the microscope. Using molecular techniques such immunohistochemistry, invasive breast cancers can further be classified according to molecular subtypes (Makki 2015; Feng, Spezia et al. 2018).

This chapter highlights literature reviewed on the epidemiology, risk factors, histologic type, histologic grading, as well as the molecular subtypes of breast cancer. It also highlights the importance of human epidermal growth factor receptor 2 in breast cancer.

2.2 Epidemiology of breast cancer

Breast cancer is the second most common cancer in the world and by far the most frequent cancer among women with an estimated 2.4 million new cases diagnosed in 2015 and 25% of all types of cancer with a slight majority of cases reported in women in less developed regions. The incidence rates vary fourfold across the world regions with rates ranging from 27 per 100,000 in middle Africa and Eastern Asia to 96 per 100,000 in Western Europe. It ranks as the fifth cause of death from cancer overall (522,000 deaths) and while it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%). The range in mortality rates between world regions is less than that for incidence because of the more favourable

survival from breast cancer in high-incidence developed regions (Parkin, Bray et al. 2001; Torre, Bray et al. 2015).

In Zambia, the incidence of breast cancer remains high (22.4 per 100,000) due to lack of screening programs especially among the underprivileged women in rural areas. It is the second most common cancer accounting for 11.4% of the cancer cases in women and kills approximately 400 women each year (Zyaambo, Nzala et al. 2013; Torre, Bray et al. 2015).

2.3 Risk Factors for breast cancer

2.3.1 Non-hormonal risk factors

Age

Breast cancer incidence rises sharply with age. The overall incidence rate of breast cancer is low at young ages 20-24 at 1.4 per 100,000. As women begin to transition through menopause, the rates of breast cancer increase substantially (Anderson, Rosenberg et al. 2008). Data from the American National Cancer Institute's Surveillance Epidemiology and End Results (SEER) program between 1999 and 2003 showed the incidence rate of 119.3 per 100,000 for women ages 40 to 44, 249.0 per 100,000 for women ages 50 to 54, 388.3 per 100,000 for women ages 60 to 64 and the highest rate was observed among women ages 75 to 79 (490 per 100,000) (Altekruse, Kosary et al. 2010). In a systemic review and meta-analysis of estimating the incidence of breast cancer in Africa by Adeloye *et al* (2018), the incidence rate was highest among persons older than 60 years at 36.6 per 100,000 compared to an incidence rate of 3.3 per 100,000 among persons aged 30-39 years.

In a retrospective study on the epidemiology of cancers in Zambia by Kalubula *et al* (2018), breast cancer peak was observed in the age range 40-49 years at 9.46 per 100,000, followed by the age range 60-69 years at 9.09 per 100,000. The incidence rates of age ranges 20-29, 30-39, 50-59, and 70-79years were 1.29, 4.03, 8.53, and 4.38 per 100,000 respectively.

Race

Breast cancer rates also differ by race and ethnicity. Although African American women have a lower overall incidence of breast cancer than white women, African Americans have a higher

incidence of breast cancer before age 35. Although breast cancer incidence is higher in black women than in white women among women younger than 40 years, the reverse is true among those aged 40 years or older. In the American National Cancer Institute SEER database, there are qualitative interactions between age and race. Age-specific incidence rates overall are higher among black women than among white women younger than 40 years (15.5 vs. 13.1 per 100,000 women/year), and then, age-specific rates crossed with rates higher among white women (281.3) than among black women (239.5) aged 40 years or older. The black to white incidence rate crossover is observed for all tumor characteristics than for high-risk tumor characteristics. In addition, breast cancer mortality is substantially greater at all ages among African Americans than it is among whites, 34 vs. 25 deaths per 100,000 women, respectively (Altekruse, Kosary et al. 2010). Estimates of the prevalence of breast cancer risk factors indicate that African American and white women differ in terms of their ages at menarche, menstrual cycle patterns, birth rates, lactation histories, patterns of oral contraceptive use, levels of obesity, frequency of menopausal hormone use, physical activity patterns, and alcohol intake (Bernstein, Teal et al. 2003).

2.3.2 Hormonal risk factors

Oestrogen exposure

It has been shown repeatedly that estrogen exposure is directly associated with risk for developing breast cancer. A prolonged or increased exposure to estrogen is associated with an increased risk for developing breast cancer, whereas reducing exposure is thought to be protective. Therefore, factors that increase the number of menstrual cycles are associated with an increased likelihood for developing breast cancer, such as early age at menarche, nulliparity, and late onset of menopause. Similarly, it appears that decreasing the total number of ovulatory cycles can be protective, which can be achieved by moderate levels of exercise and a longer lactation period (Martin and Weber 2000).

Oestrogen functions as a promoter of breast cancer through different effects on the breast which includes stimulation of breast growth during puberty and pregnancy thereby increasing the number of cells that can potentially give rise to cancer (Martin and Weber 2000; Sun, Zhao et al. 2017).

Another related hormone, progesterone is also essential for normal breast development during puberty and in preparation for lactation and breast feeding. The actions of oestrogen and progesterone are primarily mediated by Oestrogen Receptors (ER) and Progesterone Receptors (PR) respectively. A subset of mammary epithelial cells (Luminal cells) in the breast express both ER and PR, and Oestrogen is usually required in order to induce the expression of PR in these ER positive cells (Lange, Sartorius et al. 2008; Lange and Yee 2008). ER and PR are also found in breast cancer cells. These cancers are termed "Luminal" as they most closely resemble normal breast Luminal cells in terms of their mRNA expression pattern, which is dominated by genes that are regulated by oestrogen. Treatment with ER blockade in these breast cancers results in improved patient survival (EBCTCG 2005). ER expression is both prognostic and predictive of response to treatment (Ludwig and Weinstein 2005; Calhoun and Collins 2015). Therefore ER testing is critical in management of patients with breast cancer.

2.3.3 Genetic susceptibility

BRCA1

The first breast cancer susceptibility gene BRCA1 was isolated in 1994. The gene was initially localized to chromosome 17q21 by genetic linkage of early-onset breast cancer families. It was later isolated by positional cloning by Miki *et al*, (1994). It is now known that germline mutations in BRCA1 represent a predisposing genetic factor in 15%-45% of hereditary breast cancer. Female mutation carriers have 60%-80% lifetime risk of developing breast cancer(Shah, Rosso et al. 2014; Sun, Zhao et al. 2017).

BRCA2

The second dominant breast cancer susceptibility gene emerged from the linkage analysis of 22 families with multiple cases of early-onset female breast cancer and at least one case of male breast cancer. A linkage was established between polymorphic markers on chromosome 13q 12-13 and BRCA2 locus. The lifetime breast cancer risk for BRCA2 mutation carriers is estimated to be in the range of 60%-85% (Shah, Rosso et al. 2014; Sun, Zhao et al. 2017).

2.4 Human Epidermal Growth Factor Receptor 2 and breast cancer

Human epidermal growth factor receptor 2 is a transmembrane tyrosine kinase encoded by a region of chromosome 17 that results in the increased activity of the cellular processes responsible for tumor growth and progression when activated (Yarden 2001).

A subset of invasive breast cancers shows HER2 gene amplification and protein overexpression and is associated with aggressive phenotypes and poor prognosis in patients who do not receive systemic HER2-directed therapy such as Trastuzumab. Trastuzumab is a humanized monoclonal antibody targeting HER2 that has been proved to significantly improve the disease free interval and overall survival in women with tumors overexpressing HER2 in both early and metastatic breast cancer (Barron, Cziraky et al. 2009; Pegram 2013).

HER2 testing is done using fluorescence in situ hybridization (FISH) to directly quantify the HER2 gene copy number or by first screening with immunohistochemistry (IHC), with confirmatory FISH for cases with equivocal results (Score 2+ expression). IHC evaluates the expression of HER2 at the cell surface using membranous staining, and FISH assesses the number of copies of the HER2 gene in the nucleus. Testing for HER2 amplification or overexpression is recommended for all primary and metastatic breast cancers to guide appropriate treatment decisions (Barron, Cziraky et al. 2009; Ballinger, Sanders et al. 2015).

The HER2 results on IHC as recommended by ASCO/CAP are reported using a 4-tier scoring system: 0, the absence of membranous staining; 1+, faint/partial membranous staining in > 10% of cancer cells, with rare or absent cells having circumferential staining; 2+, weak, circumferential membranous staining in > 10% of cells; and 3+, intense circumferential membranous staining in > 10% of cells. Absent or weak staining cells (0 or 1+, respectively) equate to < 100,000 receptors and no detectable amplification of the HER2 gene. Strong, circumferential staining (3+) correlates with > 2,300,000 receptors and a level of expression almost always associated with HER2 amplification. Equivocal results are scored as 2+, with non-intense, but complete, membranous staining of > 10% of cells (Wolff, Hammond et al. 2014).

2.5 Histologic types of breast cancer

Invasive breast carcinomas consist of several histologic subtypes. In a population based series of 135,157 women with breast cancer reported to the SEER database of the American National Cancer institute between 1992 and 2001 by Li *et al* (2005), Invasive ductal carcinoma NOS was the most common subtype (76%) followed by invasive lobular carcinoma (8%) and invasive ductal/lobular carcinoma (7%). Mucinous, tubular, medullary and papillary carcinomas were 2.4%, 1.5%, 1.2% and 1% respectively. Other subtypes included metaplastic, invasive micropapillary and cribriform carcinomas, all accounted for less than 5 % of the cases.

A similar representation has also been observed in Africa. In a study by Adebamowo CA (2008) in Nigeria, invasive ductal carcinoma NOS was 82.3% of the cases. Other histologic types reported in this study included metaplastic 4.2%, mucinous 3.7%, lobular 2.1%, medullary 1.6%, 1% cases each of tubulolobular and papillary, as well as 0.5% cases each of adenosquamous and cribriform carcinomas. In another study by Sayed *et al* (2014) in Kenya, invasive ductal carcinoma NOS was the most common histologic type at 84.2% followed distantly by invasive lobular, mucinous and metaplastic carcinomas at 2.6% each.

2.6 Histologic grading of breast cancer

Histological grading is directed principally at invasive ductal carcinomas NOS, divided into three grades based upon a combination of architectural and cytologic features assessed utilizing a scoring system developed by Elston and Ellis called the Nottingham grading system. Tumours of other types are not suitable for the particular method. The grade for an individual tumour is derived from an assessment of three morphological features namely tubular formation, nuclear pleomorphism and mitotic count.

Tubular formation

All parts of each block are scanned and the proportion of tumour displaying tubular structures is assessed. Clear lumina must be visible, and care should be taken not to mistake clefts induced by shrinkage artifact for tubular structures (this problem is diminished with good fixation). When more than 75% of the tumour area is composed of definite tubules a score of 1 point is given.

Two points are appropriate for tumours in which between 10 and 75% of the area shows tubule formation. Where tubules occupy 10% or less of the tumour 3points are assigned

Nuclear pleomorphism

In this feature both a quantitative and a qualitative judgment is made. When the nuclei are small, with little increase in size in comparison with normal breast epithelial cells, have regular outlines and uniformity of nuclear chromatin and vary little in size, 1 point is appropriate. A score of 2 points is given when the cells appear larger than normal, have open, vesicular nuclei with visible nucleoli, and there is moderate variability in both size and shape. A marked variation in size and shape, especially when very large and bizarre nuclei are present, scores 3 points. In this group nuclei are vesicular with prominent, often multiple nucleoli.

Mitotic counts

Mitotic activity is best assessed at the periphery of the tumour where active growth is most likely. A minimum of 10 fields is assessed. Strict criteria for the identification of mitotic figures must be employed and only nuclei in which clear morphological features of metaphase, anaphase and telophase are counted. Hyperchromatic and apoptotic nuclei are ignored and care is taken to avoid mistaking lymphocytes within a tumour for mitoses. Up to 9 mitoses per 10 fields scores 1 point, 10-1 9 scores 2 points and more than 20 scores 3 points.

Allocation and validation of histologic grade

To obtain the overall tumour grade, the score for each category are added together, giving a possible total of 3-9. The tumour grade is then allocated on the following basis;

3-5 points: grade 1 - well differentiated

6-7 points: grade 2 - moderately differentiated

8-9 points: grade 3 - poorly differentiated

(Elston and Ellis 1991)

Histologic grading has become widely accepted as a powerful indicator of prognosis in addition to providing an overview of the intrinsic biologic characteristics of the tumors.

Studies from Africa have shown a high prevalence of the aggressive grade 2 and 3 tumours. In a study by Sayed *et al* (2014), 39.3% and 53.7% were grade 2 and 3 respectively. The least aggressive grade 1 tumours were only 7% of the cases. Adebamowo CA (2008) reported 44.3% and 15.6% as grade 2 and 3 respectively. 9.4% of the tumours were grade 1. However 30.7% of the cases in this study were not graded. Another study by Seshie *et al* (2015) also reported a high prevalence of grade 2 and 3 tumours at 60.8% and 30.8% respectively. Grade 1 tumours were only seen in 8.3% of the cases.

2.7 Molecular subtype of breast cancer

All breast cancers are divided into four major biological subgroups based on the expression of oestrogen receptor, HER2 and the proliferation index. Luminal A breast cancers are Hormone receptor positive, HER2 negative (ER+, PR+, HER2-) and low grade (low proliferative rate), Luminal B breast cancers are similar to Luminal A but with a high proliferative rate, HER2 type breast cancers are HER2 positive (3+ score) cancers with negative ER/PR and Triple Negative Breast Cancer are negative for ER, PR, and HER2(Goldhirsch, Winer et al. 2013).

TNBC has recently been recognized as having substantial heterogeneity in tumor biology and gene expression profiles have identified six distinct TNBC subtypes (two basal-like, BSL-1 and BSL-2, Immunomodulatory- IM, Mesenchymal- M, Mesenchymal Stem cell-like MSL and Luminal androgen receptor- LAR) each associated with different treatment responsive rates and markers that may be useful for targeted therapeutic approaches(Masuda, Baggerly et al. 2013; Yu, Zhu et al. 2013).

2.7.1 A global perspective of molecular subtypes

A retrospective analysis of all African-American women diagnosed with breast cancer from 1998 to 2005 in USA by Ihemelandu *et al* (2007) revealed Luminal A subtype as the most prevalent (55.4%) compared with Luminal B (11.8%), 21.2% TNBC and 11.6% HER2 subtypes. However, when stratified into age specific groups, the TNBC subtype (57.1%) was the most prevalent in

the age group <35 years compared with Luminal A, Luminal B and HER2 subtypes at 25.0%, 14.3% and 3.6% respectively.

A more recent study done by Howlader *et al* (2014) on the USA incidence of breast cancer subtype defined by joint Hormone receptor and HER2 status assessed across the 28% of the USA population that is covered by the Surveillance, Epidemiology and End Results (SEER) registries. Age-specific incidence rates by subtypes were calculated for Non-Hispanic white, Non-Hispanic black, Non-Hispanic Asian Pacific Islander and Hispanic women. 72.7% were found to be Luminal A, 10.3% were Luminal B, 12.2% were TNBC and 4.6% were HER2 subtype. Non-Hispanic black women had the highest rate of TNBC.

A prospective study by Del Casar *et al* (2008) in Spain on 787 patients with invasive breast cancer showed 55.8% of tumors were Luminal type, 22.6% were TNBC and 21.6% were of HER2 variety. Another prospective study by Verma *et al* (2012) in India on the immunohistochemical characterization of molecular subtypes of invasive breast cancer showed Luminal A, Luminal B, HER2 type and TNBC at 47%, 15%, 21% and 17% respectively. This characterization was similar to that of the retrospective study done by Chuthapisith *et al* (2012) in Thailand which revealed Luminal A, Luminal B, HER2 type and TNBC at 59.3%, 12.3%, 13.3% and 15.1% respectively and Pervaiz *et al* (2015) in Pakistan on the evaluation of Hormone Receptor Status (ER/PR/HER2) in Breast Cancer in 345 cases which showed Luminal A, Luminal B, HER2 type and TNBC at 51%, 23.4%, 8.7% and 16.9% respectively.

2.7.2 An African perspective of molecular subtypes

The molecular subtype characterization of breast cancer in women of African ancestry remains controversial, with some studies reporting an over-representation of TNBC among African women whilst other studies concluded that the Luminal variety is the most common subtype, a representation similar to other populations.

A prospective study by Adebamowo CA (2008) in Nigeria on the immunohistochemical and molecular subtypes of breast cancer showed Luminal A, Luminal B, HER2 type and TNBC were 77.6%, 2.6%, 4% and 15.8% respectively. In this study, it was concluded that there is no difference in the patterns of hormonal receptor expression in breast cancer patients of African

ancestry compared to other populations. A similar representation was also seen in a prospective analysis by Sayed *et al* (2014) in Kenya in which ER, PR and HER2 positivity was seen in 72.8%, 64.8% and 17.6% of the cases respectively. TNBC accounted for only 20.2% of the cases.

The studies from Sub-Saharan Africa that observed an over-representation of TNBC did not adhere to the standard methodology and interpretation as recommended by the ASCO/CAP guidelines for ER, PR and HER2 immunohistochemistry. For example, in a study by Huo *et al* (2009) on population differences in breast cancer which comprised of 507 patients diagnosed with breast cancer between 1996 and 2007 at six geographic regions in Nigeria and Senegal revealed an over-representation of TNBC (56%) compared to Luminal A (27%), Luminal B (2%) and HER2 type (15%). In this study, archived formalin fixed tissue were collected from the six institutions and transported to a central laboratory for IHC. However, they grossly overlooked the effects of pre-analytical errors such as poor tissue fixation on immunohistochemistry results. Deficiencies in the pre-analytical phase of specimen handling contributed to falsely elevated receptor poor breast cancer. Nyagol *et al* (2006), in a study on routine assessment of hormonal receptor and HER2 status in Kenyan women with breast cancer described a 10% positivity cut off point for ER and PR contrary to the ASCO/CAP recommended >1% cut off point. This contributed to the high number of receptor negative cases in this study (Nyagol, Nyong'o et al. 2006). Therefore the reported high incidence of TNBC in Africa may not be accurate due to lack of quality assurance (QA) in performing and interpreting immunohistochemistry.

2.7.3 National perspective

Despite breast cancer being the second most common female malignancy in Zambia, there is no available literature on the molecular subtype characterization using ER, PR and HER2 that is critical for patient management. Most of the published studies on breast cancer in Zambia only focused on the disease epidemiology and prevention (Mukupo and Mubita-Ngoma 2007; Maree and Mulonda 2015; Mumba Ramson 2017).

CHAPTER THREE

METHODOLOGY

3.1 Study design and site

This was a laboratory based cross sectional study done in Lusaka, Zambia, at the University Teaching Hospitals, Department of Pathology and Microbiology.

3.2 Study population

All archived formalin-fixed paraffin-embedded (FFPE) tissue blocks with histological diagnosis of primary breast carcinoma between April 2016 and March 2018 at UTHs department of pathology and microbiology tissue bank.

3.3 Sampling frame

All the breast samples obtained from the Tissue Bank were sampled.

3.3.1 Inclusion Criteria

All archived FFPE cases with histological diagnosis of primary breast carcinoma in Zambian women presenting at UTHs.

3.3.2 Exclusion Criteria

All the breast cancer cases treated with either chemotherapy or radiotherapy or both.

All the cases without the record of age of the patient were also excluded from the study.

3.3.3 Sample Size

A convenient sampling method was used to select the specimen sample size of sixty-eight (68) FFPE of breast tissue diagnosed with breast cancer on histology from April 2016 to March 2018.

3.4 Sample selection

Ninety-six archived formalin-fixed tissue blocks with histologic diagnosis of primary breast cancer at UTHs between April 2016 and March 2018 were retrieved from the tissue bank. After removing cases that were treated by chemotherapy before obtaining biopsy and cases without the record of age, the sample size came to 68.

3.5 Tissue processing

A total of 68 archived FFPE blocks were retrieved and submitted for tissue processing (appendix A). Three (03) micrometer thick tissue sections were cut and stained with Hematoxylin and Eosin (H&E) for histological confirmation of breast cancer.

Immunohistochemistry for ER, PR and HER2 using the standard operating procedure (SOP) was then employed on all tissue blocks with H&E-confirmed diagnosis of breast carcinoma (Appendix B).

3.6 Immunohistochemical scoring

A semi-quantitative histochemical score was used to record results of ER and PR staining according to the system established by Allred *et al* (2010). This system considers both the proportion and intensity of stained cells. The intensity score (IS) ranges from 0 to3, with 0 being no staining, 1 weak staining, 2 intermediate staining, and 3 intense staining. The proportion score (PS) estimates the proportion of positive tumor cells and ranges from 0 to 5, with 0 being non-reactive, 1 for 1% reacting tumor cells, 2 for 1-10% reacting tumor cells, 3 for one-third reacting tumor cells, 4 for two-thirds reacting tumor cells, and 5 if 100% of tumor cells show reactivity. The PS and IS are added to obtain a total score (TS) that ranges from0 to 8. Tumor cells with a total score of 3 to 8 were considered positive, whereas those with TS less than 3 were considered negative cases. IHC was considered valid only in the presence of positive internal controls.

For HER2 IHC, the CAP/ASCO guidelines of 2014 were used which recommend positive HER2 (3+) being >10% of invasive tumor cells with complete intense membranous staining. Negative IHC HER2 included cases with no staining or membrane staining that is incomplete and faint/barely perceptible and within <10% of invasive tumor cells (score 0), and incomplete

membrane staining that is faint/barely perceptible and within >10% of invasive tumor cells (score 1+). Equivocal cases scored as 2+ defined as circumferential membranous staining that is incomplete and/or weak/moderate and within >10% of invasive tumor cells, or complete and circumferential membrane staining that is intense and within <10% of invasive tumor (Wolff, Hammond et al. 2014). These equivocal cases were considered negative since FISH was not available for this study.

Luminal A cancers were defined as ER/PR positive, HER2 negative with a Nottingham Mitotic score of 1, while those cancers with ER/PR, HER2 negative with mitotic scores of 2 and 3 were classified as Luminal B. Cancers with HER2>10% or 3+ score with negative ER/PR were classified as HER2 type and cancers negative for ER/PR and HER2 were classified as Triple Negative Breast Cancer (TNBC).

These slides were examined using a binocular light microscope, Olympus^{R} CX 21.

3.7 Data management and analysis

3.7.1 Data collection tools

Data was collected from DISA and after microscopic examination and IHC results entered onto a data collection tool (appendix C).

The variables included;

1. Age as the independent variable
2. Histological type, histological grade and molecular characterization were the dependent variables.

3.7.2 Data analysis

Data was analyzed using SPSS version 22.

Cross table analysis and Chi-square test were done to analyze association between age and dependent variables (histologic type, Nottingham grade and molecular subtype).

3.8 Expected outcome

The Luminal A molecular subtype will be the most prevalent.

Invasive ductal carcinoma NOS will be the most common histologic type and Nottingham grade III will be the commonest tumor grade.

3.9 Ethical considerations

This was a laboratory based cross sectional study on archived formalin fixed paraffin embedded tissue blocks without any patient contact. Waiver of consent and approval to carry out the study was sought from ERES CONVERGE IRB (Appendix E).

Data extracted from the laboratory database (DISA) was treated with confidentiality as per SOP of electronic LIMS.

CHAPTER FOUR

RESULTS

4.1 Introduction

A total of 68 cases of invasive breast cancer identified from the University Teaching Hospital histopathology laboratory tissue bank from April 2016 to March 2018 were included in the final analysis of this study. All the patients enrolled in the study were of African ancestry. Baseline characteristics of the participants namely; age, histologic type and grade including molecular tumour subtype are shown in Table 1.

Table 1: **Baseline characteristics**

Patients characteristics	No. of participants (%) (n=68)
Age (years)	51.2 ± 14.5
Histologic type	
Invasive ductal carcinoma NOS	65 (95.5)
Mucinous carcinoma	01 (1.5)
Cribriform carcinoma	01 (1.5)
Micropapillary carcinoma	01 (1.5)
Histologic grade	
Grade 1 (well differentiated)	08 (11.8)
Grade 2 (moderately differentiated)	35 (51.5)
Grade 3 (poorly differentiated)	25 (36.7)

Molecular subtype	
ER+	42 (61.8)
PR+	28 (41.2)
HER2+	25 (36.8)
ER/PR+, HER2-	09 (13.2)
ER/PR+, HER2+	33 (48.5)
ER/PR-, HER2+	06 (8.8)
ER/PR-, HER2-	20 (29.4)

4.1 Age distribution

The youngest and oldest patients in this study were 29 and 93 years old respectively with a mean age of 51.2 years (SD 14.5) at the time of diagnosis. Majority of the patients18 (26.5%) were between the ages of 30-39 years, followed by the age group 50-59 years 15 (22.1%), 14 (20.6%) were in the age group 60-69, 12 (17.6%) presented in the age group 40-49 years, 4 (5.9%), 3 (4.4%) and 2 (2.9%) presented in the 8^{th}, 9^{th} and 3^{rd} decades of life respectively as shown in figure 1.

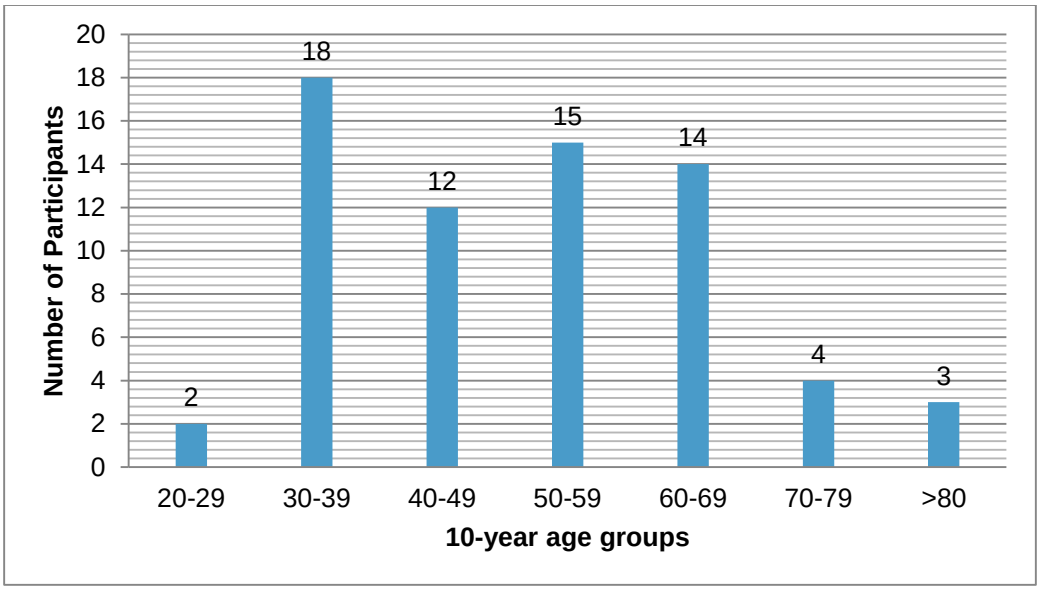


Figure 1: Age distribution of the 68 participants

4.2 Histologic findings

4.2.1 Histologic types

Four histologic types were seen in the 68 cases that were evaluated as shown in figure 2.

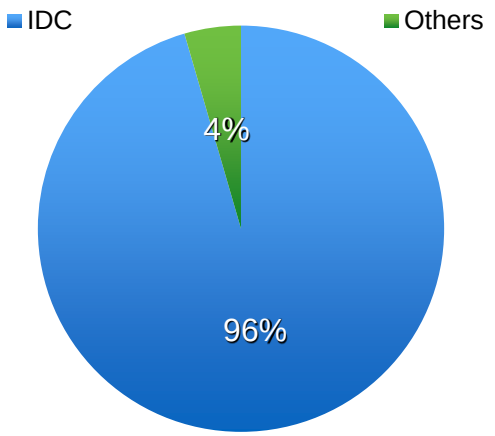
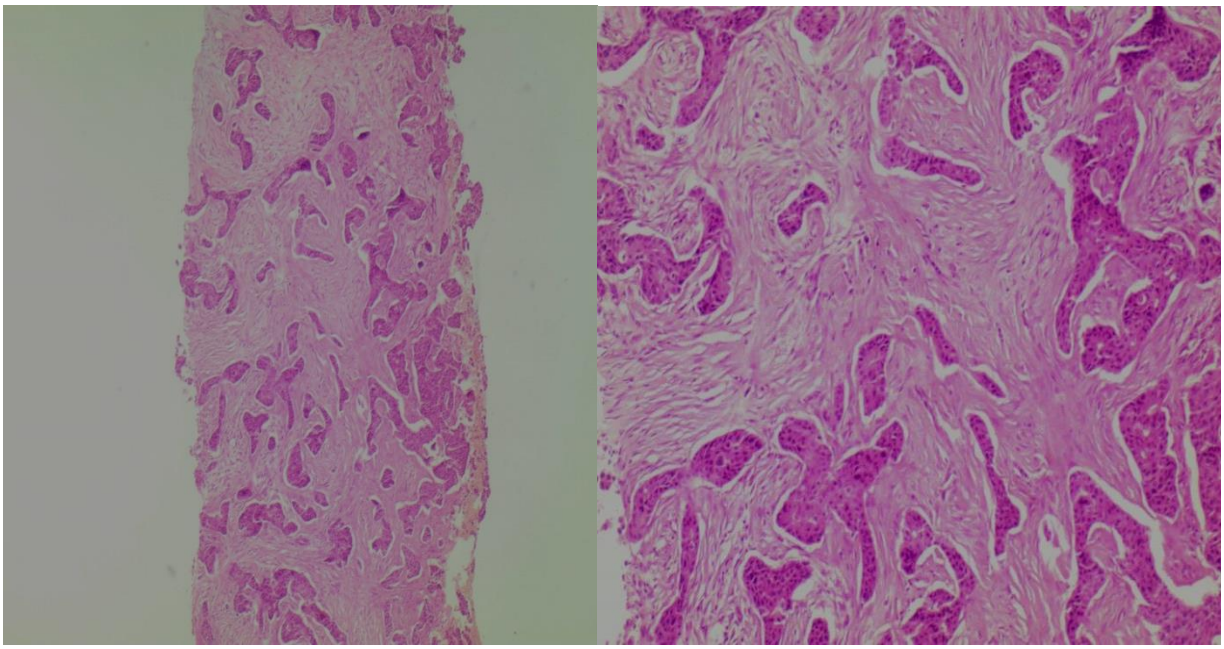


Figure 2: Percentage of histologic types of breast cancer

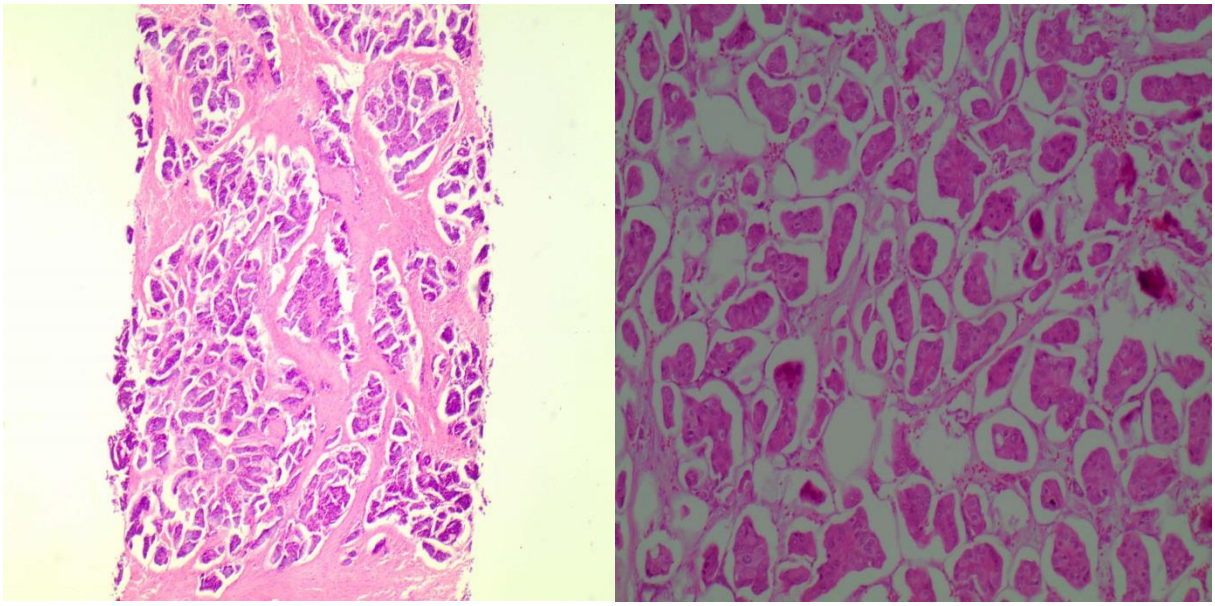
65 (95.5%) cases were of invasive ductal carcinoma NOS (Figure 3). Micropapillary (Figure 4), cribriform (Figure 5), and the mucinous varieties (Figure 6) contributed 1 (1.5%) case each. The mucinous carcinoma stained strongly positive on PAS stain (Figure 7). There was significant statistical association between histologic type and the age groups of the patient **(p value = 0.002)**. Histologic type by age group distribution is shown in Table 2.



(X40 magnification) **(X100 magnification)**

Figure 3: H&E stain invasive ductal carcinoma, NOS Nottingham grade 2

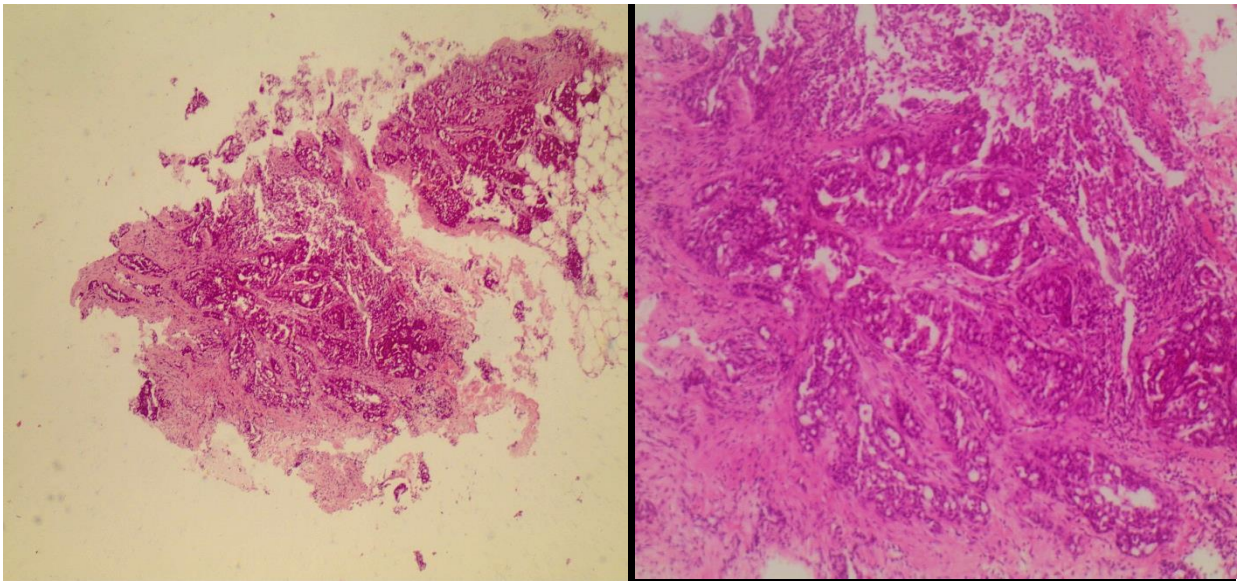
Tumor cells are seen infiltrating fibroconnective tissue as cords, tongues and nests. There is moderate pleomorphism with no areas of tubule formation.



(x40 magnification) **(x100 magnification)**

Figure 4: H&E stain micropapillary carcinoma

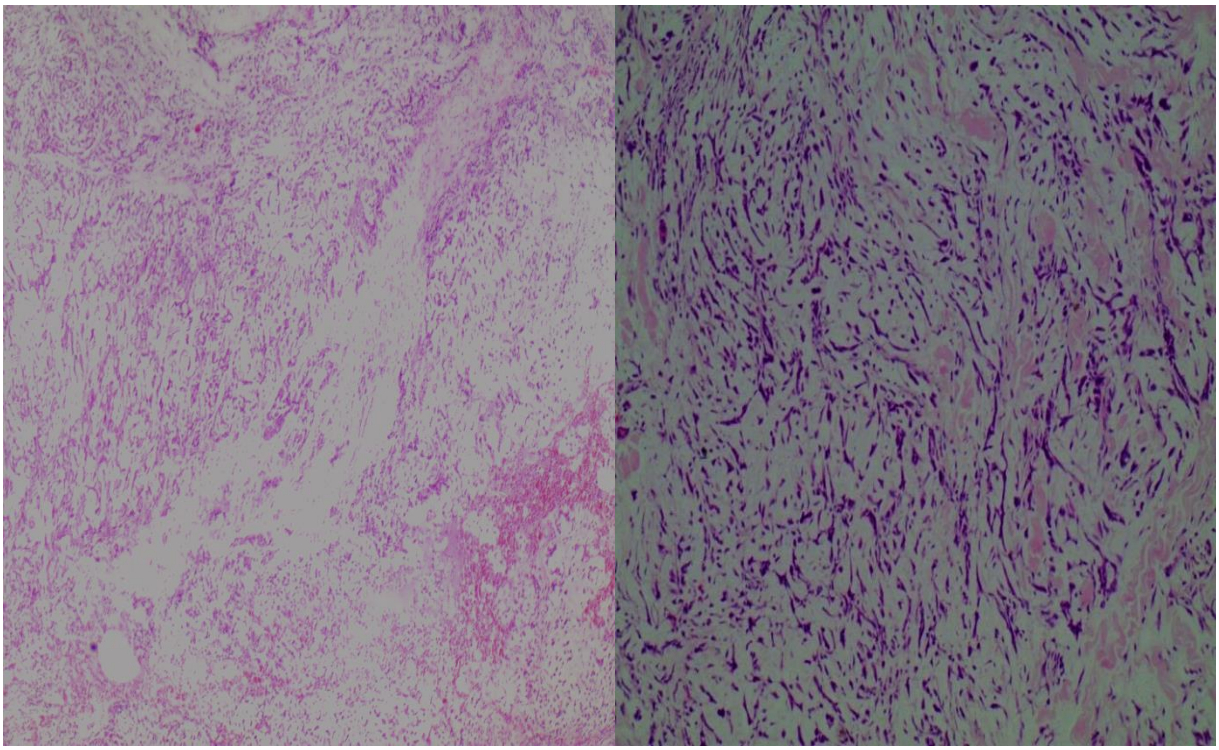
Morule-like micropapillary clusters of tumor cells separated by a network of fibroconnective stroma.



(x40 magnification) **(x100 magnification)**

Figure 5: H&E stain cribriform carcinoma

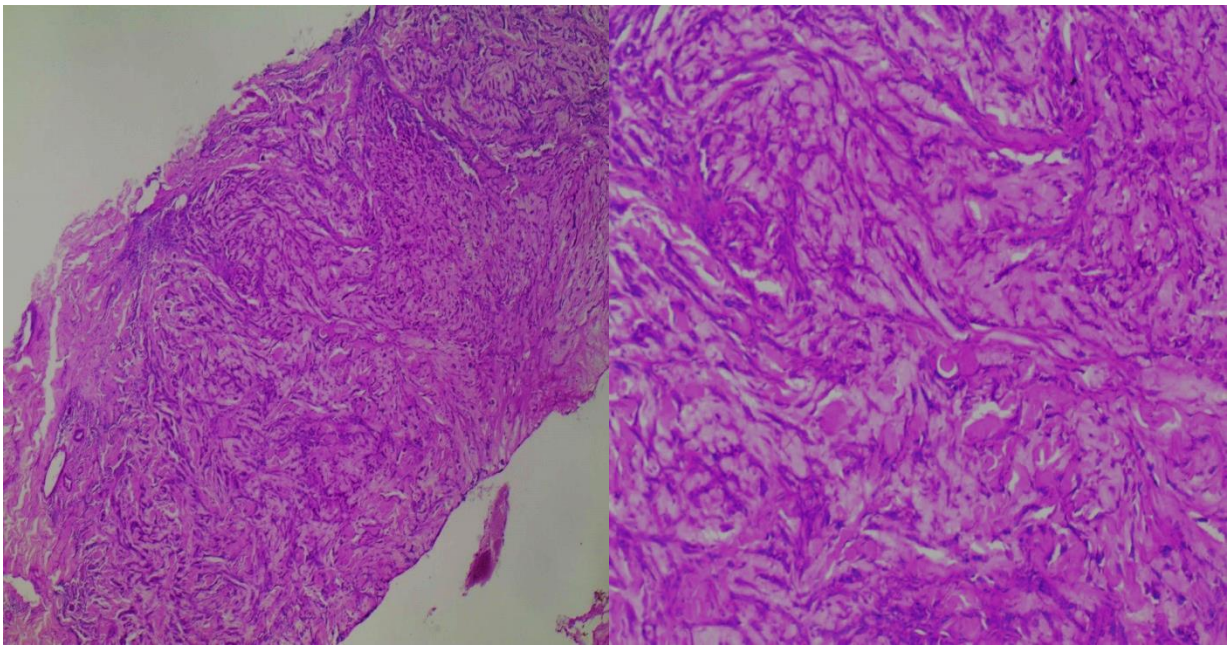
Tumor cells infiltrating fibroconnective tissue in a cribriform pattern; sharply outlined, round and oval glandular spaces distributed throughout tumour aggregates, creating a fenestrated appearance (sieve-like growth pattern). Background chronic inflammation composed predominantly of lymphocytes is also discernible.



(x40 magnification) (x100 magnification)

Figure 6: H&E stain mucinous carcinoma

Tumor cells infiltrating fibroconnective tissue with accumulation of abundant pools of mucin (clear areas).



(x40 magnification) (x100 magnification)

Figure 7: PAS positive stain mucinous carcinoma

Demonstrating tumor cells infiltrating fibroconnective tissue with accumulation of abundant pools of mucin. The mucin stained pink in color.

Table 2: Distribution of histologic type by age groups

	Histologic Type (%)			
Age Groups	IDC	Cribriform	Mucinous Carcinoma	Micropapillary Carcinoma
20-29	1.5	100	0	0
30-39	26.2	0	0	100
40-49	18.5	0	0	0
50-59	23.1	0	0	0
60-69	20.0	0	100	0
70-79	6.2	0	0	0
80+	4.6	0	0	0
Total	100	100	100	100

4.2.2 Histologic grade (Nottingham grade)

More than half of the cases (51.5%) were Nottingham grade II tumors, 11.8% of the cases were the least aggressive grade I tumors and 36.7% of the cases were grade III tumours. Majority of grade III tumours (32%) were seen in age group 30-39 years whereas majority of grade I (37.5%) tumors were seen in the age group 60-69 years. The tumor grade distribution and tumor grade by age group distributions are shown in Figure 8 and Table 3 respectively. There was no association between age and histologic grade **(p value=0.734)**

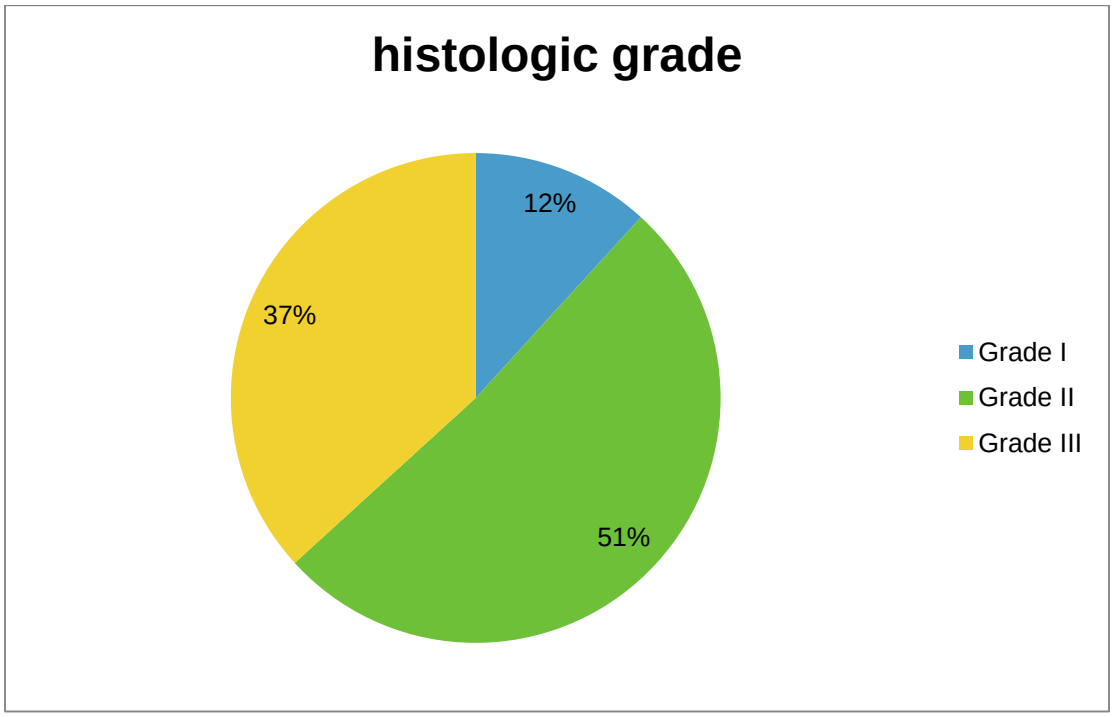


Figure 8: Nottingham histologic grade by percentage

Table 3: Tumor grade distribution by age group

	Histologic grade, N (%)			
Age Groups	I	II	III	Total
20-29	1(12.5)	1(12.9)	0	2(2.9)
30-39	2(25.0)	8(22.9)	8(32.0)	18(26.5)
40-49	1(12.5)	7(20.0)	4(16.0)	12(17.6)
50-59	1(12.5)	10(28.6)	4(16.0)	15(22.1)
60-69	3(37.5)	6(17.1)	5(20.0)	14(20.6)
70-79	0	2(5.7)	2(8.0)	4(5.9)
80+	0	1(2.9)	2(8.0)	3(4.4)
Total	8 (100)	35(100)	25(100)	68(100)

4.3 Molecular subtypes

4.3.1 Luminal type

ER/PR positivity was seen in 61.8% of the cases, 48.5% of which were Luminal B and 13.2% were Luminal A subtype (Figure 9). 77.7% of the Luminal A cases were found in women younger than 50 years, majority (33.3%) of which was in the age group (40-49 years). The youngest and the oldest patients with Luminal A in this study were 29 and 67 years old respectively. Whereas, 30 and 77 years old were the youngest and oldest respectively for patients with the Luminal B subtype. Slightly more than half (51.5%) of the Luminal B cases were found in women younger than 50 years, 36.4% of which were in the age group 30-39 years (Table 4). 62.5% of grade 1 cases were Luminal A. There was no grade 3 tumour that was of Luminal A variety. Conversely, there was no grade 1 tumour that was Luminal B. 54.5% of the Luminal B cases were grade 2 and 45.5% were grade 3 (Table 5). 1/9 of the Luminal A cases were a cribriform carcinoma and the rest (8/9) of the cases were invasive ductal carcinoma, NOS.

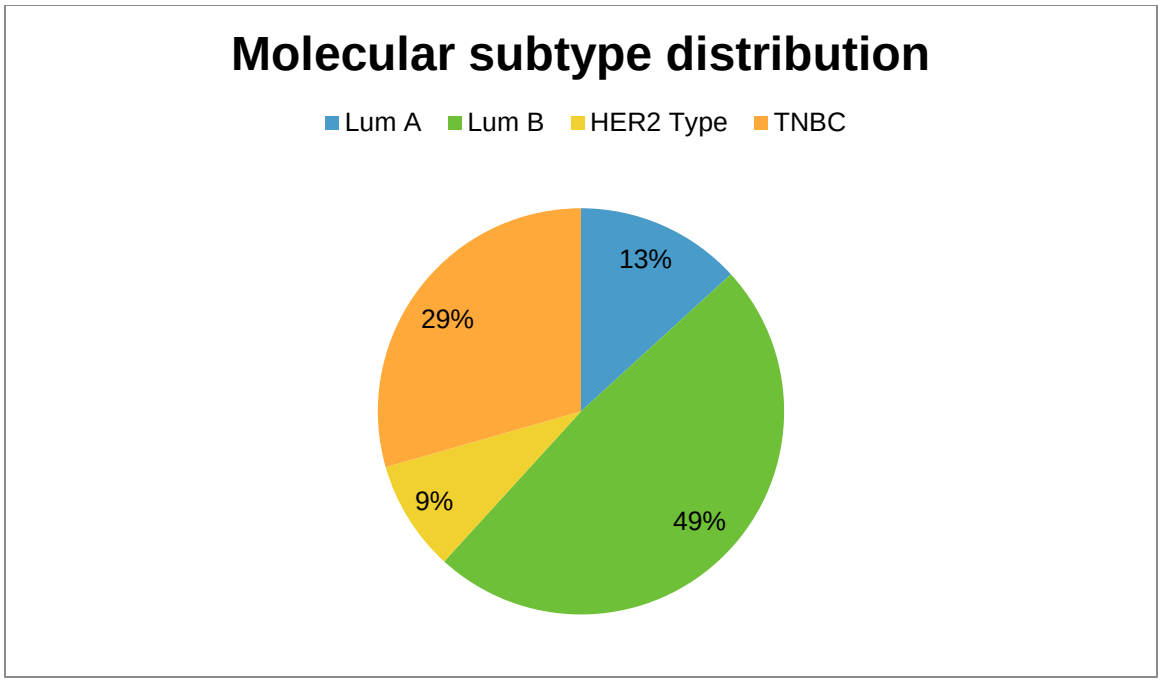


Figure 9: Molecular subtype distribution percentage of 68 participants

Table 4: Distribution of molecular subtype by age group

Age Groups	Molecular Classification				Total
	Luminal A	Luminal B	HER 2 Type	TNBC	
20-29	2(22.2)	0	0	0	2
30-39	2(22.2)	12(36.4)	2(33.3)	2(10.0)	18
40-49	3(33.3)	5(15.1)	0	4(20.0)	12
50-59	1(11.1)	11(33.3)	0	3(15.0)	15
60-69	1(11.1)	2(6.1)	3(50.0)	8(40.0)	14
70-79	0	3(9.1)	1(16.7)	0	4
80+	0	0	0	3(15.0)	3
Total	9(100)	33(100)	6(100)	20(100)	68(100)

Table 5: **Distribution of molecular subtype by tumor grade**

Tumor grade	Molecular Classification				Total
	Luminal A	Luminal B	HER 2 Type	TNBC	
Grade I	5 (62.5)	0	2(25)	1(12.5)	8
Grade II	4(11.4)	18(51.4)	2(5.7)	11(31.4)	35
Grade III	0	15(60.0)	2(8.0)	8(32.0)	25
Total	9(13.2)	33(48.5)	6(8.8)	20(29.4)	68(100)

4.3.2 HER2 type

Out of the 68 cases analyzed for HER2, 25 (36.8%) were HER2 score 3+, 9 were HER2 score 2+(equivocal), 7 were HER2 score 1+ and 27 were HER2 score 0. 28% of the HER2 positive cases (HER2 3+) were also positive for ER/PR. Thus, only 8.8% of the cases were classified as HER2 type (ER/PR-, HER2 +). The youngest and oldest participants with HER2 positivity were 33 and 72 years old respectively. However 66.7% of HER2 positivity was found in patients above the age of 60 years (Table 4). The majority of HER2 positive tumors were higher grade tumours. 52% were grade 2 and 40% were grade 3. Only 8% were grade 1(Table 5). Out of the

25 HER2 positive cases, 24 were invasive ductal carcinoma, NOS and 1 case was of micropapillary carcinoma variant.

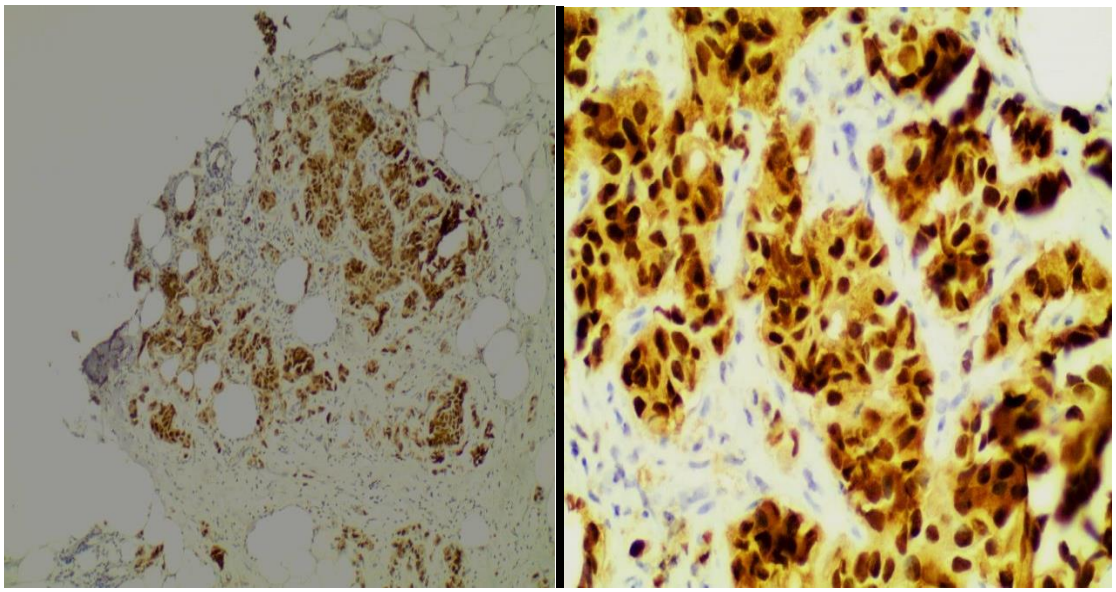
4.3.3 TNBC

TNBC was seen in 20 (29.4%) out of the 68 cases analyzed (Figure 9). The youngest patient was 37 years old and the oldest was 93 years of age. 70% of the cases were seen in women older than 50 years and the majority of the cases (40%) were seen in the age group 60-69 years (Table 4). Of the 20 cases, 11 (55%) were grade 2, 8 cases (40%) were grade 3 and only 1 (5%) case was seen in grade 1 tumours (Table 5). 19 (95%) out of the 20 TNBC cases were invasive ductal carcinoma, NOS and 1 case was of the mucinous carcinoma variety.

There was a significant association between age group and molecular subtype (**p value = 0.001**) and an association between histologic tumor grade and molecular subtype (**p value < 0.001**) was also found.

4.3.4 Immunohistochemistry

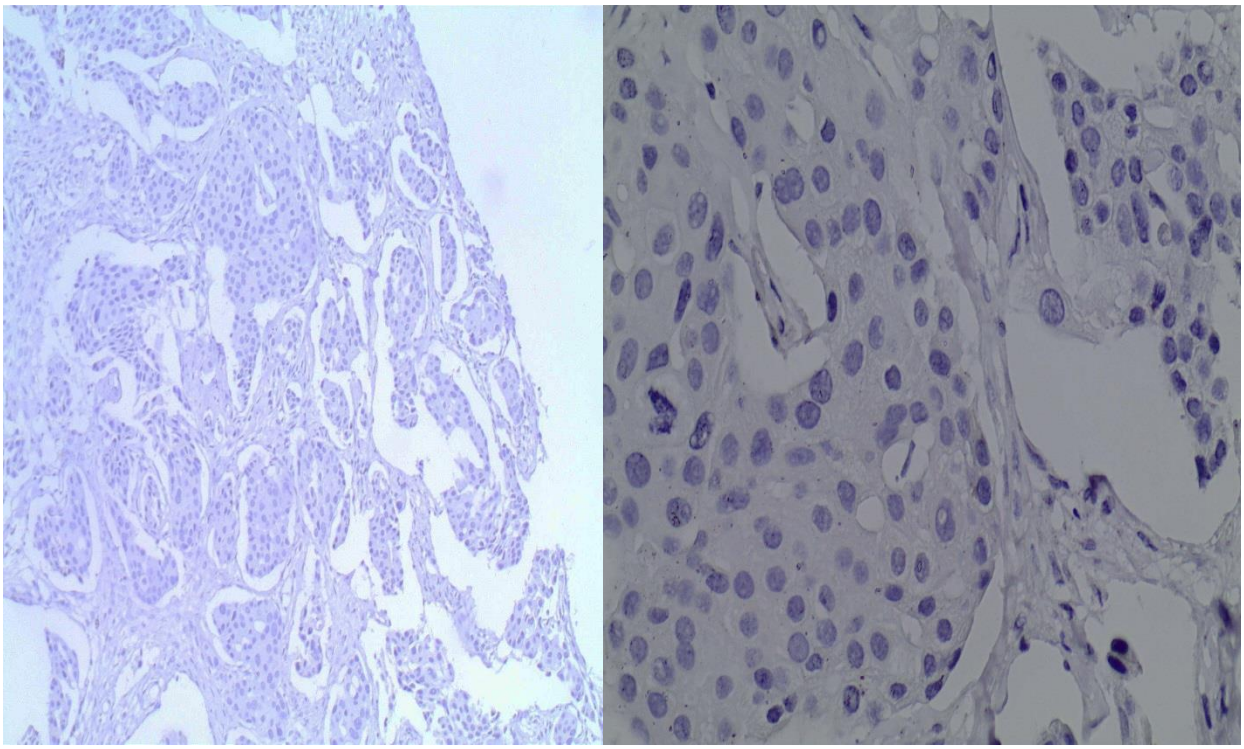
Positive and negative IHC stains for ER, PR, and HER2 are shown in Figure 10 to Figure 15.



(x100 magnification) (x400 magnification)

Figure 10: ER IHC positive nuclear staining

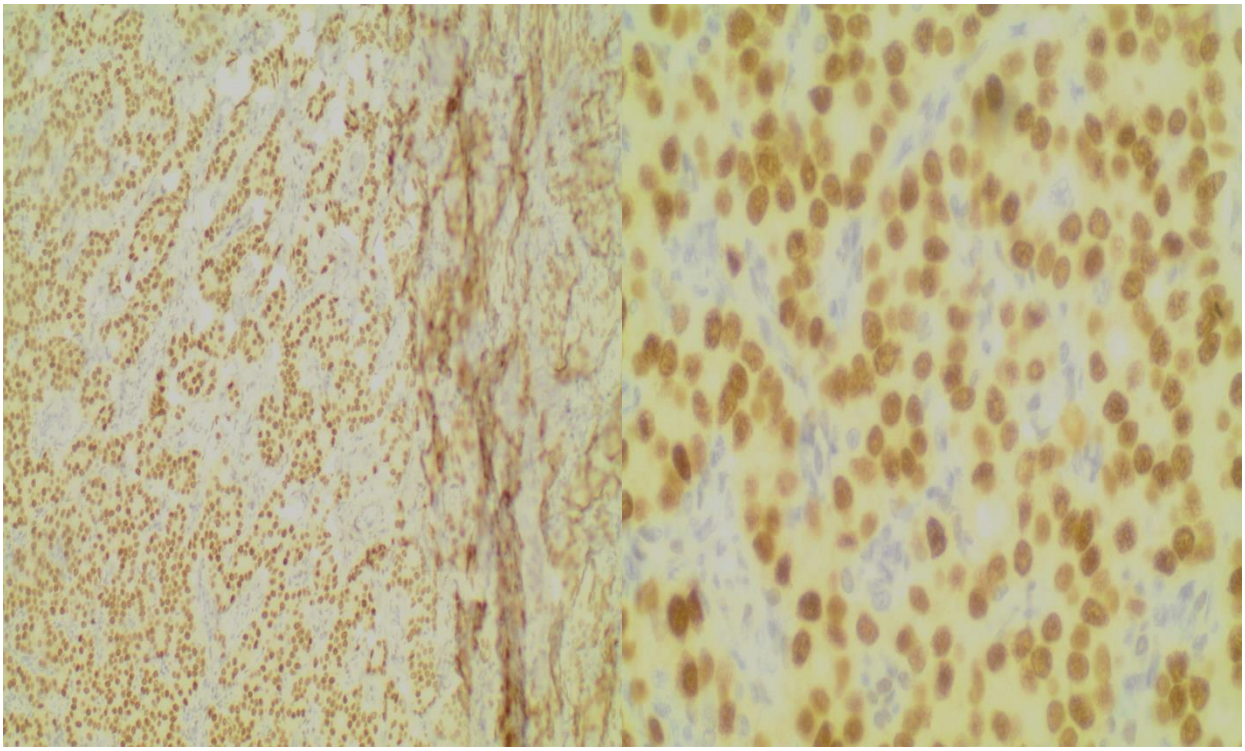
Invasive ductal carcinoma, NOS Nottingham grade 2 showing a strong ER nuclear staining (3+) in approximately 100% of the tumour cells



(x100 magnification) (x400 magnification)

Figure 11: ER IHC negative nuclear staining

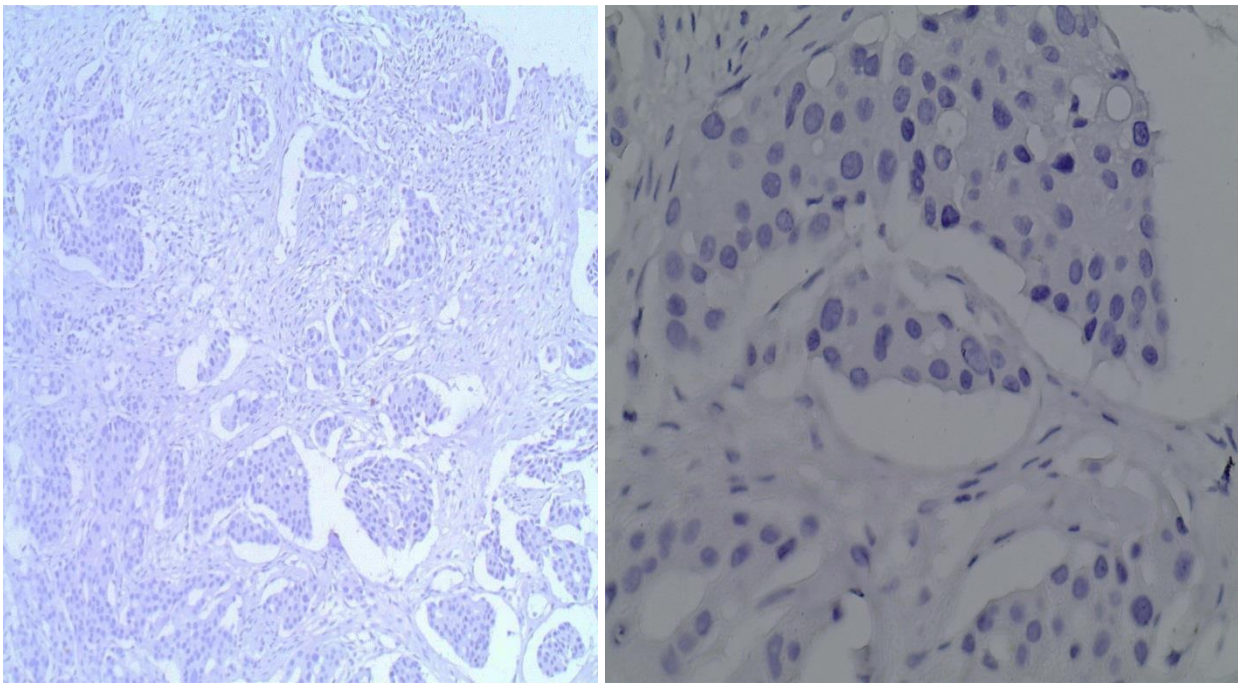
Invasive ductal carcinoma, NOS Nottingham grade 2 showing non-expression of ER in tumor cell nucleus



(x100 magnification) (x400 magnification)

Figure 12: PR IHC positive nuclear staining

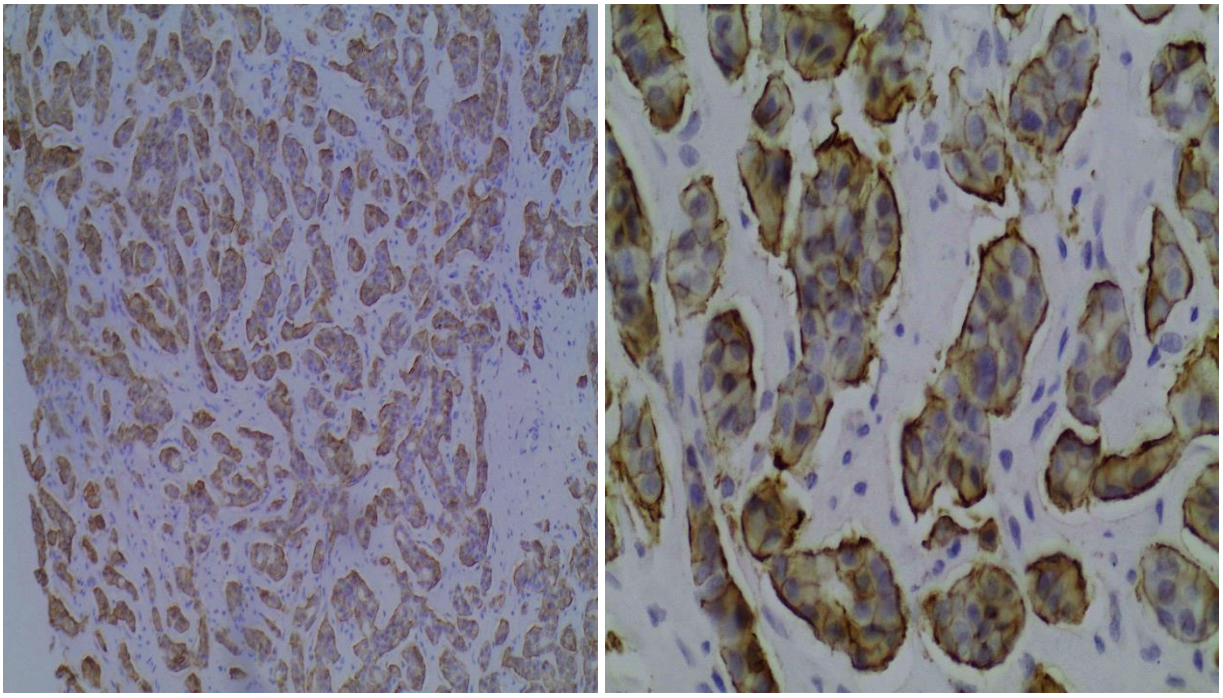
Invasive ductal carcinoma, NOS Nottingham grade 2 showing PR staining of moderate intensity (2+) in approximately 100% of the tumor cell nucleus



(x100 magnification) (x400 magnification)

Figure 13: PR IHC negative nuclear staining

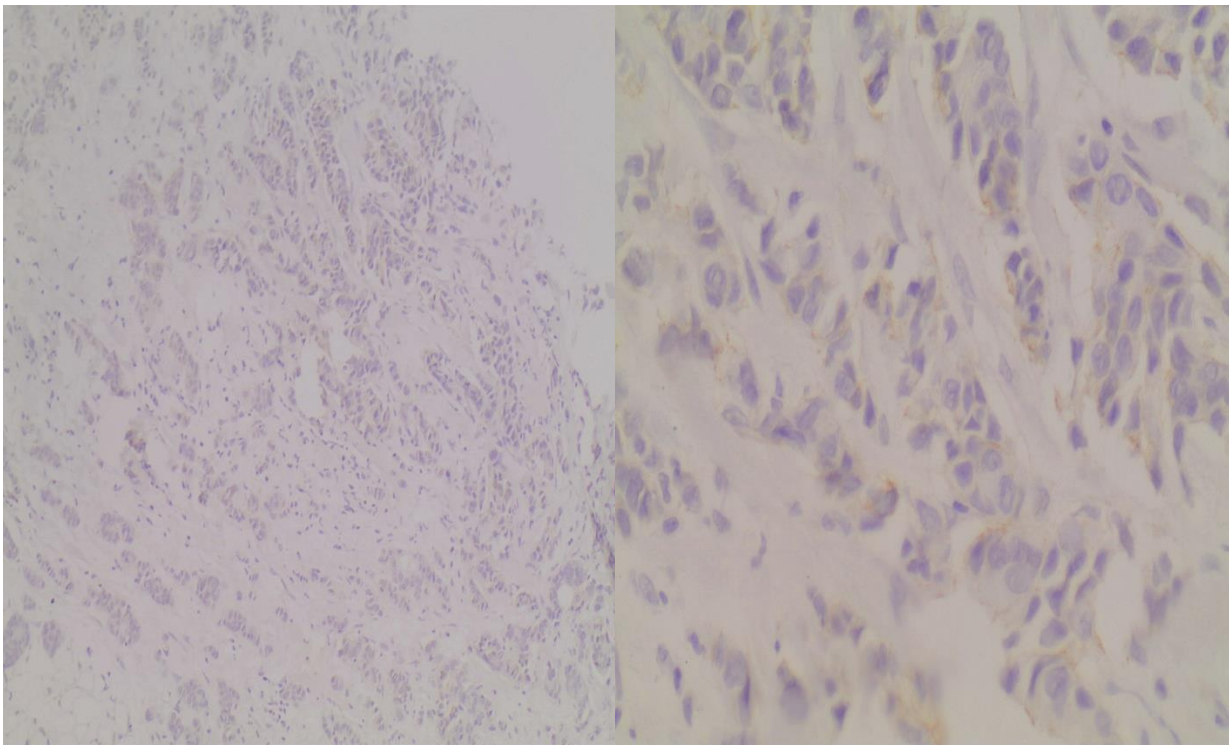
Invasive ductal carcinoma, NOS Nottingham grade 2 demonstrating non expression of PR in tumor cell nucleus



(x100 magnification) (x400 magnification)

Figure 14: HER2 IHC positive membranous staining

Invasive ductal carcinoma, NOS Nottingham grade 2 demonstrating a strong circumferential membranous HER2 staining in approximately 100% of the tumor cells



(x100 magnification) (x400 magnification)

Figure 15: Tumor cell membrane negative for HER2

Invasive ductal carcinoma, NOS Nottingham grade 2 demonstrating incomplete membrane staining that is faint in > 10% of the invasive tumour cells (score 1+)

CHAPTER FIVE

DISCUSSION

5.1 Introduction

In the recent past, there has been a significant advance in understanding breast cancer biology using IHC and other molecular techniques resulting in improved management and prognosis of the disease. Breast cancer is no longer considered as single disease entity but a heterogeneous group of disease falling in different histologic types, grades and molecular subtype categories(Sorlie 2004; Dolled-Filhart, Ryden et al. 2006; Glass, Lacey et al. 2007; Weigelt, Geyer et al. 2010).

This study examined the histologic types, histologic grade and molecular subtypes of breast cancer at the University Teaching Hospitals. Using standard IHC, breast cancers were classified into four groups based on ER, PR and HER2 immunohistochemistry reactivity as Luminal A, Luminal B, HER2 type and TNBC. Age, histologic type, grade and molecular subtype were the variables under analysis. Chi-square test for association with categorical variables was done, all analyzed at 95% confidence interval and p value was considered significant if <0.05.

5.2 Age distribution

The youngest and oldest patients in this study were 29 and 93 years old respectively at the time of diagnosis. This age range was consistent with findings from other studies on women of African ancestry with breast cancer. In a study by Sayed *et al* (2014) in Kenya, the youngest and oldest patients were 19 and 94 years old respectively with a median age of 47.5 years. Another study by Seshie *et al* (2015) in Ghana revealed an age range of 24 to 77 years. A similar age distribution has been observed in studies from other populations. A study by Onitilo *et al* (2009) in the USA revealed an age range of 27 to 95 years. This wide age range reemphasizes the need for breast cancer screening in all age groups post puberty.

The predominant age group in this study was 30-39 years. This finding is keeping with a study by Zyaambo *et al* (2012) on the distribution of cancers in Zambia in which most of the female cancers were reported in the age group of 34-44 years and Kalubula *et al* (2018) in a

retrospective study on the epidemiology of cancers in Zambia in which breast cancer peak was observed in the age range 40-49 years.

Invasive ductal carcinoma, NOS was seen in all the age groups from 29 to 93 years. This finding is consistent with other studies from Africa (Adebamowo CA 2008; Huo, Ikpatt et al. 2009; Sayed, Moloo et al. 2014; Seshie, Adu-Aryee et al. 2015).

The mucinous, micropapillary and cribriform carcinomas were seen in 60, 38 and 29 year olds respectively. Mucinous and micropapillary carcinomas have been reported to occur throughout all the age ranges. Di Saverio *et al* (2007) evaluated SEER data for 11,422 patients with pure mucinous carcinomas in which the age range was 25 to 85 years. Several studies have reported an age range of 25 to 89 years at diagnosis of invasive micropapillary carcinoma(Siriaunkgul and Tavassoli 1993; Middleton, Tressera et al. 1999; Kuroda, Sakamoto et al. 2004; Pettinato, Sparano et al. 2004).

A study by Page *et al* (1983) found that women with classical invasive cribriform carcinoma tended to be younger with a range from 7 to 91 years.

5.3 Histologic findings

5.3.1 Histologic types

This study has shown that invasive ductal carcinoma NOS is by far the most common histologic type comprising 95.6% of the cases. Other histologic types seen were micropapillary, mucinous and cribriform carcinoma with each contributing 1.5%. These findings were similar to those by Adebamowo, 2008 in which majority of the cases (82.3%) were invasive ductal carcinoma NOS, tubulolobular and papillary contributed 1% each, whereas adenosquamous and cribriform carcinomas contributed 0.5% each. The other subtypes seen were metaplastic, medullary and lobular carcinomas.

Invasive ductal carcinoma NOS is known to be a heterogeneous collection of carcinomas and each carcinoma may have its own unique clinical and biological behavior (Lu, Masood et al. 2002). Although identification and characterization of these clinically relevant subsets plays a

significant role in patient management, predicting the prognosis of the majority of the breast cancer at UTHs based on histology alone is therefore challenging.

Micropapillary carcinomas are particularly aggressive form of invasive breast cancer that has a proclivity for lymph node metastasis even when small in size (Walsh and Bleiweiss 2001). However one of the limitations of our study was the unavailability of the lymph node status in the patients under study.

Mucinous carcinomas are known to represent a prognostically favorable subtype of invasive breast cancer (Li, Moe et al. 2003). In comparison to invasive ductal carcinoma NOS in a study by Di Saverio *et al* (2007), several differences were apparent. In particular, women diagnosed with mucinous carcinoma were older by an average eight years, were more likely to experience two or more primary cancers perhaps due to their longer survival, were more likely to be diagnosed at the most favorable stage (i.e. localized) and histologic grade (i.e. grade 1), and less likely to die from breast cancer as opposed to other causes than women diagnosed with invasive ductal carcinoma NOS.

Invasive cribriform carcinoma is also associated with favorable prognosis and likely to occur in younger patients. In a study by Page *et al* (1983) deaths attributed to invasive cribriform carcinoma did not occur among the 34 patients studied, with follow up intervals of 10 to 21 years.

A significant association between age group of patients and histologic type was found in this study.

5.3.2 Histologic grade

Histologic grade is an assessment of degree of differentiation (i.e. tubule formation and nuclear pleomorphism and proliferative activity (i.e. mitotic index) of a tumour and mirrors its aggressiveness with grade 1 being the least aggressive and grade 3 as the most aggressive (Elston and Ellis 1991). Higher grade tumours usually show higher mitotic rates, and by definition less differentiation, factors usually associated with a less favourable outcome and more aggressive behavior.

This study has demonstrated that women presenting with breast cancer at the UTHs are likely to have the aggressive grade 2 or 3 tumours at diagnosis and a less favourable outcome. This is consistent with other studies that have shown higher tumor grades at diagnosis in African women. A study by Seshie *et al* (2015) in a retrospective analysis of breast cancer subtype in Ghanaian patients reported grades 1, 2 and 3 at 8.3%, 60.8% and 30.8% respectively. Another study by Sayed *et al* (2014) in Kenya showed grades 1, 2 and 3 at 7%, 39.3% and 53.7% respectively. A similar representation was also reported by Adebamowo CA (2008) in which 9.4%, 44.3% and 15.6% were grade 1, 2 and 3 respectively. However 30.7% of the cases in this study were not graded.

5.4 Molecular subtypes

5.4.1 Luminal type

The majority of the cases in this study were of Luminal type (ER/PR+) and constituted 61.8%, of which 48.5% were of Luminal B variety. Only 13.2 of the cases were the least aggressive Luminal A subtype. Similar rates of ER/PR positivity were reported by Sayed *et al* (2014) in Kenya in which 72.8% of the cases of Luminal type and in a study by Adebamowo CA (2008) in which majority of the tumours were of Luminal type at 80.2%. However, in comparison with the two studies there was an over-representation of the Luminal B subtype (48.5% vs 10.8% and 2.6% respectively) in this study due to higher rates of HER2 positivity which contributed almost 2/3 to the Luminal B cases. This therefore implies that between the two Luminal subtypes, women presenting with breast cancer at UTHs are likely to be diagnosed with the more aggressive Luminal B than the Luminal A subtype and would benefit from both hormonal and anti-HER2 therapies.

5.4.2 HER2 type

HER2 positivity was seen in 36.8% of our cases. However, only 8.8% were classified as HER2 type (ER/PR-, HER2+). These findings were similar to those by Sayed *et al* (2014) in which the HER2 type was 7.7% of the cases. However, in comparison to the Kenyan study, an overall higher prevalence of HER2 positivity (36.8% vs 17.6) was found in this study. This implies that approximately a third of the breast cancers seen at UTHs may be associated with increased

tumour aggressiveness, increased rates of recurrence, and increased mortality in node positive patients but can have predictive response to anti-HER2 therapy, Trastuzumab whereas ¼ of the patients can benefit from both hormonal and anti-HER2 therapy.

5.4.3 TNBC

TNBC was the second most common subtype at 29.4% of the cases. This is in keeping with the findings by Sayed *et al* (2014) in which 20.4% of the cases were TNBC. This study has also shown that women presenting with TNBC variety at UTHs are likely to be older than 50 years. This is in contrast with several population-based studies that have demonstrated that TNBC often presents at young age in women of African ancestry(Carey, Perou et al. 2006; Bauer, Brown et al. 2007; Ihemelandu, Leffall et al. 2007; Huo, Ikpatt et al. 2009). In a study from the California Cancer Registry by Bauer *et al* (2007) revealed that African American women with TNBC were likely to be aged 40 years. Genetic and perhaps environmental factors could explain this difference in age at diagnosis of TNBC.

TNBC are usually more aggressive, harder to treat and more likely to recur than cancers that are hormone receptor positive and/or HER2 positive (Gonzalez-Angulo, Timms et al. 2011).

TNBC has recently been recognized as having substantial heterogeneity in tumour biology and gene expression profiles have identified six distinct TNBC subtypes namely; Basal-like 1-BSL1, Basal-like 2 - BSL2, Immunomodulatory-IM, Mesenchymal-M, Mesenchymal stem cell-like-MSL, and Luminal androgen receptor- LAR each associated with different treatment response rate (Aysola, Desai et al. 2013). For example, there is evidence that the basal-like phenotypes associated with BRCA1 and BRCA2 mutations respond well to platinum based drugs such as Cisplatin or Carboplatin (Gonzalez-Angulo, Timms et al. 2011). However, the contribution of each of the six to the TNBC subtype was not evaluated in this study.

5.5 Study limitations

This study is limited by the lack of complete clinical dataset such as tumour stage and lymph node status. This would have allowed for demonstration of association between molecular subtype with tumour stage and lymph node status. Another limitation was the unavailability of

FISH to confirm the positivity of equivocal HER2 (2+) which was considered negative in our study. However, it is unlikely that this would have significantly changed the results.

Due to financial constraint, we were also unable to profile the six distinct TNBC subtypes (two basal-like, BSL-1 and BSL-2, Immunomodulatory- IM, Mesenchymal- M, Mesenchymal Stem cell-like MSL and Luminal androgen receptor- LAR) by gene expression. Therefore the contribution of each to the TNBC was not evaluated.

Markers of proliferations were not available for our study therefore the rate of proliferation was only determined by the manual mitotic count which has high levels of inter-observer disagreement compared to the automated mitosis detection method.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

More than half were ER and/or PR positive. However only 13.2% of the tumors examined were the least aggressive Luminal A subtype. 48.5% were of the Luminal B subtype which was the most frequently occurring subtype followed by TNBC. The aggressive HER2 type was only seen in 8.8% of the cases. Invasive ductal carcinoma, NOS was the most common histologic type and majority of tumors were grade 2.

The prevalence of aggressive subtypes HER2 and TNBC is in keeping with older age at diagnosis at UTHs.

6.2 Recommendations

We recommend a routine ER, PR and HER2 IHC panel for breast cancer in selection of patients for targeted therapy. We also recommend the evaluation of the six distinct TNBC subtypes via gene expression profiles. This will enable for personalization of treatment based upon the molecular signature of breast cancer patients with TNBC.

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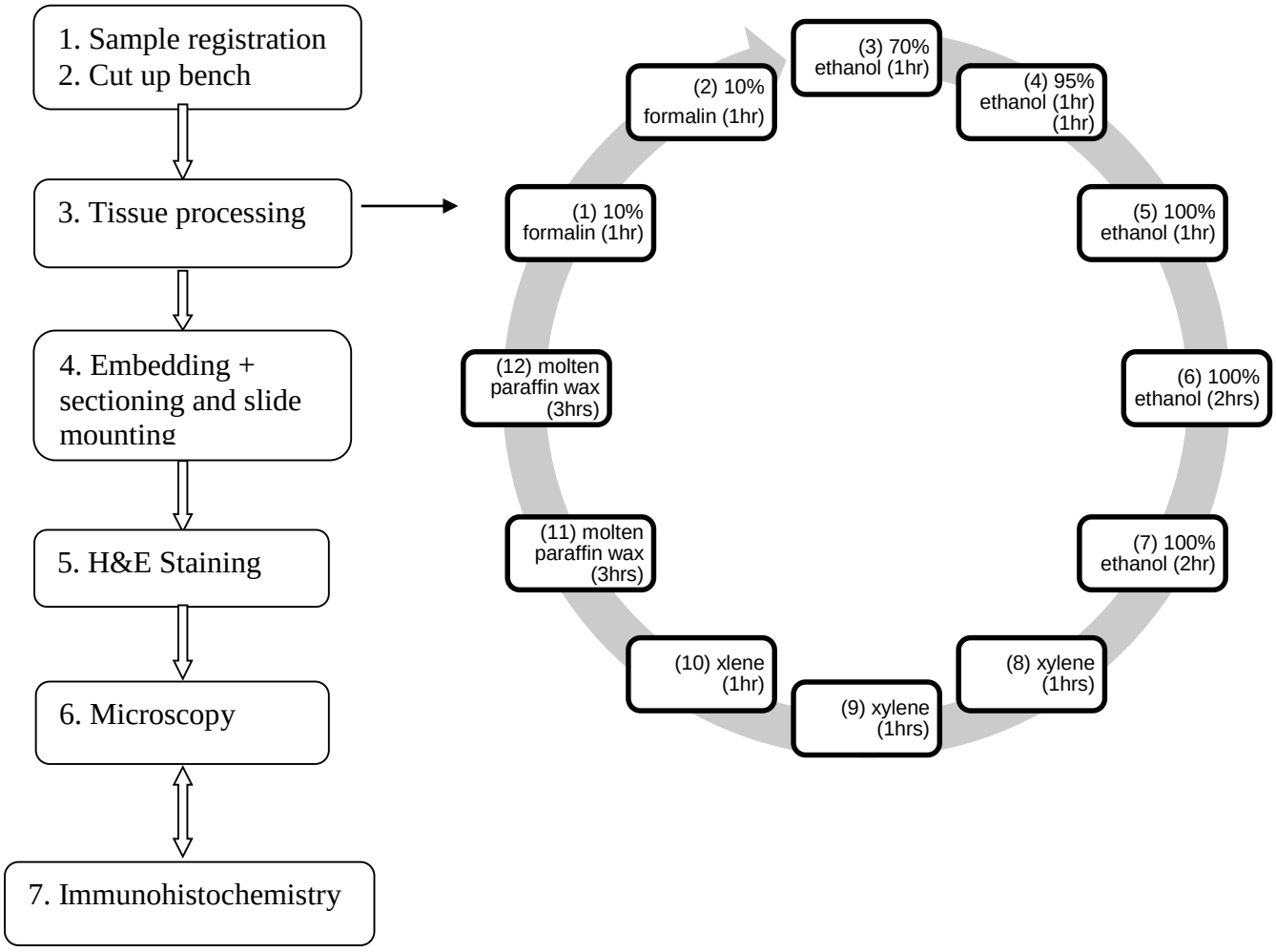
APPENDICES

A. Tissue processing flow chart



B. Standard operating procedures (SOP)

HEMATOXYLIN AND EOSIN STAINING PROTOCOL:

Principle

The oxidation product of haematoxylin is haematin, and is the active ingredient in the staining solution. Haematoxylin is not classified as a dye since the molecule possesses no chromophore. The *in situ* oxidation of haematoxylin is effected by the addition of a strong oxidant to the stain, in this case sodium iodate.

Haematin exhibits indicator-like properties, being blue and less soluble in aqueous alkaline conditions and red and more soluble in alcoholic acidic conditions. In acidic conditions, haematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant, in this case aluminium. To ensure saturation of chemical binding sites, the stain is applied longer than necessary, resulting in the over staining of the tissues with much non-specific background colouration. This undesirable colouration is selectively removed by controlled leaching in an alcoholic acidic solution, (acid alcohol), the process being termed "differentiation". Differentiation is arrested by returning to an alkaline environment, whereupon the haematin takes on a blue hue, the process of "blueing-up". The haematin demonstrates cell nuclei.

Full cellular detail is obtained by counterstaining with the eosin mixture. Colour enhancement is achieved by fortifying the stain with phloxine, a chemical member of the same family as eosin (halogenatedfluorosceins). The mechanism of their staining is not fully understood, but is believed to be of an electrostatic nature. Visualizations most acceptable to the histologist are obtained by applying the dyes in acidic conditions, whereby more intense specific colourations are obtained, the more acidic tissue components taking up the dye to a greater intensity, hence the addition of acetic acid.

Technical Points

1. (step 2) - The length of time necessary to over-stain the tissues will depend upon fixation and the type of alum haematoxylin employed. Lillie Mayer's alum haematoxylin-formalin fixed tissues should take 5 minutes.

Tissue Type	Haematoxylin	Acid alcohol 0.3%	Eosin	Comment
Routine tissues	4 minutes	See technical point 2	2 minutes	
Breast biopsies	4 minutes	1-2 seconds	2-4 minutes	Check staining

2. (Step 4) - Differentiation with acid alcohol requires some practical experience to ascertain the correct end-point, since the acid solution alters the colour of the tissue to red. The correct end-point is when, after blueing up, the background is almost colourless.

3. (Step 6) - If Scott's tap water substitute is employed; blueing up is achieved in a much shorter time.

4. (Step 8) - Eosin is highly soluble in water. Over-staining is removed by washing in running water.

5. Fixation - Not critical. Acidic fixatives will give a more eosinophilic result. Picric acid containing fixatives give an overall enhanced result. Acidic decalcifying fluids give poor nuclear staining.

6. Biopsies - 10% buffered formalin. Sections cut at 2micrometres

Method

1. Bring sections to distilled water

2. Stain nuclei with the alum haematoxylin (see note)

3. Rinse in running tap water

4. Differentiate with 0.3% acid alcohol (see note)

5. Rinse in running tap water

6. Rinse in Scott's tap water substitute (see note)

7. Rinse in tap water

8. Stain with eosin 2 mins

9. Dehydrate, clear and mount.

Results

Collagen.....pale pink

Muscle.....deep pink

Acidophilic cytoplasm.....red

Basophilic cytoplasm.....purple

Nuclei.....blue

Erythrocytes.....cherry red

Reagent Formulae

1. Lillie Mayer alum haematoxylin

aluminium ammonium sulphate ----- 200 g
haematoxylin (CI 75290) ---------- 20 g
ethanol ---------- 40 ml
sodium iodate ---------- 4 g
acetic acid ---------- 80 ml
glycerol ---------- 1200 ml
distilled water ---------- 2800 ml

In a 4L Ehrlenmeyer flask, to 1000mls of the distilled water add the aluminium ammonium sulphate. Place the flask on a heater/stirrer, turn on the heater and allow mixing until the alum dissolves - this takes about 15 minutes. Remove the flask from the heater/mixer, allow cooling, and then adding the remaining 1800mls distilled water - this will further cool the solution. Add the haematoxylin powder to the alcohol and dissolve as much of the powder as possible by shaking for a few minutes. Pour the strong alcoholic solution of haematoxylin into the cooled alum solution and stir to ensure all the powder is dissolved, preferably overnight. Add the sodium iodate, acetic acid, and finally the glycerol. Mix well, plug loosely and store. It is appropriate to make up a batch of the required amount, dependent upon the usage rate.

2. Acid alcohol 0.3% Acid Alcohol

commercial grade ethanol ----- 2800 ml

distilled water ---------- 1200 ml

conc. hydrochloric acid --------- 12 ml

In a sufficiently large container, add the acid to the water, then add the alcohol and mix thoroughly. The generation of fine bubbles is an indication that mixing is thorough.

3. Scott's tap water substitute

sodium hydrogen carbonate --- 10 gm

magnesium sulphate ---------- 100 gm

distilled water ---------- 5 L

Dissolve the salts in the water. Store the stock solutions at room temperature.

4. alc. acetified eosin/phloxine TQEH

1% eosin Y (CI 45380) ---------- 400 ml

1% aqphloxine (CI 45405) ----- 40 ml

95% alcohol ---------- 3100 ml

gl acetic acid ---------- 16 ml

Mix the above reagents together, and stir well. The solution keeps well.

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Immunohistochemistry protocol

The study will use the HiDef detection HRP polymer system. This is a visualization system that is ready-to-use in immunohistochemical protocol. This two-step system uses an indirect method resulting in an antibody-enzyme complex that universally detects mouse and rabbit primary antibodies. The resulting chromogenic reaction can be visualized by HRP-compatible chromogens using light microscope. It is biotin free and eliminates non-specific staining that could result from endogenous biotin. This visualization system consists of two detection reagents and is based on the sequential application of HiDef Detection Amplifier (Mouse and Rabbit) followed by HiDef Detection HRP polymer Detector, amplifying the detection of low expressing antigens. This detection system is compatible with both manual and open automated staining platforms

1. Trilogy/pressure cooker set on 120 degrees Celsius for 15 minutes.
2. Trilogy hot rinse for 2 minutes
3. Wash with distilled water for 30 seconds.
4. Peroxidase block for 10 minutes.
5. Wash with TBS buffer for 1 minute.
6. Apply primary antibody for 30 minutes
7. Wash with TBS buffer for 1 minute.
8. HRP HiDef Amplifier for 10 minutes.
9. Wash with TBS Buffer for 1 minute.
10. HRP HiDef Detector for 10 minutes (incubate on the slide).
11. Wash with distilled water for 1 minute.
12. DAB Chromogen for 3 minutes (incubate on slide)
13. Wash with distilled water for 1 minute
14. Counter stain with hematoxylin , incubate 1 minute on the slide
15. Wash with distilled water for 1 minute.
16. Cover slipping the slide
17. Dehydrate the slide using 95% alcohol for 3 minutes, followed by 100% alcohol for minutes, then 3 times with Xylene for 5 minutes each

Reference

Ministry of Health Standard operating Procedure for level III Hospitals (2008 Revision), Lusaka, Zambia

C. Data collection tool

Biopsy No	Age	Histologic type	Histologic Nottingham grade			ER	PR	HER2	Molecular subtype
			Mitosis	pleomorphism	Tubular formation				

D. Contact information

Should you have any questions, you can contact the principle investigator Dr. Allen Chomba Chama or Dr. Aaron Lunda Shibemba at the University Teaching Hospital or Dr. Evans Malyangu at Maina Soko military Hospital or ERES CONVERGE IRB.

1. **Dr. Allen Chomba Chama**

   Project Principal Investigator.

   Mobile 097-6-679-878

2. **Dr. Aaron Lunda Shibemba**

   Project Consultant Pathologist/ Supervisor

   Head of Department

   Department of Pathology and Microbiology

   University Teaching Hospital

   Lusaka, Zambia

   096-8-469-198

3. **Brig. Gen. Dr. Evans Malyangu**

   Project Consultant Pathologist/ Supervisor

   Commandant

   Maina Soko Military Hospital

   Lusaka, Zambia

   097-1-582-933

4. **ERES CONVERGE IRB**

   33 Joseph Mwila Road, Rhodes Park

   Lusaka, Zambia

   095-5-155-633

E. Ethical approval letter

33 Joseph Mwilwa Road
Rhodes Park, Lusaka
Tel: +260 955 155 633
+260 955 155 634
Cell: +260 966 765 503
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

13th April, 2018

Ref. No. 2018-Mar-015

The Principal Investigator
Dr. Allen Chomba Chama
University of Zambia
Department of Pathology,
LUSAKA.

Dear Dr. Chama,

RE: MOLECULAR SUBTYPE CHARACTERIZATION OF PRIMARY BREAST CANCER AT UNIVERSITY TEACHING HOSPITAL, LUSAKA ZAMBIA.

Reference is made to the above subject matter dated 4th April, 2018. The IRB resolved to approve this study, your application for waiver of consent and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2018-Mar-015**
Approval and Expiry Date	Approval Date: 13th April, 2018	Expiry Date: 12th April, 2019
Protocol Version and Date	Version - Nil	12th April, 2019
Information Sheet, Consent Forms and Dates	• English.	12th April, 2019
Consent form ID and Date	Version - Nil	12th April, 2019
Recruitment Materials	Nil	12th April, 2019
Other Study Documents Questionnaires,	Data Collection Sheets.	12th April, 2019
Number of participants approved for study	155	12th April, 2019

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- ERES Converge IRB does not "stamp" approval letters, consent forms or study documents unless requested for in writing. This is because the approval letter clearly indicates the documents approved by the IRB as well as other elements and conditions of approval.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us. Late submission of these will attract a penalty.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Prof. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON