BREAST CANCER CLASSIFICATION ACCORDING TO IMMUNOHISTOCHEMICAL MARKERS: CLINICOPATHOLOGIC FEATURES IN WOMEN TREATED AT PIETERSBURG HOSPITAL, LIMPOPO

by

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Submitted in partial fulfilment of the requirements for the degree

MASTER OF MEDICINE

in

RADIATION ONCOLOGY

in the

FACULTY OF HEALTH SCIENCES

(School of Medicine)

at the

UNIVERSITY OF LIMPOPO

SUPERVISOR: Dr. F.Ooko

2022

DECLARATION

I , **RAMADIMETJE JOYCE MPHAHLELE** declare that Breast Cancer Classification According To Immunohistochemical Markers: Clinicopathologic Features In Women Treated At Pietersburg Hospital, Limpopo

Is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references and that this work has not been submitted before for any other degree at any other institution.

MPHAHLELE RAMADIMETJE JOYCE .....

FULL NAMES **DATE**

DEDICATION

To my late parents who raised me to be the person that I am today.

And to my husband and three children for their constant love and support throughout my studies. My appreciation also goes to God almighty that made all things possible. Lastly to my siblings, who constantly cared for children due to my hectic schedule.

ACKNOWLEDGEMENTS

- A special thank you to my supervisor, Dr. Ooko for his guidance, support and encouragement, and reviewing the whole research project from proposal to this report.
- To the stuff and colleagues at Pietersburg Oncology unit for their support.
- The Limpopo Province: Department of Health, for giving me permission to the study

ABSTRACT

Background

Breast cancer is known to be a heterogeneous disease that demands patient centered care. Establishing the clinicopathological characteristics of breast cancer patients is a vital step in an effort to individualize their treatment.

Aim

The aim is to evaluate the clinicopathologic features of the different subtypes of breast cancer when classified according to immunohistochemistry markers in women attending Pietersburg hospital.

Methods

A retrospective review of medical records of women treated at Pietersburg hospital between 2010 and 2011 was done. Data collection was extracted on a customized data collection sheet. Chi square was used to determine association between clinicopathologic features and molecular subtypes. Analysis of variants was used to assess association between molecular types and age.

Results

The mean age of the population was 55.3 years (+/-14 standard deviation). The majority of patients were in stage III (46.9%) and IV (33.5%). The ER, PR, HER2/neu positive rate was 50.6%, 30% and 14,3 % respectively with a negative rate of 13,4%, 19,5% and 23,4% respectively. ER, PR and HER2/neu was unknown in 18%, 19,
5% and 23,4% respectively. The most common molecular subtype was luminal A (53,6%) followed by triple negative (27.2%), HER2/neu (11, 4%) and luminal B (7. 9%).There was no association between the subtypes and tumour stage (p=0.578).The rate of distant metastasis was similar across the subtypes being 37,9%,35%, 32,4% and 31,9% in HER2/neu, luminal B ,luminal A and TNBC, respectively. All four molecular subtypes had high rate of axillary lymph node involvement (p=0.886) Luminal A had the least percentage of high grade tumours with TNBC having the highest. Five-year overall survival for the cohort was 25, 6% with luminal A and B having a better 5 year overall survival of 27,2%

and 25% respectively, whereas HER2/neu and TNBC had lower 5 year OS of 24% and 23,3%.

Conclusion

The findings of this study suggest that luminal A subtype is the most predominant and the majority might benefit from hormonal therapy. However, some patients could not be classified due to missing IHC marker test results. The outcome across all four subtypes is poor and more effort should be put towards improving the diagnosis and treatment individualization and follow-up in these patients.

Key words: molecular subtypes; luminal A; luminal B; triple negative; HER2/neu.

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LIST OF ABBREVIATIONS

ER	Oestrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor receptor 2
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
BRCA 1	Breast cancer gene 1
BRCA 2	Breast cancer gene 2
CK 5/6	Cytokeratin 5/6

DEFINITION OF CONCEPTS

Breast cancer: Breast cancer is a diverse group of neoplasms arising from epithelial cells lining the milk ducts in the breast with different histological and clinical characteristics (Polyak, 2011). In this study the term will be used as defined above.

Immunohistochemistry: Immunohistochemistry is the laboratory technique that uses monoclonal and polyclonal antibodies for assessment of specific antigens in tissue sections (Duraiyan, Govindarajan, Kaliyappan, and Palanisamy, 2012). In this study the term will be used as defined above.

Clinicopathologic features: These are a combination of signs and symptoms that are observed by the clinician and the results of laboratory examination (Sepe, Piscuoglio, Quintavalle, Perrina, Quagliata, Formisano et al., 2016). In this study clinico-pathologic features means both clinical and laboratory features associated with breast cancer that includes tumour size, lymph node status, age at diagnosis, histological grade, estrogen receptor status, and progesterone receptor status.

Oestrogen receptor (ER) positivity: This refers to tumour cells with 1% or more of the tumour cells showing nuclear staining of any intensity for oestrogenic receptors (Allison, Hammond, Dowsett, McKernin, Carey, Fitzgibbons et al., 2010). In this study the term will be used as defined above.

Progesterone receptor (PR) positivity: It refers to tumour cells with 1 % or more showing nuclear staining of any intensity for progesterone receptors (Allison, et al, 2020). In this study the term will be used as defined above.

Human epidermal growth factor receptor 2 (HER2) positivity: It is defined as consistent membrane staining of HER2 in 10 % of the tumour cells expressed as tumour score of 3+ by Immunohistochemical method; or in case of unequivocal results (2+) (Wolff, Hammond, Schwartz, Hagerty, Allred, Cote et al., 2007). In this study the term will be used as defined above.

Menopause: Menopause refers indefinite termination of menstruation that symbolises the end of reproductive life occurring in women at any time from 40 to 60 years with a mean age of 51 (Torino, Barnabei, De Vecchis, Appetecchia, Strigari, and Corsello, 2012). In this study postmenopausal will be defined as 55 years or older.

Cancer staging: It is a way of determining the extent of cancer using both clinical features and findings on radiological imaging of the disease (Edge and Compton, 2010) In this study cancer staging is defined as above, using the 7th edition of the American Joint Committee on Cancer Tumour-Node-Metastasis (TNM) system (Edge , Byrd , Compton , Fritz , Greene ,2010)

Overall survival: This is the time span from histological diagnosis to death of a patient due to any cause (Kunheri, Raj, Vijaykumar, and Pavithran, 2020.) In this study it is the time from histological diagnosis to the last consultation with the patient.

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CHAPTER ONE

1. INTRODUCTION

1.1 INTRODUCTION AND BACKGROUND

Breast cancer is the most commonly diagnosed cancer in females worldwide with an estimated 2.3 million new cases annually (Sung, Ferlay, Siegel, Laversanne, Soerjomataram, Jemal, and Bray, 2021). In South Africa, breast cancer contributes up 23.11% (n=9624) of all histologically diagnosed neoplasms (National Institute for Communicable Diseases [NICD], 2020). The life-time risk of developing breast cancer in South Africa is 1 in 25 women of ages 0-74 years (NICD, 2020). Although breast cancer can occur in both males and females, it is predominantly diagnosed in females. Breast cancer is the first among causes of death due to cancer in women (Sung et al, 2021).

The development of new therapies for breast cancer has resulted in significant increase in disease-free survival and reduction of cancer specific mortality (Florescu and Nistor, 2019). However, appropriate selection and administration of therapies according to the patient and disease characteristics is critical not only for prolonging disease-free survival and overall survival, but also for preventing late treatment related complications such as anthracycline-related cardiac toxicities, myelodysplastic syndrome, leukaemia and taxane-associated neuropathy (Güler, 2017)

Historically, the confirmatory diagnosis and classification of invasive breast cancer has been made by the use of histological report based on the World Health Organization morphological classification according to the appearance of the malignant cells. The main histological types according to this classification are invasive ductal carcinoma – not otherwise specified (IDC-NOS), and lobular carcinoma. However, breast cancer is now known to be a highly heterogeneous cancer type, made up of distinct phenotypes and morphologic appearances with diverse clinical behaviour (Tang, Wang, Kiani, and Wang, 2016). Present knowledge confirms that breast cancer consists of a broad spectrum of histological lesions that are regarded as highly heterogeneous in terms of its presentation, morphological characteristics, prognosis and therapeutic outcome

(Grant, Myburgh, Murray, Pienaar, Kidd, Wright, and Kotze, 2019). The first microarray-based gene expression profiling was conducted nearly two decades ago (Perou, Sørlie, Eisen, Van De Rijn, Jeffrey, Rees, Pollack, Ross, Johnsen, Akslen, and Fluge ,2000).It resulted in the discovery of intrinsic molecular subtypes which helped to explain the diversity in biological behavior and response to treatment amongst breast cancer patients (Sørlie, Tibshirani, Parker, Hastie, Marron, Nobel, Deng, Johnsen, Pesich, Geisler, and Demeter, 2003; Reis-Filho, and Pusztai, 2011).

Hierarchical cluster analysis of genes that vary between breast tumours, also referred to as the intrinsic genes, reveals the existence of at least four molecular subtypes of breast cancer, namely: luminal A, luminal B, HER2-enriched, and basal-like tumours (Güler, 2017). Following the class-discovery studies that have unmasked the heterogeneity of breast cancers, microarray-based gene expression profiling was further developed and are now routinely used for predicting the outcome for individual breast cancer patients with the aim of identifying patients with cancers of reasonably good prognosis to allow for the safe omission of adjuvant chemotherapy (Weigelt, Baehner, and Reis-Filho, 2010). Examples of commercially available microarray-based gene testing kits for predicting outcomes in patients with breast cancer include MammaPrint (MP), OncotypeDX, PAM-50 risk recurrence score, Breast Cancer Index, and EndoPredict (Güler, 2017).

Microarray-based tumour profiling using the 70-gene MP profile has been available in South Africa since 2007, and as from 2009, local referral criteria were introduced for payment by medical aid providers (Grant, Apffelstaedt, Wright, Myburgh, Pienaar, De Klerk, and Kotze, 2013;Grant et al, 2019). However, gene testing kits are expensive and not universally available to many, especially in low and middle income countries including a majority of patients in South Africa (Vallejos, Gómez, Cruz, Pinto, Dyer, Velarde, Suazo, Neciosup, León, Miguel, and Vigil, 2010; Grant, Myburgh, Murray, Pienaar, Kidd, Wright, and Kotze, 2019). Additionally, most of the commercially available kits require fresh frozen tissue samples that usually make it necessary to repeat a biopsy to obtain the sample for testing (Vallejos et al. 2010). Initially in South Africa, analysis was performed on fresh tissue only, but since 2012, the use of formalin fixed paraffin embedded (FFPE) tissue became available and has now become the only method used (Grant et al, 2019).

The highly heterogeneous nature of breast cancer requires that the treatment for each patient be individualized. The process of individualizing therapy starts with the assessment of clinical parameters such tumour size and grade, lymph node involvement, patient demographics, and several molecular markers found within the tumour. These parameters are also known as clinicopathologic characteristics. The most significant molecular markers considered in the treatment decision-making are the oestrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). ER, PR, and HER2 are usually identified by conducting immunohistochemistry studies on the biopsy specimens of the cancer. Immunohistochemistry (IHC) profiles in breast cancer are defined by the analysis of ER, PR, HER2, cytokeratin 5/6, and HER 1 (Vallejos et al, 2010). The most accepted way of assessing the status of these biomarkers is by immunohistochemistry (IHC).

IHC uses antibodies specific for each biomarker, with the estimated number of positively staining cells in the tumour correlating to positive or negative result. IHC is considered adequate for testing ER and PR. However, IHC alone for HER2 protein expression is not perfect and leads to approximately 10% false negative rates (de Ronde, Hannemann, Halfwerk, Mulder, Straver, Peeters, Wesseling, van de Vijver, Wessels, and Rodenhuis, 2010). Therefore, confirmatory testing by performing fluorescent or chromogenic in situ hybridization (FISH or CISH) to confirm HER2 gene amplification or to establish its presence or absence when IHC results are ambiguous is often required.

IHC and ISH show high concordance with microarray-based gene profiling and are often used as surrogates for gene expression analyses as they reflect the genetic subtypes in a nearly similar way to the advanced genetic testing techniques for molecular markers. Thus, the laboratory results of IHC and ISH, can be used to identify histologic subtype or molecular phenotype in an accessible, affordable and easier way (Engstrøm, Valla, and Bofin, 2017). All cases of breast cancer can be classified into molecular subtypes based on surrogate markers as shown in Table. 1.1.

In the absence of microarray-based gene profiling, classification of breast cancers by immunohistochemistry into molecular subtypes is still crucial because the sub-types

markedly influence the kind of treatment a patient is likely to respond to, and the prognosis (Vallejos et al., 2010).

MOLECULAR SUBTYPE	IHC MARKER	PROLIFERATION CLUSTER
Luminal A	ER+ and/or PR+ HER2-	Ki67<15%
Luminal B (HER2-)	ER+ and/or PR+ HER2-	Ki67≥15%
Luminal B (HER2+)	ER+ and/or PR+ HER2+	Any
HER2 enriched	ER- and PR- HER2+	Any
Basal type (triple negative)	ER- and PR- HER2-	

Table 1.1 Molecular subtyping based on surrogate IHC markers

1.2 RESEARCH PROBLEM

1.2.1 Source and background of the research problem

Breast cancer is a highly heterogeneous disease and the need for individualized therapy is now universally acknowledged (de Ronde et al 2010). Microarray-based gene profiling has resulted in the identification of distinct breast cancer molecular subtypes, thereby explaining the diversity in biological behavior and response to treatment of different patients with apparently the same morphological type of cancer. Identification of subtypes is very crucial in determining the prognosis of each patient, and in the selection of the most effective therapy, while minimizing unnecessary use of chemotherapy with its associated side effects.

1.2.2 Statement of the research problem

Patients with breast cancers of similar morphological appearance on histology exhibit diverse clinical presentation, course of the disease, and response to treatment. At Pietersburg Hospital Medical Oncology clinic, selection of breast cancer treatment is often based on the histological report according to the WHO morphological appearance such as ductal carcinoma or lobular carcinoma, in addition to the ER and PR, and HER2 profile resulting in a significant number of patients receiving chemotherapy. Additionally, anti- HER2 therapy, trastuzumab is not available, thus

no patient has ever received the benefit of receiving it. By re-classifying previously treated patients into their respective breast cancer molecular subtypes, the researcher is able to re-evaluate the treatment each patient received, the behavior of their tumour, and how they responded to the administered treatment. This will assist in designing quality improvement initiatives to obtain the best outcomes for breast cancer patients in Limpopo. Therefore, this study retrospectively re-classified breast cancer patients treated at Pietersburg Hospital using their immunohistochemical test results to categorize them into different breast cancer molecular sub-types.

1.3 PURPOSE OF THE STUDY

1.3.1 Aim

The aim of this study is to re-classify the tumours in women with breast cancer treated at Pietersburg Hospital in a particular year into the molecular subtypes based on their immunohistochemistry marker, and compare the molecular sub-types with clinico pathologic characteristics of these patients.

1.3.2 Objectives

The specific objectives are:

- To assess the prevalence of breast cancer molecular sub-types among breast cancer patients treated in a particular year with subsequent follow-up.
- To determine the clinicopathologic characteristics (i.e. age of patient at diagnosis, histology, grade, stage of cancer and treatment outcome) of these patients.
- To determine any association that may exist between clinicopathologic characteristics and the breast cancer molecular sub-types in this cohort of patients.

1.4. RESEARCH QUESTION

What are the different molecular sub-types in women with breast cancer treated at Pietersburg Hospital, when classified according to immunohistochemistry marker results?

1.5 RESEARCH METHODOLOGY

1.5.1 Research design

This is a retrospective review of the medical records of women with breast cancer treated at Pietersburg hospital medical oncology clinic in the years 2010 and 2011.

1.5.2 Sampling

The entire population of consecutive women diagnosed with breast cancer and referred to the medical oncology clinic in 2010 and 2011 were eligible to enter the study (n=329). Sample consisted of part of the population who satisfied the inclusion criteria (n=254).

1.5.3 Data collection

Data collection took place in the month of August 2021. The relevant information on IHC and clinicopathological features were extracted from the patients' medical records and entered into a Microsoft Excel data sheet prepared for this study.

1.5.4 Data analysis

Data was cleaned and transferred to the SPSS software version 27 (created by SPSS, Inc. United States of Chicago IL) for analysis. Descriptive statistics (mean, proportions and frequency) are used to analyze the categorical variables.

1.5.5 Reliability, validity

Immunohistochemistry analysis was performed on samples using well defined protocols on calibrated laboratory equipment and the recommended ingredients. Manufacturer's guidelines were strictly followed. The data is analyzed and reported as found without any alteration. These were medical records collected during the actual treatment of patients and so may be considered reliable and valid.

1.5.6 Bias

These are records from patients who were referred to the public hospital. Selection bias may have occurred as some patients with breast cancer from Limpopo may have been referred to other facilities in the private sector or outside the province for treatment.

1.6 ETHICAL CONSIDERATIONS

Ethical clearance was obtained from the Turfloop Research Ethics Committee (TREC), certificate number TREC/127/2021: PG (annexure C). The TREC also waived the requirement for informed consent from each patient, as the risk of harm was considered low in this retrospective study.

1.7 SIGNIFICANCE OF THE STUDY

The results of this study have revealed the prevalence of the breast cancer molecular subtypes among the cohort of patients treated at this Centre. It also highlights the clinicopathologic characteristics of the patients as related to their breast cancer subtypes. The survival data demonstrates the outcome of treatment for patients with each molecular subtype. The results highlight the need to improve on the immunohistochemistry testing for important immunohistochemical markers such as Ki67, and incorporating them into the molecular classification of breast cancer for the purpose of providing individualized care to the patients.

1.8 OUTLINE OF THE RESEARCH REPORT

The introduction and background, and orientation to the study is highlighted in chapter 1. Chapter 2 is a concise review of the literature on the topic of breast cancer heterogeneity, breast cancer molecular subtypes, and its implications for therapy in individual patients. Chapter 3 describes the research methodology. In chapter 4, the findings of this research are presented. The results are further interpreted and discussed with respect to the research aims and objectives and compared with reports of findings from other studies available in the literature. A summary of the study and its finding, implications for care of breast cancer patients in Limpopo, and recommendations based on the findings are also presented

CHAPTER TWO

2. LITERATURE REVIEW

2.1 INTRODUCTION

Literature review is a well-coordinated presentation of knowledge gained from reading and analyzing selected articles, books and other resources, which provide a summary on the topic of the subject under consideration (Grove, Burns and Gray,2010). Chapter 2 discusses literature review on the subject of immunohistochemical markers and its association with clinicopathological features in women with breast cancer. The researcher reviews the literature on the burden of breast cancer– internationally and in the low and middle-income setting; methods of diagnosis and classification; and the role of immunohistochemical markers and their implication to the treatment of breast cancer in the clinical setting.

2.2 EPIDEMIOLOGY OF BREAST CANCER

Breast is an organ found in both men and women where it lies on the anterior chest wall over the pectoralis muscles. However, breast tissue is rudimentary in the males but well developed in the females. Breast consists of milk producing glandular tissue arranged in lobes composed of lobules connected in ducts, areolar tissue and blood vessels.

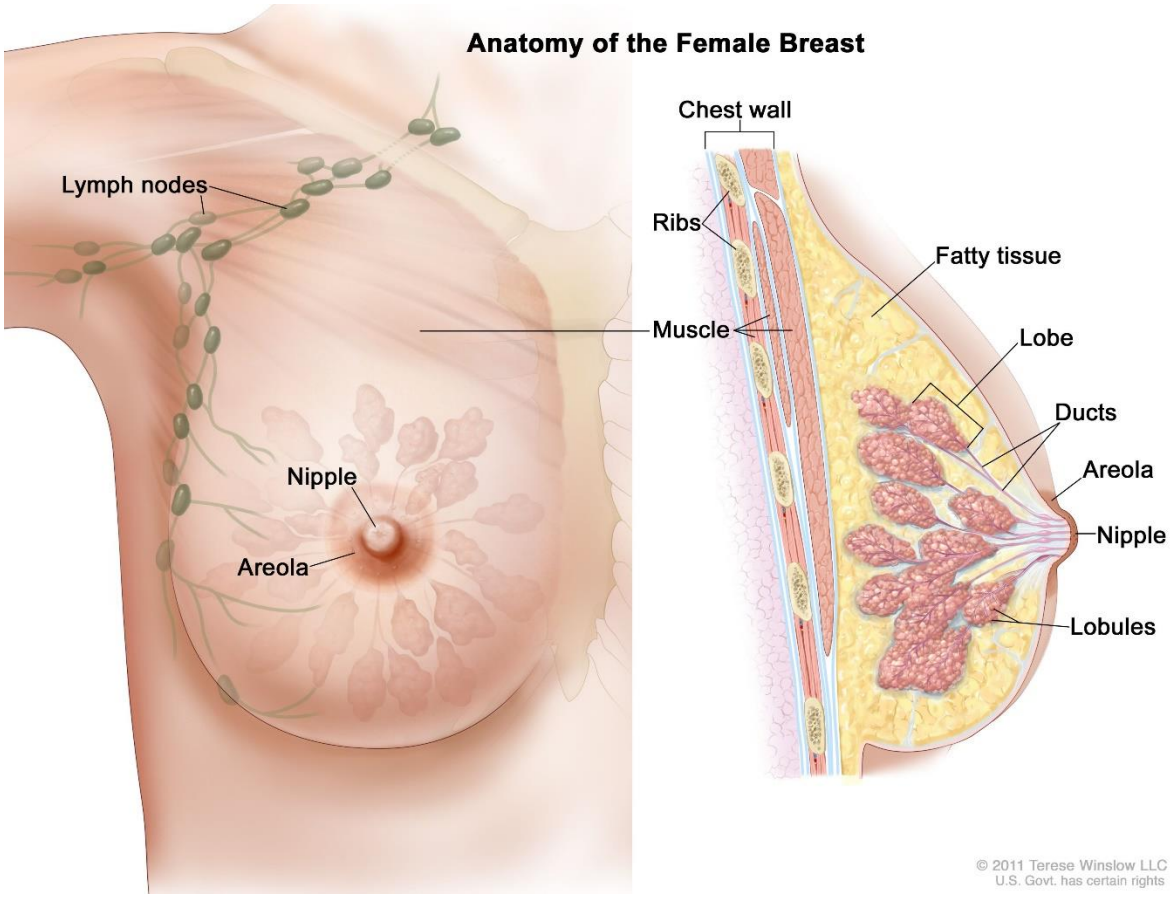


Figure 2.1 Anatomy of the female breast. Source: https://www.cancer.gov/types/breast/patient/breast-treatment-pdq (accessed 4/9/21)

Breast cancer is a cancer that forms in the cells of the breast tissue. It can occur in both males and females, but is more common in females.

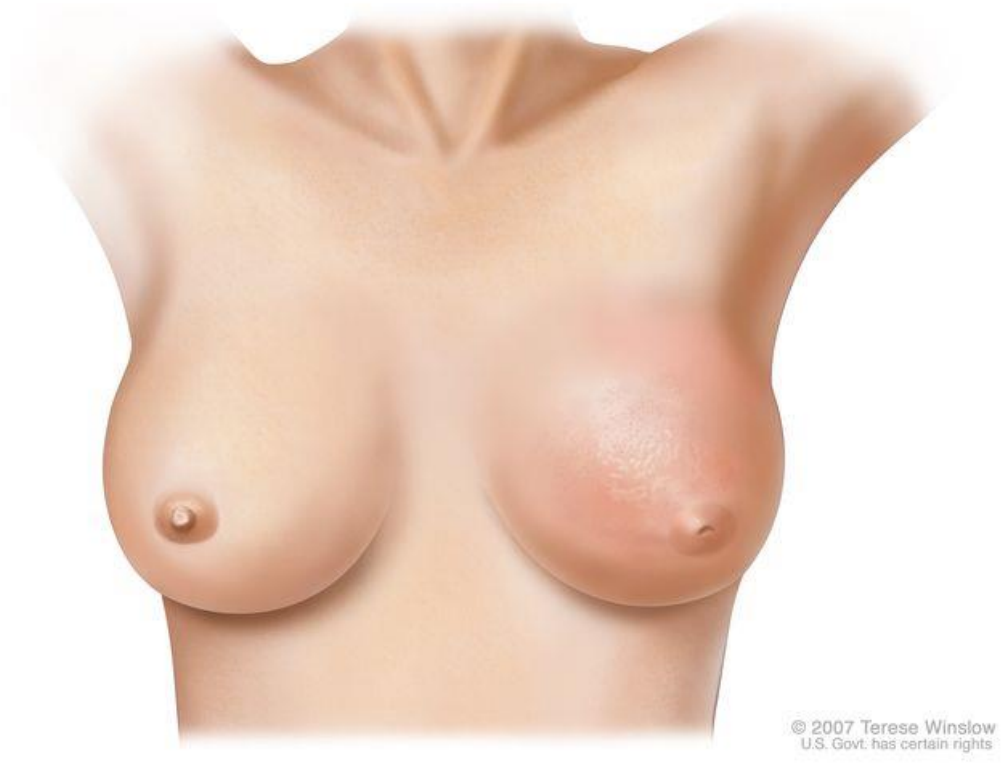


Figure 2.2 Image of breast cancer. Source: https://www.cancer.gov/types/breast/patient/breast-treatment-pdq (accessed 4/9/21)

2.2.1 Global burden of breast cancer

Globally, breast cancer is the most frequent cancer in women, followed by colorectal cancer in transitioned countries, and cervical cancer in transitioning countries (Sung, et al, 2021).Female breast cancer has now surpassed lung cancer as the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases (Sung et al., 2021). Cancer of the breast is the fifth leading cause of cancer mortality worldwide, resulting in 685,000 deaths. Among women, breast cancer also accounts for 1 in every 4 cancer cases, and for 1 in every 6 cancer deaths, ranking first for incidence in many countries (Sung, et al. 2021).

Incidence rates are higher in transitioned countries than in transitioning countries (55.9 and 29.7 per 100,000, respectively) with the highest incidence rates (>80 per 100,000) in Australia/New Zealand, Western Europe (Belgium has the world's highest incidence), Northern America, and Northern Europe. The lowest incidence rates (<40 per 100 000) are in Canada, Eastern and Middle Africa, and South Central Asia (Sung. et al., 2021)

The European Cancer Information notes that there are 404,920 new cases of breast cancer in Europe, with age standardized rate of 144.9 per 100 000 (Dafni, Tsourti, and Alatsathianos, 2019).The American Cancer Society (2021) estimates that there will be 281,550 new cases of breast cancer in the United States of America by 2021, with an increased incidence rate of 0.5% per year.

The prevalence of breast cancer continues to increase worldwide. This has mainly been attributed to several factors such as higher prevalence of reproductive and hormonal risk factors (early age at menarche, later age at menopause, advanced age at first birth, less number of children, less breast feeding, hormone replacement therapy, oral contraceptives).Others are life-style risk factors (alcohol intake, less physical activity, excess body weight); increased rate of detection by intentional mammographic screening; aging; and increase of the population (Bray et al 2018; Sung,et al 2021). Nevertheless, there is a wide range of geographical variation with high prevalence in Australia, New Zealand and Northern Europe (Bray et al 2018). Research on migrants confirmed that the geographical distinction is attributed to acquired factors such as time of onset of menstruation, history of reproduction, exogenous oestrogen replacement, alcohol use and obesity (Ziegler, Hoover, Pike, Hildesheim, Nomura, West, Wu-Williams, Kolonel, Horn-Ross, Rosenthal, and Hyer, 1993; Bray et al 2018).

2.2.2 Burden of breast cancer in Africa

Breast cancer incidence is associated with geographical and ethnic variation in the African continent that previously had low incidence rates. Breast cancer incidence has increased significantly in the continent (Joko-Fru, Jedy-Agba, Korir, Ogunbiyi, Dzamalala, Chokunonga, Wabinga, H., Manraj, Finesse, Somdyala, and Liu, 2020).There are suggestions that the recently increasing incidence of breast cancer in developing countries could be due to increasing life expectancy, improved control of infectious diseases, change in life style, adoption of western diet, reduced physical activity, and change in obstetric practices (Akarolo-Anthony, Ogundiran and Adebamowo, 2010). According to GLOBOCAN 2018, the age standardized incidence rate of breast cancer was 39.7 per 100 000 for the whole of Africa. However, even

within Africa there is still some geographical variation reflected by a higher age standardized incidence rate (ASIR) of 29.9 per 100 000 in Eastern Africa, and a lower ASIR of 4.9 per 100 000 in Northern Africa (Bray, et al, 2018).

Despite a relatively lower incidence and prevalence rate of breast cancer, transitioning countries have 17% higher mortality rates compared with women in transitioned countries (15.0 and 12.8 per 100,000, respectively) with the highest mortality rates found in Western Africa (Sung, et al 2021).

2.2.3 Burden of breast cancer in South Africa

According to GLOBOCAN 2018 female breast cancer and cervical cancer are the most frequently diagnosed cancer in South Africa (Bray, et al, 2018), with female breast cancer being the most common cancer in whites and Asians, and cervical cancer being common in blacks and coloured (Singh, Joffe, Cubasch, Ruff ,Norris, and Pisa, 2017).

The National Breast Cancer Registry in South Africa was founded in 1986 to provide cancer statistics .It was last updated in 2017. Based on statistics from National breast cancer registry an average of 9624 new cases were diagnosed in 2017 with an average age standardized rate of 35.35 per 100 000.The age standardized rates varied from as high as 89.58 in white women to as low as 20. 8 in African females (National cancer registry, 2017).An urban population-based registry (Ekurhuleni) published in 2018 reported an overall lifetime risk of 1:28 women with highest risk in white and Asian women of 55 and 36 per 100 000 respectively in a specific region of Ekhuruleni in Gauteng province of South Africa. In this provincial registry, black females had age standardized rate of 25 per 100 000 which was higher than the national cancer registry rates of 20 per 100 000 (Ndlovu, Khoali, Motsuku, Abraham, Chen, Sengayi-Muchengeti, and Singh, 2018). In a study that assessed breast cancer trends in South Africa, there was an increase in incidence with age standardized rates similar to those found in other African countries, but still lower than those in the western countries. (Singh, Joffe, Cubasch, Ruff, Norris, and Pisa, 2017). The country specific survival is 20 - 60 % in South Africa compared to 55% in United States (Connecticut), and 57% in Norway (Sung, Ferlay, Siegel, Laversanne, Soerjomataram, Jemal, and

Bray, 2021). A systematic review and meta-analysis of 87 studies conducted in Africa reported that 77% of all patients were diagnosed with stage III and IV breast cancer (Jedy-Agba, McCormack, Adebamowo and dos Santos-Silva, 2016.).

Research from United States and Denmark reported a rise in oestrogen positive breast cancer with decrease in oestrogen negative breast cancer, which was attributed to increase use of mammographic screening and obesity (Sung et al 2021). The high prevalence of oestrogen positive breast cancer is consistent with data from hospital based study done in Soweto and a cross-sectional study done in Potchefstroom (McCormack, et al., 2013 and Kakudji, Mwila, Burger, du Plessis & Naidu, 2021). Research on prevalence of breast cancer in Limpopo province is limited. A prospective study done in Limpopo reports a low prevalence of breast cancer in reduction mammoplasty specimens (Mzezewa, Setati, Netshiongolwe, and Sinoamadi, 2020).

2.3 RISK FACTORS

Female gender and advanced age are the most consistent and remarkable breast cancer risk factors (Halpern, Wazer, Perez, Brady, 2013). Familial breast cancer accounts for 5% to 10 %, with most cases being due to BRCA1 and BRCA2 mutations, which increase lifetime risk from approximately 65% to 85 % and 60 %, respectively (Halperin et al, 2013). Other factors associated with development of breast cancer include early menarche, nulliparity and delayed child birth. Breast cancer in women of native African decent and African American has been characterized by younger age at onset, advanced stage at diagnosis and poor prognosis (Carey, Perou, Livasy, Dressler, Cowan, Conway et al, 2006). An observational study done in Uganda reported a mean age of presentation at 45 years, with 70 % of patients having stage III and IV disease, and a median survival of 28 months (Galukande, Wabinga ,Mirembe, Karamagi & Asea, 2014).

2.4 CLINICAL PRESENTATION

Early stage breast cancer presents with a breast lump, which could be associated with nipple discharge. Physical examination includes evaluation of the breast and the regional lymph nodes. Advanced stage breast cancer presents with larger tumours, skin changes including presence of peau de orange, satellite skin nodules, ulceration, invasion of the chest wall, and axillary nodal enlargement. Research has shown that breast cancer patients in African countries frequently presented with advanced disease. A hospital based study in Soweto reports that 54% of patients had advanced disease (stage III/IV) (Mc Cormack et al, 2013). An observational study done in Uganda reports a mean age of presentation at 45 years, with 70 % of patients having stage III and IV disease (Galukande et al., 2014). This correlates to a retrospective study done in Angola that reports that 77.8% of their patients presenting with stage III/IV breast cancer (Lopes, Miguel, Freitas, Tavares, Pangui, Castro, Lacerda, Longatto-Filho, Weiderpass & Santos, 2015).

A systematic literature review and meta-analysis that included 87 studies from 17 Sub-Saharan Africa countries reports that 77% of black study population presented with stage III and IV disease. Advanced stage presentation is associated with a larger tumour size but independent of age at presentation and tumour grade (Jedy-Agba 2016).This is in contrast to data from the National Cancer Institute Surveillance Epidemiology and End results program from USA (DeSantis, Fedewa, Goding Sauer, Kramer, Smith, and Jemal, 2016) which shows that the incidence of advanced stage breast cancer decreased from 1973 to 2011 from 50% to 27 % in white women, and from 60 % to 32 % in black women. Late stage at presentation is been linked to lack of breast cancer awareness, lack of access to health care facilities, and long distance to health provider and rural region of residence (Jedy-Agba et al., 2016; Dickens, 2014).

2.5 DIAGNOSIS AND TREATMENT

The diagnosis of breast cancer involves the triple assessment protocol, including breast physical examination, diagnostic mammogram and ultrasound, and biopsy of the breast lump (Buccimazza, 2010). Biopsy is the most conclusive way to make the

diagnosis and can be done using a trucut biopsy, core biopsy, or fine needle biopsy of the associated axillary lymph node followed by a histological evaluation (Hammond, Hayes, Dowsett, Allred, Hagerty, Badve et al., 2010).

Breast cancer treatment depends on whether the disease is early or locally advanced. Early breast cancer generally refers to AJCC stage I to IIA (Ward, Tendulkar, and Videtic, 2020). Treatment of early breast cancer includes surgical resection followed by adjuvant chemotherapy, radiotherapy and endocrine therapy, depending on the presence of high risk features and pathological factors such as ER, PR, HER2 and Ki 67 status (Ward, et al 2020).Locally advanced breast cancer, as defined by the presence of clinical stage IIB (T3N0) to stage III and inflammatory disease is generally treated with neoadjuvant chemotherapy followed by surgery and radiotherapy with or without endocrine therapy (Gradishar, Anderson, Abraham, Aft, Agnese, Allison et al., 2020).

2.6 GENETIC PHENOTYPING

Microarray based gene expression analysis involves evaluation of a large number of genes in a single experiment where the labelled target (a sample RNA, complementary DNA, complementary RNA) is cross examined with probes that are immobilized to a solid matter (Weigelt, Baehner, and Reis-Filho, 2010). Microarray based gene expression class discovery studies have led to the concept that breast cancer is a heterogeneous disease. In the era of individualized medicine, being able to determine which patients will benefit from treatment and those who will not become significant. Gene expression profiling is used in predicting outcome of patients, especially in identification of good prognosis to allow exclusion of adjuvant chemotherapy (Guler et al., 2017). Commercially available multigene signature include both the first generation signatures (Mammaprint, Oncotype Dx and Genomic Grade Index), and the second generation prognostic signatures such as Prosigna, EndoPredict, Breast Cancer Index (Győrffy, Hatzis, Sanft, Hofstatter, Aktas, and Pusztai, 2015).

Breast cancer can be classified into five intrinsic subtypes based on microarray-based gene expression profiling with the difference in gene expression reflecting the difference in tumour at molecular level (Sorlie, Tibshirani, Parker, Hastie, Marron,

Nobel, et al., 2003). However, gene expression profiling is expensive and not readily available in most public hospitals in the low and middle income countries (Vasconcelos, Hussainzada, Berger, Fietze, Linke, Siedentop & Schoenegg, 2016). Gene expression profiling divides breast cancer into four identifiable sub-types: luminal subtype A, luminal subtype B, HER2 overexpressed, triple negative and normal breast like (Sorlie et al., 2003). Immunohistochemistry (IHC) is often used as surrogate for molecular sub-types (Vasconcelos et al., 2016). IHC tests for the following markers of expression: oestrogen receptors, progesterone receptors, HER2 overexpression or amplification, which can also be tested using in-situ hybridization if found to be equivocal (Vasconcelos et al 2016).

2.7 IMMUNOHISTOCHEMISTRY CLASSIFICATION

Since the introduction of molecular classification of breast cancer by Perou et al, there has been variation in the classification of breast cancer in terms of the dialect and biological markers in use (Blows, Driver, Schmidt, Broeks, Van Leeuwen, Wesseling, Cheang, Gelmon, Nielsen, Blomqvist, and Heikkilä, 2010). Breast cancer is divided into luminal-like, basal like, HER2 enriched and normal like (Perou, Sørlie, Eisen, Van De Rijn, Jeffrey, Rees, Pollack, Ross, Johnsen, Akslen, and Fluge, 2000). In a population-based case-controlled study done in the Carolina Breast Cancer study, breast cancer is divided into basal like, HER2 enriched, and unclassified, with the luminal like being further subdivided into luminal A and luminal B (Carey, Perou, Livasy, Dressler, Cowan, Conway, Karaca, Troester, Tse, Edmiston, and Deming, 2006). The basal type is defined by lack of expression of ER, PR and HER 2 and positive for cytokeratin 5/6 and/or HER1. Unclassified is defined by lack of expression of all the markers (Carey et al,2006).

The St Gallen 2011 International conference on breast cancer endorsed the classification of breast cancer into the luminal A, luminal B, HER2 enriched and triple negative (Goldhirsch, Wood, Coates, Gelber, Thürlimann, and Senn, 2011). The St Gallen 2011 further propose a use of a proliferation marker (Ki 67) in addition to the other immunohistological markers, to differentiate between luminal A and luminal B. The conference further suggest the use of histological grade in case Ki 67 is not available and further recognizes the lack of standard methodology and

cutoff of Ki 67 (Goldnirsh et al 2011).

A study done at a Peruvian hospital on immunohistochemical classification uses a more simplified classification as follows: Luminal A(ER^+ and/or, PR^+ and $HER2^{-)}$; Luminal B(ER^+ and /or PR^+ and $HER2^{-)}$; HER2 enriched(ER^- , PR—and $HER2^+$) ; and Basal (ER^-, PR^-, $HER2^{-)}$. Basal like and triple negative are considered to be synonymous (Vallejos, Gómez, Cruz, Pinto, Dyer, Velarde, Suazo, Neciosup, León, Miguel, A. and Vigil, 2010).

Generally, immunohistochemical classification divides breast cancer in luminal A, luminal B, HER2 enriched, and triple negative sub-types. Luminal subtype A is defined by expression of hormonal receptors (estrogen and progesterone receptor positive). Luminal A subtype is the most common among the intrinsic subtypes accounting for 64.3 % in the Carolina breast cancer study and is associated with good prognosis (Carey et al., 2006). Luminal subtype B, defined by expression of hormonal receptors (estrogen and progesterone receptor) and HER2 positive, or a high Ki 67 proliferation index accounts for 15 to 20% of breast cancers, is associated with higher tumour grade and worse prognosis compared to luminal A (Creighton, 2012). HER2 overexpressed is defined by a lack of expression of hormonal receptors (estrogen and progesterone receptor negative), accounts for 15 - 20% of breast cancers, and is associated with high nuclear grade and poor prognosis (Yersal and Barutca, 2014).

Triple negative (basal-like) breast cancer is defined by immunohistochemical staining being negative for ER, PR and HER2, and a high expression of myoepithelial markers like cytokeratin 5/6 (Yersal & Barutca, 2014). Triple negative breast cancers account for 3-37% of breast cancer and is associated with high nuclear grade and high chances of metastasis to the brain and lung (Heitz, Harter, Lueck, Fissler-Eckhoff, Lorenz-Salehi, Scheil-Bertram et al., 2009).

In the Carolina breast cancer study, basal like subtype accounts for 20%, with significant variation among premenopausal African American women accounting for 39% versus 14% in postmenopausal African Americans, and 16 % in non-African Americans (Carey et al, 2006). The prevalence of breast cancer intrinsic subtypes within Africa is controversial (Eng, McCormack, and Dos-Santos-Silva, 2014; Bird, Hill

and Houssami, 2008). Data suggests that there is high prevalence of triple negative subtypes, but systematic meta-analysis done reports a high number of hormone receptor positive cancer (Eng et al., 2014). In a systematic review and meta-analysis review of 80 studies which analyzed more than 17 000 patients with breast cancer from North Africa and sub-Saharan Africa, more than 50 % of breast cancers were ER positive with no subtype predominance (Eng et al., 2014).

In a study done in Lagos, Nigeria, the luminal subtype A accounted for 39.6% followed by triple negative which accounted for 29%, luminal subtype B with 18.8%, and HER 2 with 12.5% (Nwafor and Keshinro, 2015). These authors concludes that triple negative breast cancer subtype was quite common in their environment, affecting young female patients (Nwafor et al., 2015). A cross-sectional study done in Uganda reportes a high number of triple negative and luminal subtype A, with triple negative subtype accounting for 34%, luminal A 38%, HER2 accounting for 22%, and luminal B, 5 % (Galukande, Wabinga, Mirembe, Karamagi, and Asea, 2014).

In a study done in Soweto South Africa at a public hospital in 2013, the majority of tumours (63%) are ER positive in black breast cancer patients with an overall ER positivity of 65 % (McCormack, Joffe, van den Berg, Broeze, dos Santos Silva, Romieu, et al., 2013). Similar trend is observed in African American women older than 50 years in which the triple negative subtype constituted 20% and the late stage tumours are ER negative (McCormack, et al., 2013). The only known study from Limpopo province reports the prevalence of HER 2 overexpression of 26%, triple negative of 27.9%, and basal like subtype of 10.5% (van Bogaert, 2008; van Bogaert, 2013).

2.8 CLINICOPATHOLOGICAL FEATURES

Clinicopathological features represent a combination of both signs and symptoms found on clinical examination, and laboratory findings associated with the course of the disease (Sepe, Piscuoglio, Quintavalle & Perrinal, 2015). The clinicopathological features of breast cancer (including nodal status, tumour stage, tumour size, ER status, HER2 molecular classification, and histological grade) are often used in the management of breast cancer patients. Available data suggest that there is a

correlation between the breast cancer subtypes and some clinicopathological features.

2.8.1 Molecular subtypes and lymph node involvement

Lymph node involvement indicates invasion beyond the primary disease and determines treatment outcome. The association between lymph node and molecular subtypes is not well defined. A cross sectional study done in Potchefstroom reports no association between molecular subtype and node involvement (p=0.362) with a preponderance of positive axillary lymph node irrespective of molecular sub-type (Kakudji et al., 2021). This is consistent with a large European-based study involving 1339 women with invasive breast cancer that also found no association(p=0.886) between the molecular subtypes and involvement of axillary lymphnode (Spitale et al., 2009). However, Spitale et al (2009) observes the highest percentage of negative lymph node cases occurs in TNBC (57.5%) and luminal A (62.2%) tumors in contrast to patients with the Her2/neu subtype who has the highest prevalence of positive lymph nodes (49.2%). Nevertheless, a hospital-based study done in Peru reports a highly significant association (p=0.001) between molecular subtypes and axillary node status(Vallejos et al, 2010).

2.8.2 Molecular subtypes and clinical stage

Breast cancer stage determines treatment and outcome. Researchers report significant association between molecular subtypes and cancer stage. Spitale et al (2009) reports a significant association between molecular subtype and stage . They compared mean tumour diameter at diagnosis and found significant difference among the different sub-types ($P < 0.0001$). TNBC and HER 2/neu had a larger tumour diameter (T-stage in TNM) than both luminal A and luminal B subtypes (Spitale et al. 2009). Vallejos et al. (2010) in Peru also reports significant association of the tumor size according to AJCC clinical classification with molecular subtype. In their series, a high percentage of stage T3 tumours occur in HER2/neu subtype (54.4%), and most of T4 tumours (38.6%) occor in TNBC subtype.

2.8.3 Molecular sub-types and tumour grade

Histological grade and molecular subtypes are both independent markers that

determine the patients' outcome. Breast cancer histological grade is based on the degree of differentiation, level of nuclear pleomorphism, glandular tubule formation and mitotic count. The association between tumour grade and subtype was first found in a systematic review and meta-analysis of publications reporting on the frequency of breast cancer receptor-defined subtypes in indegenous population in Africa (Eng et al. 2014). This literature review reports that the presence of TNBC subtype is often associated with high grade, reflecting loss of estrogen expression in more advanced form of the disease. This is consistent with most studies that have consistently shown significant association between different molecular subtypes and histological grade (Spitale et al. 2009; Vallejos et al., 2010; Kakudji et al., 2021). Spitale et al., (2009) in a European study observe significant differences among molecular subtypes in which TNBC and HER2/neu cases show the highest prevalence of poorly differentiated phenotype (75.9% and 66.7%, respectively), whereas luminal A tumors are more frequently well/moderately differentiated (84.6%). Vallejos et al.(2010) in a South American study also found that histologic grade is significantly associated with immunohistochemical subtypes ($P < .0001$) with well- or moderately differentiated tumors (grade 1 and 2) appearing most frequently in the luminal A subtype (76.6%), while a greater percentage (70.3%) of poorly differentiated tumors (grade 3) occurred in TNBC subtype. In South Africa, Kakudji et al., (2021) reports a statistically significant association where both luminal ($p<0.001$) and non-luminal molecular subtypes ($p<0.001$) are significantly associated with tumour grade 2 and 3.

2.9. IMPLICATIONS OF IMMUNOHISTOCHEMISTRY CLASSES

Prognostic factors such as presence of axillary lymph nodes, tumour size, histological type and grade are normally used in the management decisions of breast cancer (Fragomeni, Sciallis, and Jeruss, 2018). The intrinsic subtypes are known to be associated with difference in response to treatment and overall survival (Millar, Graham, O'Toole, McNeil, Browne, Morey, et al, 2009). Luminal A is associated with a favorable overall survival and response to endocrine therapy like selective estrogen receptor modulator (tamoxifen), aromatase inhibitors (exemestane and anastrosole), with little benefit from chemotherapy (Li and Ma 2020). Luminal B shows benefit to chemotherapy with or without hormonal therapy with, no improvement in disease free

survival (Yin, Duan, Bian, and Yu, 2020; Carey et al., 2006). HER2 enriched breast cancer benefit to combination of chemotherapy and anti-HER2 therapy such as Trastuzumab and Pertuzumab (Cheang, Chia, Voduc, Gao, Leung, Snider et al., 2009). Triple negative breast cancer is associated with less efficacy to endocrine and targeted therapy, young age and poor prognosis (Yin et al., Carey et al 2006).Ideally, patients with different breast cancer sub-types should have different approach to treatment even if they have the same histological appearance by cancer cell morphology. This has significant implication in terms of their response to treatment and prognosis.

CHAPTER THREE

3. RESEARCH METHODOLOGY

3.1 INTRODUCTION

This chapter describes the research method, research design, study site, study population, sampling and sample size. The process of data collection, data analysis, ways of minimizing bias, and measures taken to ensure validity and reliability are discussed. Ethical considerations and how they were attended to are also presented.

3.2 RESEARCH DESIGN

The study method used is quantitative. Quantitative study refers to a process of describing an issue or phenomenon by collecting data in numerical form and analyzing

it using statistical methods (Aliaga and Gunderson, 2002). Quantitative study design allowed measurement and statistical analysis of data for immunohistochemical profile of breast cancer patients over a two year period.

This study is a descriptive retrospective study. Descriptive study is a non-experimental design when the researchers want to describes the variables of interest as it naturally occurs (Botma, Greef, Mualudzi, and Wright, 2010). Retrospective study analyses data collection after the clinical event of interest or exposure has occurred (Brink H, Van der Walt, Van Rensburg, 2006) .This study was quantitative in nature and followed retrospective design where data of breast cancer patients treated between 2010 and 2011 was collected from an existing data from medical oncology clinic at Pietersburg hospital in Limpopo Province.

3.3 STUDY SETTING

The study was conducted at Pietersburg Hospital, located in Polokwane, in the Capricorn District of Limpopo, South Africa (Figure 3.1).

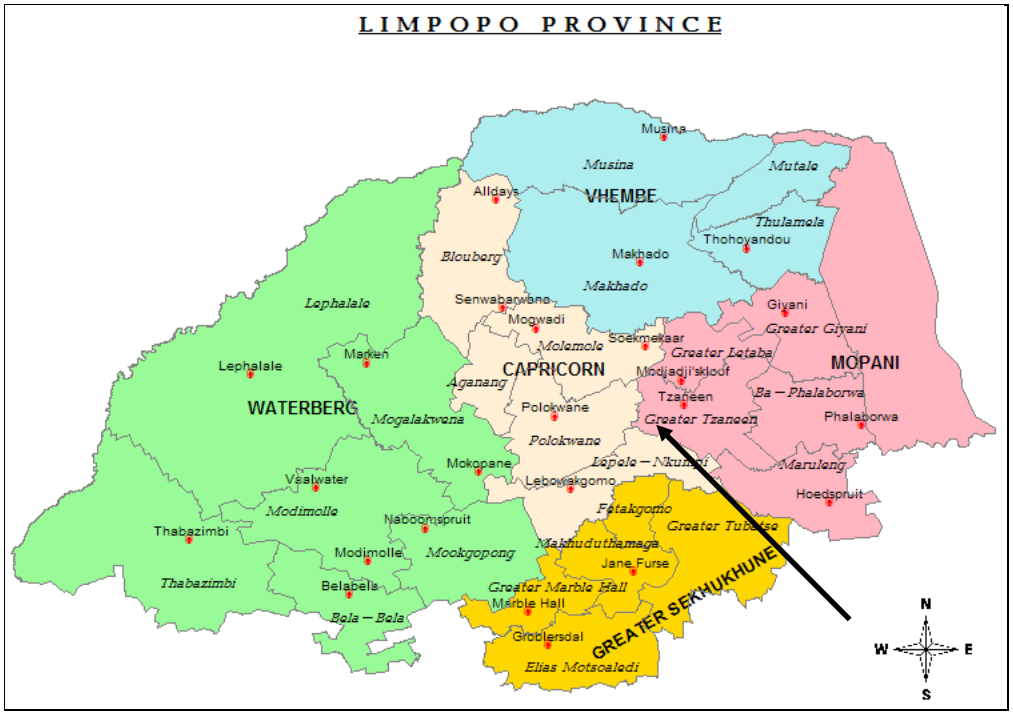


Figure 3.1. Map showing districts of Limpopo Province and the study site (Malangu & Legothoane, 2013:45). Arrow indicates study site

Pietersburg Hospital is a government tertiary referral and teaching hospital that has radiation therapy and chemotherapy facilities for treating cancer patients. All patients receiving cancer treatment in the government sector are referred from other hospitals in the 5 districts of Limpopo province to Pietersburg Hospital as it is the only facility in the state sector that has cancer treatment facilities in the province.

3.4 SAMPLING

3.4.1 Study population

Study population refers to all items that meet the sample criteria (Nancy, & Grove, 2012). Approximately 1500 to 2000 new cancer patients are seen annually at Pietersburg Hospital with nearly 150 to 250 of them diagnosed with breast cancer.
In this study the population is all female breast cancer patients treated at Pietersburg hospital over a 2-year period from 01 January 2010 to 31 December 2011, and subsequently followed up for 5 years or until they were lost to follow up.

3.4.2 Sampling method

This study used consecutive sampling of all female breast cancer patients who presented to medical oncology from January 2010 to December 2011. Consecutive sampling is a sampling technique that includes all patients with certain features of interest who are attainable within the defined study period (Mathieson, 2014).This technique is used to avoid sampling bias that can result from choosing certain patients.

3.4.3 Inclusion criteria

All women with breast cancer who presented to Medical oncology between 2010 and 2011 treated at Pietersburg Provincial hospital were included in this study. Only women with complete immunohistochemical data were included in the final analysis.

3.4.4 Exclusion criteria

Patients with incomplete immunohistochemical data were excluded in the final analysis.

3.4.5 Sample

A sample refers to a subgroup of the population which has been linked to the study (Botma et al 2010). The sample consisted of all patients with histologically proven breast cancer and available immunohistochemical results recorded within the study period

3.5 DATA COLLECTION

3.5.1 Data collection tool

A customized data collection sheet is used to capture the data for the study (Annexure 1). The information that is collected include age at diagnosis, menopausal status, ER, PR, HER2 results, tumour grade, tumour size (T stage), involvement of regional lymph nodes (N), metastasis (M), WHO histology type, time duration in months from histological diagnosis to the last recorded hospital visit or up to 60 months of follow up.

3.5.1.1 Development testing of data collection instrument

A customized data collection sheet was created using information extracted from Medical oncology patient digital records were relevant information was entered into the data collection form. There was no need to test the data collection instrument as the information was extracted from the patients' records.

3.5.1.2 Characteristics of the data collection instrument

The information collected include age at diagnosis, race, menopausal status, ER, PR, HER2 results, tumour grade, tumour size (T), involvement of regional lymph nodes (N), metastasis (M), WHO histology type, time duration in months from histological diagnosis to the last recorded hospital visit or up to 60 months of follow-up. There was no need to test reliability and validity of the data collection instrument as it was used to collate data since the study is retrospective. Validity was ensured for the immunohistochemistry data as daily quality assurance measures were done by the laboratory personnel according to the company's (National Hospital Laboratory Service) protocols. External tissue controls were also used to standardize and

optimize the reagents used in immunohistochemistry. Validity of data collection is also by assuming that the laboratory collected appropriate samples and run the tests accurately since this information was used to treat the patients.

3.5.2 Data collection process

Data collection was done from medical Oncology Department out-patient digital records. Relevant information was captured and entered into the data collection form. Medical oncology clinic has a password protected computer program where all the new patient information was recorded by the doctor who saw the patient. After the initial visit, patients were given return dates for their subsequent appointment. Subsequent follow up visits findings and outcomes are also recorded for every visit. The return dates are given at intervals of between 2 months to 6 months. During follow-up visit the consulting doctor asked to the patient specific questions about their illness such as presence of body pain, lesions in the breast, and their physical activity. This is followed by a complete physical examination of the whole body but focusing on the breast, the neck, axillary lymph node regions, and any other site that the patient has complained about. Any specific complaint or a positive finding by the doctor was followed by targeted laboratory or imaging investigations such as breast cancer tumor marker (C53.3) or imaging (plain X-rays, CT scan or ultrasound), or hematological tests (complete blood counts, liver function tests, or differential blood counts). The doctor then prescribes medicines needed such as endocrine therapy (tamoxifen, anastrazole, or goserelin) for eligible patients. The digital data is protected by password known only to the consulting doctor and subsequent follow up was recorded. Consecutive records of breast cancer patients which met the inclusion criteria were retrieved and analyzed. Age at presentation, menopausal status, histological type and pathological grade, disease stage, hormonal receptor status (estrogen and progesterone receptor status) and HER2 receptor status were collected. The type of treatment patient received such as surgical operation, chemotherapy, radiotherapy or hormonal therapy were retrieved. The last patient contact date that appeared in the clinical notes before 60 months was completed was used as a surrogate for survival time. Names of the patients and hospital numbers were not captured to ensure anonymity and protect their confidentiality.

3.5.2.1 Determination of breast cancer sub-types by immunohistochemistry

The determination of immunohistochemical breast cancer subtype are made by combining the results of estrogen receptor, progesterone receptor, and HER2 receptor (Vallejos, Gómez et al. 2010). This classification is defined as follows:

- Luminal A: ER+ and/or PR+, HER2-
- Luminal B: ER+ and/or PR+, HER2+
- HER2 like: ER-, PR-, HER2 +
- Triple negative (basal): ER-, PR-, HER2-

3.5.2.2 Determination of AJCC breast cancer staging

AJCC group staging is determined by combining the findings of the size of tumour size (T), and or presence of the regional lymph nodes (N), and presence or absence of distant metastasis (M) (Koh and Kim 2019). This classification system is defined in Table 3.1

Table 3.1 AJCC 7th Edition. Adapted from (Koh and Kim 2019

Stage	Tumor	Node	Metastasis
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0	N1mi	M0
	T1	N1mi	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0

IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any	N3	M0
IV	Any T	Any N	M1

3.6 ETHICAL CONSIDERATIONS

3.6.1 Ethical clearance and permissions

Ethical clearance was requested as this study involves humans. Ethics clearance was obtained from the Turfloop Research Ethics Committee (TREC) certificate number TREC/127/2021: PG (Annexure C).The TREC also waived the requirement for informed consent from each patient as the risk of harm was considered low. Permission to conduct the study was further obtained from the Chief Executive Officer of Pietersburg Hospital and the Head of Department of Health, Limpopo province to allow the study to be conducted in the hospital and the province respectively (see appendix).

The conduct of the study adhered to the Declaration of Helsinki code of conduct that serves to protect the rights of participants and ensure that they are not exposed to unnecessary harm, and also ensuring the methodological practices were appropriate to the study aim.

3.6.2 Anonymity

Anonymity of the patients in this study was ensured by using code numbers and not capturing the any identifying information such as patient names and hospital numbers. The use of study codes ensured that no patient could be linked to the variables used in this study and its findings

3.6.3 Confidentiality

Data collected was not revealed to anyone outside the research team. The patient files were handled by authorized people only and was kept under lock and key when not in

use by the research team. Electronic data was kept under protected password available only to the researcher and supervisor. Information captured was aggregated and no individual person was named or could be linked to any particular finding.

3.6.4 Minimizing harm

The study was conducted in such a way that no physical harm was done to any patient whose record was used. Harm due information being misused was reduced by allowing the files to be accessed only by authorized people and safe keeping of information.

3.7 DATA ANALYSIS

Data was entered in Microsoft Excel sheet and cleaned. It was then entered into SPSS software version 27 (created by SPSS, Inc. United States of Chicago IL) and analysed. Descriptive statistics such as mean, proportions and frequency was used to analyze the variables. Data is presented as graphs and charts.

Analysis of variants (ANOVA) is used to assess the association between breast cancer subtypes and age (continuous variables) the different variables. The Chi squared is used to determine the association between the clinicopathological features and breast cancer subtypes (discontinuous variables). A p value of 0.05 will be considered statistically significant

The overall survival refers to time from histological breast cancer diagnosis and death and/or last follow up (Beena et al 2020) Survival curves were generated using Microsoft Excel® application. Kaplan- Meier method was used to calculate the survival.

3.8 VALIDITY, RELIABILITY, AND BIAS

3.8.1 Validity

Validity refers to the degree to which a measurement represents the true value of a parameter (Botma et al., 2010). Internal validity refers to the extent to which the

observed effects can be attributed to the independent variable, whereas external validity is the extent to which the findings of the research can be generalized from the sample to the population(Frambach, van der Vleuten et al. 2013). Validity was ensured for the immunohistochemistry data as daily quality assurance measures were done by the laboratory personnel according to the company's (National Hospital Laboratory Service) protocols. External tissue controls were also used to standardize and optimize immunohistochemistry.

Validity of data collection was ensured as initial tissue samples were harvested by qualified surgeons for purpose of providing accurate medical care to breast cancer patients. It is taken that the laboratory staff extracted appropriate samples and run the tests accurately since this information was used to treat the patients. The immunohistochemical tests were performed on the breast cancer pathological tissue sample obtained from the initial biopsy sample and /or from subsequent lumpectomy/mastectomy specimen in patients deemed to be operable. Leica bond III machine was used for the immunohistochemical tests. Antibodies for ER, PR, and HER2 were also from Leica bond and were used according to the manufacturer's recommendations. Automation of the process guaranteed the uniformity of immunohistochemistry and avoided variations among the laboratory staff. The information was collected and analyzed as found without any alterations. The researcher who has experience in oncology practice and is familiar with the relevant information to include for accurate study findings captured data.

3.8.2 Reliability

Reliability refers to the extent to which the results would be consistent if the study is repeated (Frambach *et al.*, 2013). Reliability was ensured in this study by explaining every step in conducting the study. A questionnaire that captured standard information was used so the same information would be captured each time if the study were to be repeated.

3.8.3 Bias

Bias is any influence that produces a distortion or misrepresentation of an outcome of

a particular finding of a study. (Botma, Greeff et al. 2010) Selection bias was minimized by including all female breast cancer patients treated at Pietersburg hospital within the study period. Information bias might occur as the data was not initially meant for this study and some details might not be available or vague. Seventy-six (76) patients were excluded from the final analysis because critical information that could have been used to determine the breast cancer immunohistochemical sub-types were missing. This may have introduced some information bias to some extent.

CHAPTER FOUR

4. RESULTS

4.1 INTRODUCTION

This chapter will present the results, and discuss the findings of this study with relevance to its objectives. The presented results are from a retrospective review of medical records of 329 women diagnosed with breast cancer who were referred to Pietersburg Hospital for the treatment of their cancer. Seventy six(76) patients were excluded from the final analysis because critical information that could have been used to determine breast cancer immunohistochemical types was missing.This reduced the sample size.Characteristics of the population is presented in Table 4.1.

4.2 DATA MANAGEMENT AND ANALYSIS

Data was entered in Microsoft Excel sheet and cleaned. It was then entered into SPSS software version 27 (created by SPSS, Inc. United States of Chicago IL) and analyzed. Descriptive statistics such as mean, proportions and frequency are used to analyze the variables. Data is presented as graphs and charts. Analysis of variants (ANOVA) is used to assess the association between the different variables.TM a p value of 0.05 is considered statistically significant.

4.3 RESEARCH RESULTS

4.3.1 Characteristics of the population

4.3.1.1 Age

The mean age of the population is 55.3 ± 14.2 standard deviation (SD) with a range of 26 to 96 years. When distribution according to age groups is considered, 12.% are younger than 40 years, 24.6% are 40 – 49 years, 28.6% are 50 - 59, 15.8% are 60 – 69, and 18.2% are 70 years or older. The difference between the age groups is not statistically significant (p = 0.991). One hundred and twenty three (37.4%) patients are younger than 50 years and 146 (44.4%) are aged between 60 – 69 years. The distribution of the population by age groups is shown in Figure 4.1.

Figure 4.1 Distribution according to age groups

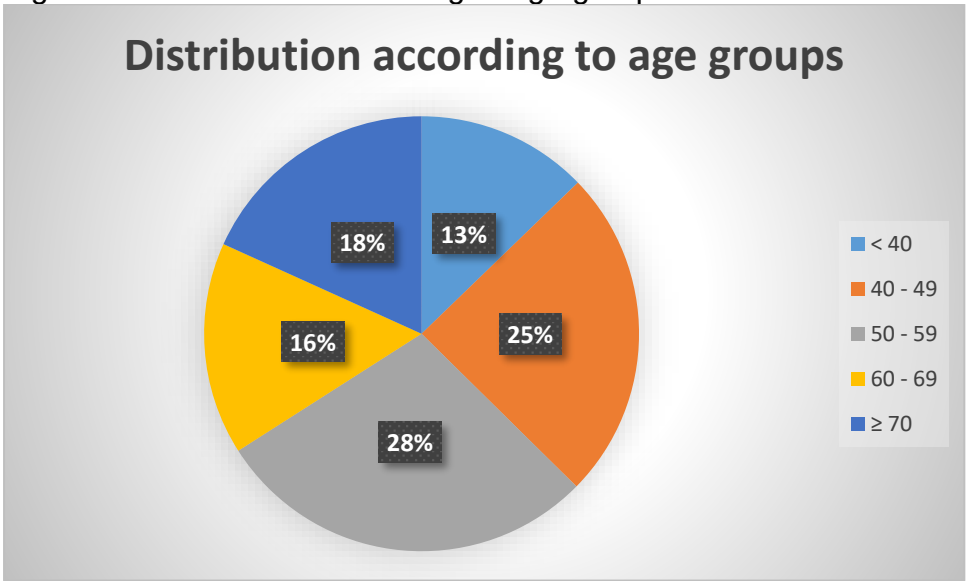


Table 4.1 Patient characteristics

Characteristic	Total (n=329)	TNBC n=69(21.0 %)	HER2/neu n=29(8.8%)	Luminal A n=136(41.3%)	Luminal B n=20(6.1%)	Unknown n=75(22.8%)	*P* value
Age at diagnosis							
Mean ± SD	55.3 ±14.2	54.4 ±13.5	54.2 ±13.0	55.6 ±15.4	52.3 ±14.9	56.1±12.8	0.755
Age by group							
< 40	42 (12.8%)	7 (10.1%)	5 (17.2%)	19 (14.0%)	5 (25.0%)	6 (8.0%)	
40 - 49	81 (24.6%)	21 (30.4%)	4 (13.8%)	33 (24.3%)	4 (20.0%)	19 (25.3%)	
50 - 59	94 (28.6%)	23 (33.3%)	9 (31.0%)	31 (22.8%)	5 (25.0%)	26 (34.7%)	0.991
60 - 69	52 (15.8%)	5 (7.2%)	7 (24.1%)	26 (19.1%)	4 (20.0%)	10 (13.3%)	
± 70	60 (18.2%)	13 (18.8%)	4 (13.8%)	27 (19.9%)	2 (10.0%)	14 (18.7%)	
Age by group							
< 50	123 (37.4%)	28 (40.6%)	9 (31.0%)	52 (38.2%)	9 (45.0%)	25 (33.3%)	
50 - 69	146 (44.4%)	28 (40.6%)	16 (55.2%)	57 (41.9%)	9 (45.0%)	36 (48.0%)	0.783
± 70	60 (18.2%)	13 (18.8%)	4 (13.8%)	27 (19.9%)	2 (10.0%)	14 (18.7%)	
Menopausal Status							
Premenopausal	179 (54.4%)	40 (58.0%)	16 (55.2%)	74 (54.4%)	12 (60.0%)	37 (49.3%)	
Postmenopausal	149 (45.3%)	29 (42.0%)	13 (44.8%)	62 (45.6%)	8 (40.0%)	37 (49.3%)	0.578
Undetermined	1 (0.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.3%)	
Tumor stage T							
T1	27 (8.2%)	6 (8.7%)	3 (10.3%)	9 (6.6%)	0 (0%))	9 (12.0%)	
T2	15 (4.6%)	3 (4.3%)	2 (6.9%)	7 (5.1%)	0 (0%)	3 (4.0%)	
T3	55 (16.7%)	13 (18.8%)	3 (10.3%)	24 (17.6%)	6 (30.0%)	9 (12.0%)	0.647
T4	64 (19.5%)	14 (20.3%)	9 (31.0%)	27 (19.9%)	2 (10.0%)	12 (16.0%)	
Tx	168 (51.1%)	33 (47.8%)	12 (14.4)	69 (50.7%)	12 (50%)	42 (56.0%)	

Estrogen receptor							
Positive	166 (50.6%)	0 (%)	0 (0%)	135 (99.3%)	20 (100%)	11 (14.9%)	0.000
Negative	103 (31.4%)	69 (100%)	29 (100%)	1 (0.7%)	0 (0%)	4 (5.4%)	
Unknown	59 (18.0)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	59 (79.7%)	
Progesterone receptor							
Positive	99 (30.1%)	1 (1.4%)	1 (3.4)	78 (57.4%)	12 (60%)	7 (9.3%)	0.000
Negative	166 (50.5%)	68 (98.6%)	27 (93.1)	58 (42.6%)	8 (40%)	5 (6.7%)	
Unknown	64 (19.5%)	0 (0%)	1 (3.4)	0 (0%)	0 (0%)	63 (84%)	
HER2							
Positive	47 (14.3)	0 (0%)	28 (96%)	0 (0%)	19 (95%)	0 (0%)	0.000
Negative	205 (62.3%)	69 (100%)	1 (3.4%)	135 (99.3%)	0 (0%)	0 (0%)	
Unknown	77 (23.4%)	0 (0%)	0 (0%)	1 (0.7%)	1 (5%)	75 (100%)	

4.3.1.2 World Health Organization histology types

The distribution according to WHO histological type among the 329 patients is shown in Table 4.2. The most common histological type is infiltrating ductal carcinoma (NOS) with 81.8%, followed by mucinous (colloid) carcinoma with 4.3%, and medullary carcinoma with 3%. Histological type is unknown in 12 cases (3.6%).

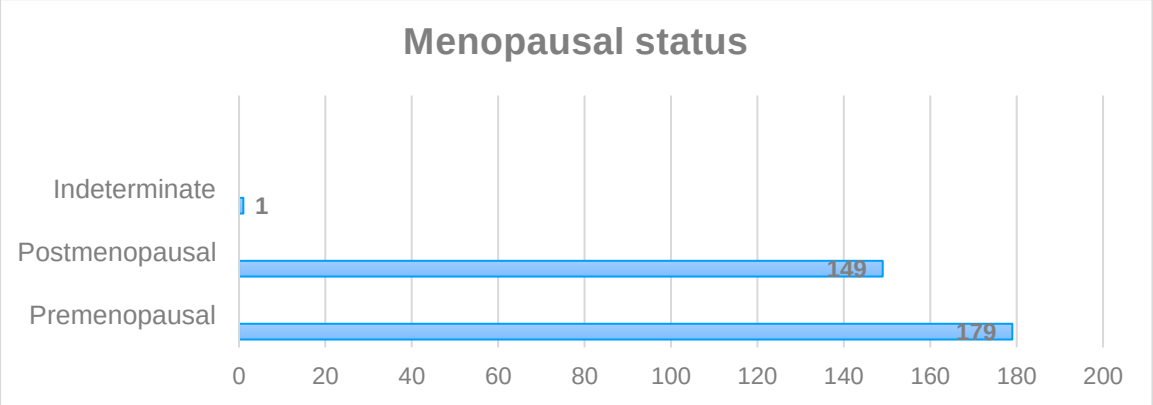
Table 4.2 Distribution according to WHO histological classification

HISTOLOGICAL CLASSIFICATION	NUMBER OF PATIENTS
Infiltrating ductal carcinoma (NOS)	269 (81.8%)
Invasive papillary carcinoma (NOS)	9 (2.7%)
Medullary carcinoma	10 (3.0%)
Invasive lobular carcinoma (NOS)	5 (1.5%)
Mixed ductal & lobular carcinoma	1 (0.3%)
Ductal carcinoma in situ (DCIS)	2 (0.6%)
Mucinous (colloid) carcinoma	14 (4.3%)
Others	5 (1.5%)
Unknown	12 (3.6%)
TOTAL	**329**

4.3.1.3 Menopausal status

With regard to menopausal status, 179 women are premenopausal (≤ 55 years) and 149 (45.3%) who are older than 55 years are classified as postmenopausal (Figure 4. 2). The difference between these two groups is not significant (p = 0.578).

Figure 4.2 Menopausal status



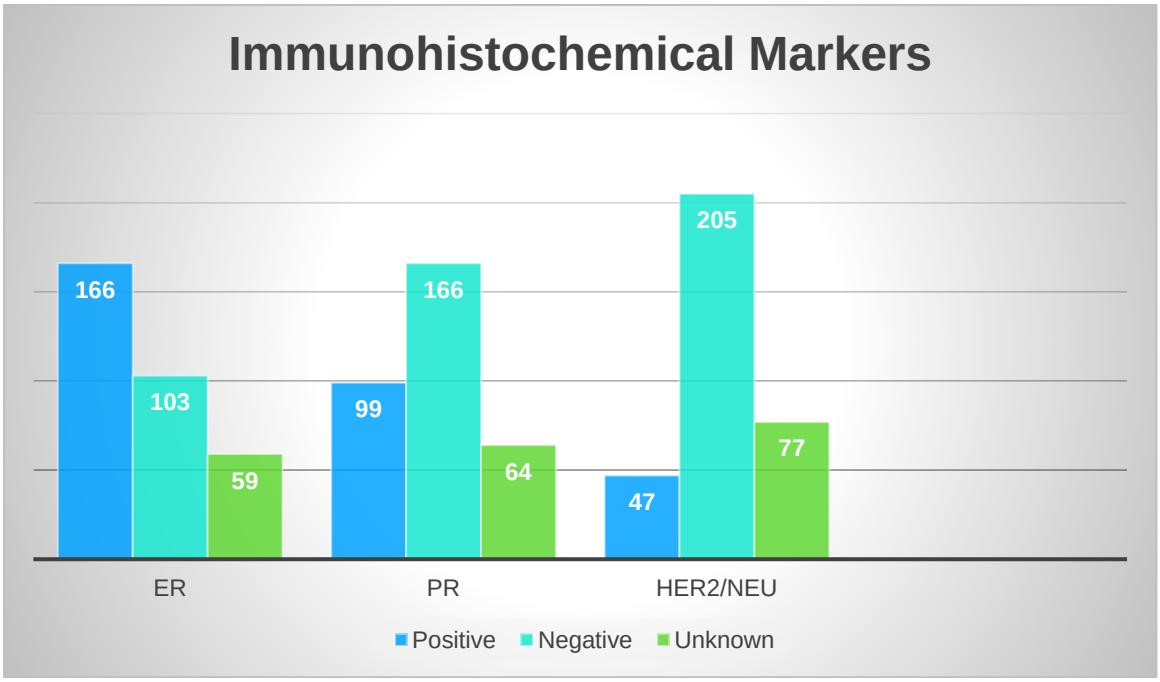
4.3.1.4 Immunohistochemistry markers

Oestrogen receptor is positive in 166 women (50.6%) , negative in 103 (31.4%), and unknown in 59 women (18%). Progesterone receptor is known in 265 women of which 99 (30.1%) and 166 (50.5%) are positive and negative respectively. Progesterone receptor result is unknown in 19.5% of the women. HER2 is positive in 47 women (14.3%), negative in 205 (62.3%), and unknown in 77 women (23.4%). The results are presented in Figure 4.3.

4.3.2 Immunohistochemical subtypes of breast cancer

Of the 329 population, breast cancer sub-types by immunohistochemistry is determined in 254 women (Table 4.3). The most common sub-type is luminal A with 136 (53.5%), followed by triple negative breast cancer type with 69 (27.2%), HER2/neu sub-type with 29 (11.4%), and luminal B with 20 (7.9%). The distribution of the patients according to the breast cancer sub-type is illustrated in Figure 4.4

Figure 4.3 Immunohistochemical markers



ER Oestrogen Receptor; PR Progesterone receptor: HER 2 Human epidermal growth factor receptor 2

Figure 4.4 Breast cancer sub-types

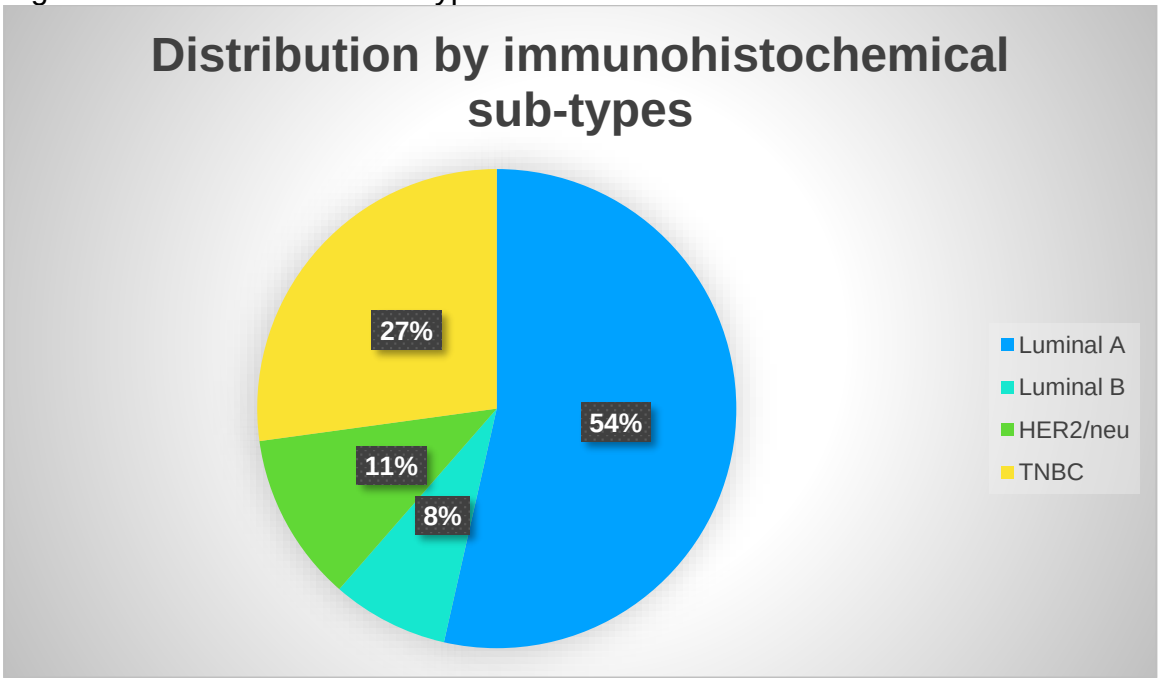


Table 4.3 Clinicopathological characteristics of immunohistochemical subtypes of breast cancer

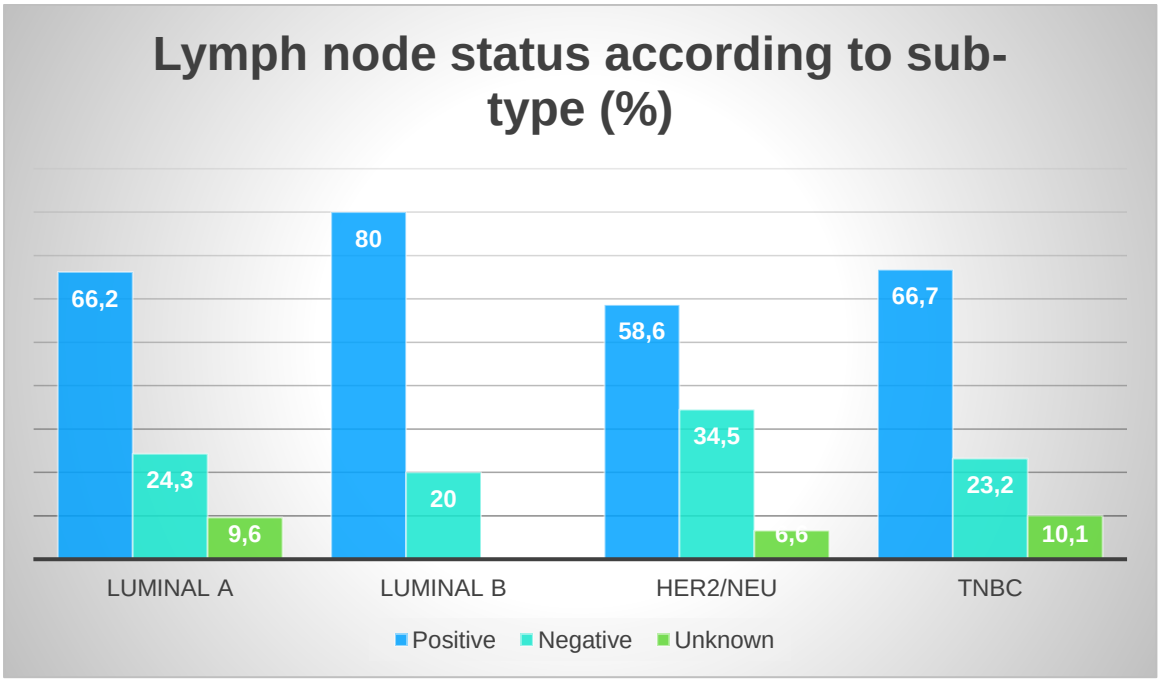
Characteristic	**Total (n=254)**	**Basal cell n=69(%)**	**HER2/neu n=29 (%)**	**Luminal A n=136 (%)**	**Luminal B n=20 (%)**	**P value**
Lymph node status						
Positive (N1-N3)	169 (66.5%)	46 (66.7%)	17 (58.6)	90 (66.2%)	16 (80.0%)	0.886
Negative (N0)	63 (24.8%)	16 (23.2%)	10 (34.5%)	33 (24.3%)	4 (20.0%)	
Not determined	22 (8.7%)	7 (10.1%)	2 (6.6%)	13 (9.6%)	0 (0%)	
Distant metastasis at diagnosis						
No metastasis (M0)	170 (66.9%)	47 (68.1%)	18 (62.1%)	92 (67.6%)	13 (65%)	0.940
Metastasis (M1)	84 (33.1%)	22 (31.9%)	11 (37.9%)	44 (32.4%)	7 (35%)	
AJCC stage						
Stage I	7 (2.8%)	1 (1.4%)	1 (3.4%)	5 (3.7%)	0 (0%)	0.578
Stage II	38 (15.0%)	13 (18.8%)	4 (13.8%)	18 (13.2%)	3 (15%)	
Stage III	119 (46.9)	31 (44.9%)	12 (41.8%)	65 (47.8%)	11 (55%)	
Stage IV	85(33.5%)	22 (31.9%)	11 (37.9%)	46 (33.8%)	6 (30%)	
Unknown	5 (2.0%)	2 (2.9%)	1 (3.4%)	2 (1.5%)	0 (0%)	
Histologic grade						
Grade 1-2	109 (42.9%)	25 (36.2%)	13 (44.8%)	60 (44.1%)	11 (55.0)	0.587
Grade 3	111 (43.7%)	41 (59.4%)	11 (37.9%)	51 (37.5%)	8 (40.0%)	
Unknown	34 (13.4)	3 (4.3%)	5 (17.2%)	25 (18.4%)	1 (5.0%)	
Surgery						
Yes	143 (56.3%)	36 (52.2%)	19 (65.5%)	75 (55.1%)	13 (65.5%)	0.997
No	107(42.1%)	32 (46.40%)	10 (34.5)	58 (42.6%)	7 (34.5%)	
Unknown	4 (1.6%)	1 (1.4%)	0 (0%)	3 (2.2%)	0 (0%)	
Chemotherapy						

Yes	215 (84.6%)	57 (82.6%)	25 (86.2%)	114 (83.8%)	19 (95.0%)	0.870
No	39 (15.4%)	12 (17.4%)	4 (13.8%)	22 (16.2%)	1 (5%)	
Adjuvant hormone therapy						
Yes	124 (48.8%)	6 (8.7%)	5 (17.2%)	98 (72.1%)	15 (75%)	0.000
No	130 (51.2%)	63 (91.3%)	24 (82.8%)	38 (27.9%)	5 (25.0%)	
Radiation therapy						
Yes	110(43.3%)	26 (37.7%)	13 (44.8%)	63 (46.3%)	8 (40.0%)	0.183
No	139 (54.7%)	41 (59.4%)	15 (51.7%)	72 (52.9%)	11 (55.0%)	
Unknown	5 (2.0%)	2 (2.9%)	1 (3.4%)	1 (0.7%)	1 (5.0%)	
Survival						
0 – 12 months	79(31.1%)	27 (39.1%)	7 (24.1%)	39 (28.7%)	6 (30.0%)	0.104
13 – 24 months	55 (21.7%)	18 (26.1%)	8 (27.6%)	25 (18.4%)	4 (20.0%)	
25 - 36 months	31 (12.2%)	6 (8.7%)	3 (10.3%)	19 (14.0%)	3 (15.0%)	
37 – 48 months	15 (5.9%)	1 (1.4%)	2 (6.9%)	11 (8.1%)	1 (5.0)	
49 – 60 months	9 (3.5%)	1 (1.4%)	2 (6.9 %(	5 (3.7%)	1 (5.0%)	
61+ months	65 (25.6%)	16 (23.2%)	7 (24.1)	37 (27.2%)	5 (25%)	

4.3.2.1 Immunohistochemical sub-type and lymph-node status

Lymph-node positivity is most common in luminal B (80.0%), followed by TNBC (66.7%), luminal A (66.2%), and HER2/neu (58.6%), respectively (Figure 4.5). Luminal B has the lowest rate of lymph node negative breast cancer (20.0%) whereas TNBC (23.2%) has a slightly higher rate. However, these re not statistically significant (p = 0.886). Lymph node status could not be determined in 22 of the 254 records.

Figure 4.5 Lymph node status by molecular subtypes



4.3.2.2 Molecular subtypes and distant metastasis

The presence of distant metastasis at diagnosis in all the breast cancer sub-types is 32.4%, 35%, 37% and 31.9% of cases in luminal A, luminal B, HER2/neu and TNBC respectively. The difference is not statistically significant (p=0.94). The diagnosis of lymph node metatstasis is depicted in Figure 4.6.

4.3.2.3 Molecular subtypes and clinical stage

The percentage distribution of the cancer stage according to American Joint Committee on Cancer (AJCC) stages is shown in Figure 4.7. The distribution according to stage is not statistically significant (p= 0.578).

Figure 4.6 Distant metastasis according to sub-types

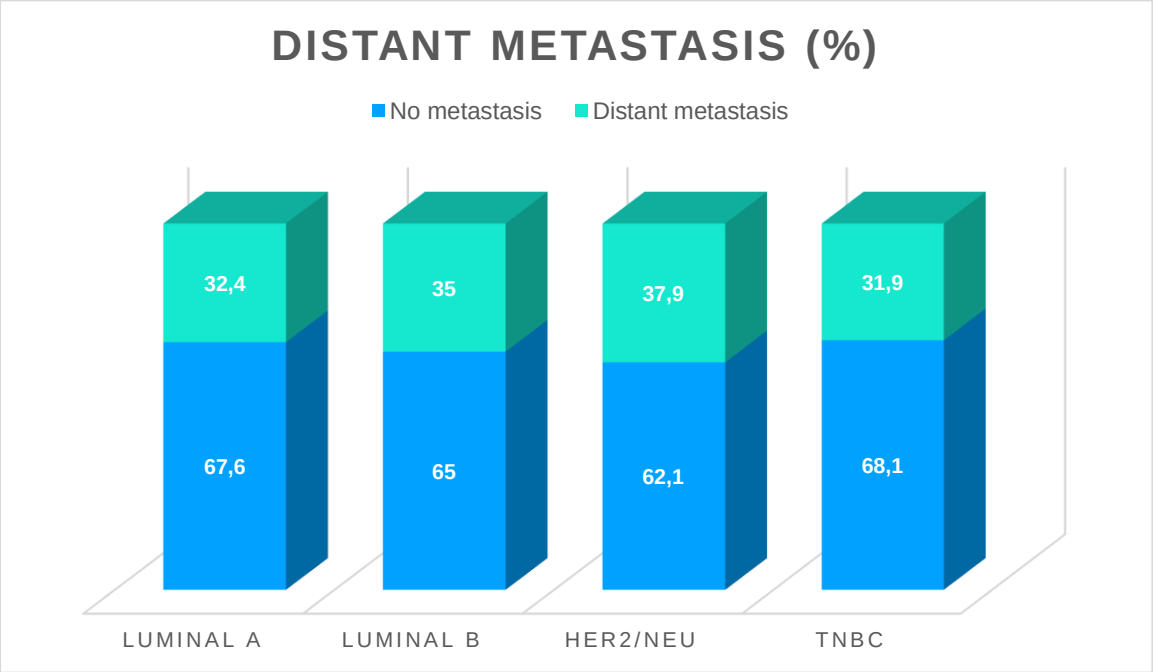
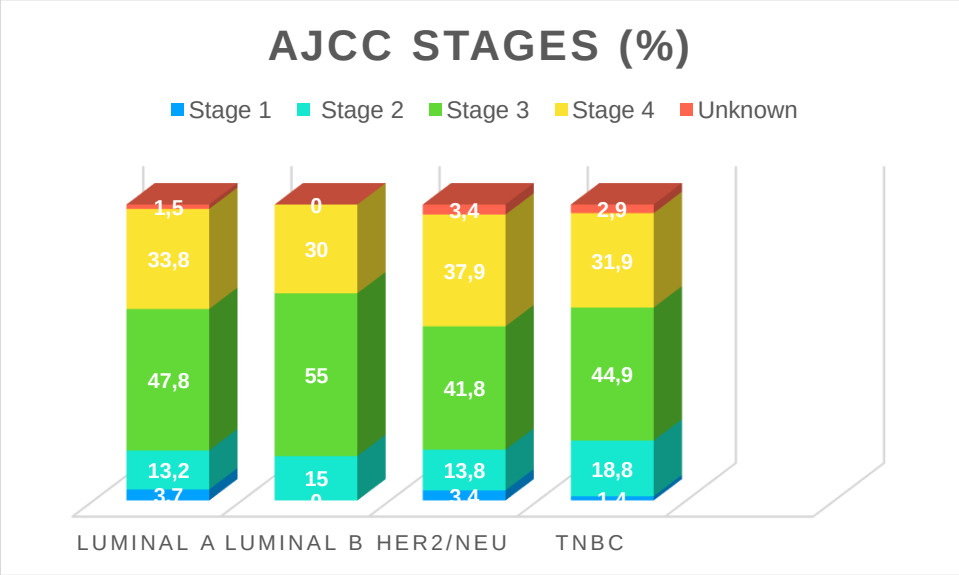


Figure 4.7 Distribution of the breast cancer sub-types according to stage



4.3.2.4 Molecular sub-type and WHO histological type

The predominant WHO histological type among the breast cancer sub-types is infiltrating ductal carcinoma (NOS). Eighty three percent of Luminal A are IDC (NOS), 90% of luminal B, 79.3% of HER2/neu, and 87% of TNBC. Other WHO histological types are also distributed among the IHC sub-types (Table 4.4)

Table 4.4 Comparison of WHO histological types and cancer sub-types

WHO HISTOLOGICAL TYPE	**IHC SUB-TYPE**			
	Luminal A	**Luminal B**	**HER2/neu**	**TNBC**
Infiltrating ductal carcinima (NOS)	113 (83.1%)	18 (90%)	23 (79.3%)	60 (87%)
Invasive papillary carcinoma (NOS)	5 (3.7%)	1 (5%)	1 (3.4%)	1 (1.4%)
Medullary carcinoma	3 (2.2%)	-	-	4 (5.8%)
Invasive lobular carcinoma (NOS)	1 (0.7%)	-	2 (6.9%)	-
Mixed ductal and lobular	-	-	1 (3.4%)	-
Mucinous (colloid) carcinoma	8 (5.9%)	-	-	3 (4.4%)
Ductal carcinoma in situ (DCIS)	1 (0.7%)	-	-	1 (1.4%)
Others	3 (2.2%)	-	1 (3.4%)	-
Unknown	2 (1.5%)	1 (5%)	1 (3.4%)	-
TOTAL	**136**	**20**	**29**	**69**

4.3.2.5 Immunohistochemical sub-types and tumour grade

Low to moderate grade (grade 1 and 2) make up 44.1% of luminal A tumours, 55% of luminal B, 44.8% of HER2/neu, and 36.2% of TNBC. High grade (grade 3) make up 59.4% of TNBC, 40% of luminal B, 37.9% of HER2/neu, and 37.5% of luminal A tumours (Figure 4.8)

4.3.3 Treatment modalities

Of the 254 patients in whom breast IHC sub-type is determined, 56.3% were operated, 84.6% received chemotherapy, 48.8% received adjuvant endocrine therapy, and 43.3% received radiotherapy in different combinations (Table 3). There is a significant difference only among the patients who received adjuvant hormonal therapy (p=0.000).

4.3.4 Survival

Survival data is shown in Table 3. The end-point for survival time considered is the number of months from the time the patient was diagnosed (according to the date on the histology report) until : the last recorded visit before 60 months; patient died before 60 months of follow-up; or patient was alive by the time 60 months from diagnosis at which point the study was stopped. Five-year overall survival is 27.2%, 25%, 24.1% and 23.2% for luminal A, luminal B, HER2/neu, and TNBC respectively. The 5-year overall survival for the whole population is 25.6%. Median survival is 28 months, 25 months, 24 months, and 16 months for luminal A, luminal B, HER2/neu, and TNBC respectively (Figure 4.9).

Figure 4.8 Distribution of molecular sub-types according to tumour grade

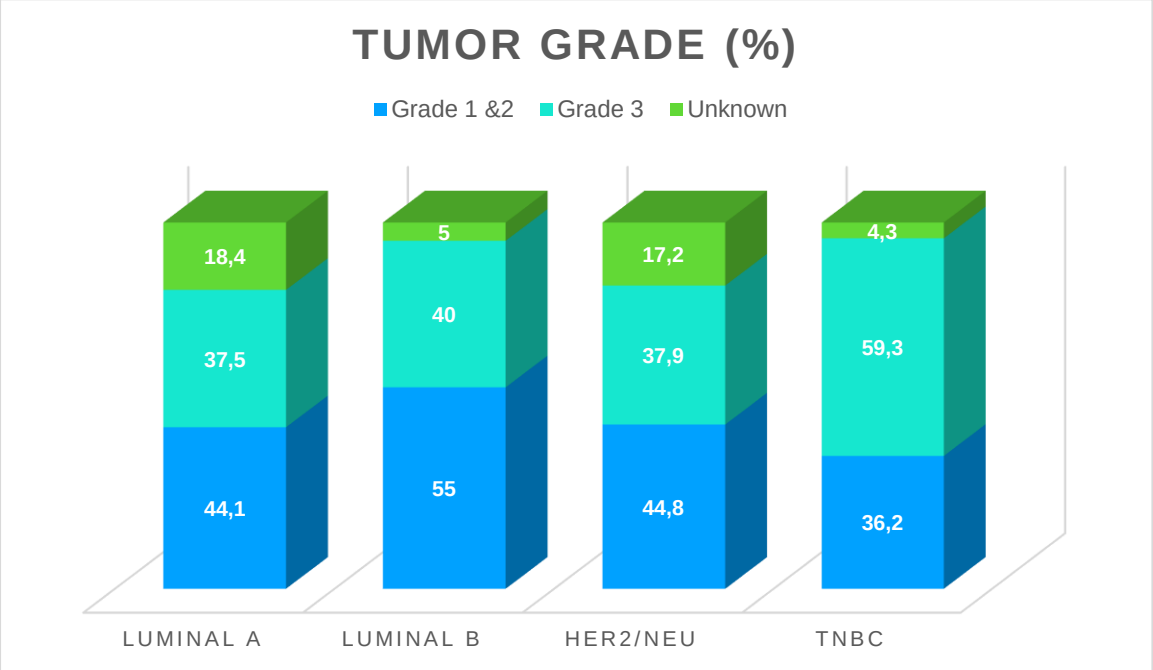
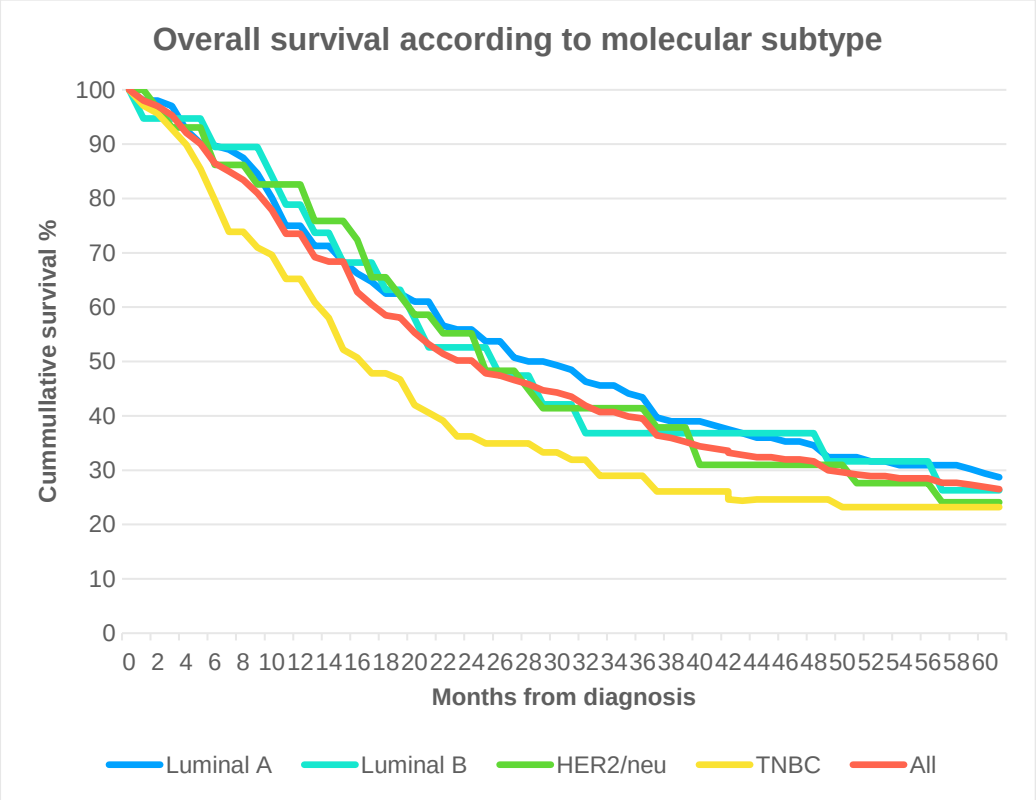


Figure 4.9 Overall survival according to molecular sub-types



4.4 CONCLUSION

The findings of the study is presented in this chapter. The findings are interpreted and compared with others from similar studies conducted both locally and internationally in the next chapter.

CHAPTER FIVE

5. DISCUSSION

5.1 INTRODUCTION

Breast cancer is the most frequent neoplasm among females in South Africa accounting for 13.1% of all cancers diagnosed in both males and females (World Health Organization, 2020). Breast cancer is not a single disease entity, but a heterogeneous disease consisting of several histological and molecular subtypes in terms of genomics, clinical presentation, risk factors and response to treatment (Hammerl, Smid, Timmermans, Sleijfer, Martens, & Debets, 2018; Bosman, 2019). The cellular composition of breast tumour is the main determinant of both the biological and clinical features of an individual disease. Breast cancer can now be characterized into five different subtypes with significant clinical implications (Sørlie, 2004). Perou et al (2000) were the first to identify this phenotypic diversity by analyzing the genetic profile of 42 breast tumours using complement DNA (cDNA) microarrays and grouping the genes into similar quantitative patterns of expression. Since then gene expression has gained wide use in oncology particularly in breast cancer where several commercially available genetic signatures have been developed for decision-making and prognostication in the clinic (Reis-Filho, & Pusztai, 2011; Güler, 2017). However, these innovative developments are expensive and currently not readily available in most public hospitals in a low to middle income country such as South Africa (Grant, Myburgh, Murray, Pienaar, Kidd, Wright, & Kotze, 2019). For this reason, immunohistochemical markers are used as surrogates to identify molecular subtypes (Vallejos et al, 2010; Kakudji, et al 2021).

In the era of personalized cancer treatment, molecular stratification will improve selection of therapy and avoid unnecessary addition of potentially toxic medications. For instance, in Luminal A breast cancer subtype, hormonal therapy alone is sufficient and chemotherapy can be omitted in some cases as these patients acquire less benefit. A number of Luminal A breast cancer patients can be saved the added toxicity of adjuvant chemotherapy. Similarly, patients whose tumours overexpress HER2 benefit from antiHER2 therapy such as trastuzumab in addition to chemotherapy.

The aim of this study is to re-classify the breast cancer patients who were treated at Pietersburg Hospital medical oncology clinic in a particular year according to the molecular sub-types using the laboratory results of their immunohistochemical markers. The sub-type groupings are further compared to the clinicopathological features, treatment approach and long-term survival over a period of 5 years.

5.2 OVERVIEW OF THE RESEARCH FINDINGS

5.2.1 Characteristics of the population.

The median age of the population in the present study is 55 years ±14.2 standard deviation, with a range of 26 to 96 years. This is similar to the findings of another study conducted among breast cancer patients attending a large referral hospital in South Africa. Out of the 602 women newly diagnosed with invasive breast cancer during the period of 2009 - 2011 at Chris Hani Baragwanath Hospital, Johannesburg, the mean age is reported to be 54.4 years ± 14.2 standard deviation (Cubasch, Dickens, Joffe, Duarte, Murugan, Chih, Moodley, Sharma, Ayeni, Jacobson, Neugut, 2018).

However, the median age at diagnosis of breast cancer is variable across different African countries and among different population groups. The African Breast Cancer Disparities in Outcomes (ABC-DO) prospective cohort study which included eight hospitals across five sub-Saharan African countries observes that the mean age at diagnosis ranges from 45 years in women from a Nigerian private hospital to 59 years in white Namibian women (McCormack, McKenzie, Foerster, Zietsman, Galukande, Adisa, Anele, Parham, Pinder, Cubasch, and Joffe, 2020). Most of the patients in this study (54.7%, n=179) are in the premenopausal group while only 149 (45.3%) are post-menopausal.

5.2.2 World Health Organization histological types

The most common histological type in the present study is invasive ductal carcinoma (81.8%, n=269). Similar pattern of WHO histological type distribution is reported among breast cancer patients in a Soweto and Potchefstroom studies in South Africa

in which ductal carcinoma is the predominant type, accounting for 80% and 96. 6% respectively (Mc Cormack et al, 2013; Kakudji, Mwila, Burger, du Plessis, and Naidu, 2021). Ductal carcinoma is followed by mucinous carcinoma, (4, 3%), medullary carcinoma (3%), invasive papillary (2.7%) and invasive lobular carcinoma (1.5%).

5.2.3 Breast cancer immunohistochemistry receptor status

The oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER) positivity in the present study is 50.6% , 30% , and 14.3% (p=0.000) respectively. McCormack et al (2013) reported a positivity rate of 64.7%, 52.8% and 26% for ER, PR and HER2, respectively. This is similar to the findings by Kakudji et al (2021) who report a receptor positivity rate of 71.6%, 64.7% and 24.1% for ER, PR, and HER 2, respectively.

Marked regional heterogeneity in the ER, PR and HER2 positivity rates between studies is reported in a large literature review of studies published between January 1980 and April 2014 involving 80 studies from North Africa and sub-Saharan Africa (Eng et al. 2014). The proportion of ER+ disease in this review is 10% (4%–17%) lower for studies based on archived tumor blocks compared to freshly collected specimen. However, this review has limitations as there were no standardized procedures across the different studies for collection, fixation, and receptor testing; and poor methodology in some of the studies.

5.2.4 Breast cancer molecular subtypes

In this study conducted at Pietersburg Hospital, luminal A is the most frequent molecular subtype (54%) among women with breast cancer. The second most frequent molecular sub-type is triple negative breast cancer (TNBC) at 27%, followed by HER 2/neu (11%), and lastly, luminal B (8%). Luminal A has a peak incidence in relatively younger patients between the ages 40-49 years, whereas luminal B, TNBC, and HER2/neu all have peak incidence at 50 – 59 years. The mean age at diagnosis for each of the molecular sub-types are 55.6±15.4 SD, 54.4±13.5 SD, 54.2±13.0 SD, and 52.3±14.9 SD for luminal A, TNBC, HER 2/neu, and luminal B patients, respectively. However, the difference is not statistically significant (p = 0.755). In

contrast to the findings of the current study, Kakudji et al (2021) reported that 29.3% of their patients have luminal A breast cancer, followed by 24.1% with luminal B HER2-negative breast cancer, 22.4% with triple-negative breast cancer, and 18.1% with luminal B HER2- positive breast cancer, while 6% have HER2/neu breast cancer. The main difference between this study and the Pietersburg Hospital one is that they factored in the values of the proliferation index Ki-67 in determining the molecular sub-types. However, the findings of the present study are consistent with those from the Soweto study that report 53.7%, 14.6%, 20.4%, and 11% of their patients with luminal A, luminal B, TNBC, and HER2/neu breast cancer, respectively (McCormack et al. 2013). The finding in the Pietersburg series is also consistent with Peruvian study which looks at medical records of 1524 breast cancer patients treated between January 2000 and December 2002 (Vallejos et al. 2010). One factor that may have contributed to disparity in the prevalence of molecular sub-types with the Potchefstroom study but similar finding with the Peru study is that the molecular sub-types in the present study are defined using a combination of ER, PR and HER2 results without considering the Ki-67 factor, similar to the procedure in the Peruvian study.

A comparative study looks at the prevalence of molecular sub-types of breast cancer between women diagnosed in Sudan and those in Germany. These researchers found that there is less occurrence of luminal A in the Sudanese and the Germans (36.9% versus 68.4%), nearly the same proportion of luminal B (13% versus 10.7%), twice the proportion of HER 2/neu (15.7% versus 6.8%), and twice the proportion of TNBC at 34.5% versus 14.2% in German women (Sengal, Haj, Mukhtar, Vetter, Elhaj, Bedri, Hauptmann, Thomssen, Mohamedani, Wickenhauser, and Kantelhardt 2017). They postulate that the disparity in the proportion of molecular subtypes between Sudanese and German women could be a result of both environmental and inherent biologic factors, which could modulate immune and tumour microenvironment. Some of these factors include exposure to insecticides and agricultural pesticides, and viral and parasitic infections, which could possibly induce Sudanese women to develop disease that is more aggressive.

5.2.4.1 Molecular subtypes and menopausal status

Although there is no significant (p=0.578) association between menopausal status and molecular subtype, a high percentage of premenopausal patients are found with luminal B and triple negative (58%) subtype. Lack of association of molecular subtype and menopausal status has also been reported elsewhere (Ihemelandu, Leffall Dewitty, Naab, Mezghebe, Makambi, Adams-Campbell, and Frederick, 2007).

5.2.4.2 Molecular sub-type and lymph-node status

Lymph node involvement indicates tumour infiltration beyond the primary disease and determines treatment outcome.The findings in this study reveal predominantly high rates of lymph node involvement across all the breast cancer subtypes. Luminal B, TNBC, luminal A, and HER2/neu subtypes have lymph node positivity rate (N1 – N3) of 80%, 66.7%, 66.2%, and 58.6%, respectively. However, the differences between the sub-types did not reach statistical significance (p = 0.886). This is consistent with a cross sectional study done in Potchefstroom where there is no association between molecular subtype and node involvement (p=0.362) with a preponderance of positive axillary lymph node irrespective of molecular sub-type (Kakudji et al., 2021). A large European-based study involving 1339 women with invasive breast cancer also report no association (p=0.886) between the molecular subtypes and involvement of axillary lymphnode (Spitale et al., 2009). However, Spitale et al (2009) observed the highest percentage of negative lymph node cases in TNBC (57.5%) and luminal A (62.2%) tumors in contrast to patients with the Her2/neu subtype who have the highest prevalence of positive lymph nodes (49.2%). Contrary to these findings, a hospital based study done in Peru report a highly significant association (p=0.001) between molecular subtypes and axillary node status(Vallejos et al, 2010).

5.2.4.3 Molecular subtypes and clinical stage

In this study , no association is found between the molecular subtype and clinical stage (p=0.578). Nevertheless, some researchers have found significant association between molecular subtypes and cancer stage. Spitale et al (2009) reports a significant association between molecular subtype and stage . They compared mean

tumour diameter at diagnosis and found significant difference among the different sub-types (P < 0.0001). TNBC and HER 2/neu had a larger tumour diameter (T-stage in TNM) than both luminal A and luminal B subtypes (Spitale et al. 2009). Vallejos et al. (2010) in Peru also found significant association of the tumor size according to AJCC clinical classification with molecular subtype. In their series, a high percentage of stage T3 tumours occurred in HER2/neu subtype (54.4%), and most of T4 tumours (38.6%) was found in TNBC subtype.

5.2.4.4 Molecular subtypes and distant metastasis

A number of studies have found no association between molecular subtypes and distant metastasis (Spitale et al. 2009; Ekpe, Shaikh, Shah, Jacobson, and Sayed, 2019; Kakudji et al. 2021). This is consistent with the findings of the present study, despite lack of statistical significance (p = 0.940). The rate of distant metastasis is similar among the 4 subtypes being 37.9%, 35%, 32.4%, and 31.9% in HER2/neu, luminal B, luminal A, and TNBC, respectively. In contrast, the study by Vallejos et al. (2010) reports a significant association between distant metastases and molecular subtypes (P = .014), with a greater prevalence of HER2/neu type tumours.

5.2.4.5 Molecular sub-types and tumour grade

Histological grade and molecular subtypes are both independent markers that determine the patients' outcome. The association between tumour grade and subtype is first reported in a systematic review and meta-analysis of publications reporting on the frequency of breast cancer receptor-defined subtypes in indigenous population in Africa (Eng et al. 2014). The literature review found the presence of TNBC subtype was often associated with high grade, reflecting loss of estrogen expression in more advanced form of the disease. In the current study, the molecular subtype predominantly associated with high grade tumour is TNBC. Out of 69 patients, 41 (59.4%) had grade 3 tumours. Luminal A subtype has the least percentage of grade 3 tumours. However, the difference is not statistically significant (p=0.587). This is not consistent with most studies that have consistently shown significant association between different molecular subtypes and histological grade (Spitale et al.2009; Vallejos et al.2010; Kakudji et al.2021). Spitale et al. (2009) in a European study observe significant differences among molecular subtypes in which TNBC and

HER2/neu cases showed the highest prevalence of poorly differentiated phenotype (75.9% and 66.7%, respectively), whereas luminal A tumors are more frequently well/moderately differentiated (84.6%).

In the present study, low to moderate grade make up 44% of luminal A , 55% of luminal B, 44.8 % of HER2/neu and 36.2% of TNBC. High grade make up 59.4% of TNBC, 40% of luminal B, 37.9% of HER2, and 37.5% of luminal A. The association of high grade with triple negative is in keeping with data from a systematic review and meta-analysis of indigenous population in Africa that reported presence of triple negative subtype with high grade reflecting loss of estrogen expression in more advanced form of the disease (Eng, Mc Cormack and dos-Santos-Silva, 2014).The association between molecular subtype and histological grade is also shown in a study done in Potchefstroom (Kakudji et al., 2021). Vallejos et al.(2010) in a South American study also report that histologic grade is significantly associated with immunohistochemical subtypes (P < .0001) with well- or moderately differentiated tumors (grade 1 and 2) appearing most frequently in the luminal A subtype (76.6%), while a greater percentage (70.3%) of poorly differentiated tumors (grade 3) occur in TNBC subtype. In South Africa, Kakudji et al. (2021) found a statistically significant association where both luminal ($p<0.001$) and non-luminal molecular subtypes ($p<0.001$) are significantly associated with tumour grade 2 and 3.

5.2.4.6 Surgical treatment

In this study the surgical rate was 56.3 %.This surgical rate is similar to data from the retrospective review of surgical management of breast cancer in New Zealand (Lee & Vallance, 2006).This is in contrast to a population based study that reports a surgical range of 74% in Canada (Fisher, Gao, Yasui, Dabbs, & Winget, 2015).Unfortunately the researcher does not have accurate reasons for the low breast surgery rate due to the retrospective nature of the research. Ogudiran et al (2013) ascribes the low surgical rate to factors that included: inoperable disease, metastatic disease and patients' choice (Ogundiran, Ayandipo, Ademola, & Adebamowo, 2013).

5.2.4.7 Molecular subtypes and survival.

In this study, overall survival is defined as any of the following: time interval from the diagnosis of breast cancer in the patient to the last recorded visit before 60 months; or time interval from diagnosis to death of the patient before 60 month' follow- up; or if the patient was alive by the time after 60 months at which point the study was stopped. The five year overall survival for the entire cohort is 25. 5%. Luminal A and B have a better five-year survival (27.2% and 25% respectively) compared to HER2/neu and TNBC (24.1 and 23.2, respectively). TNBC has the worst survival rate and luminal A the best, while luminal B and HER2/neu have identical survival pattern. However, the difference is not significant (p=0.104).

Spitale et al (2009) followed-up 1339 females with invasive breast cancer treated in a European cancer Centre from 2003 to 2005 for 2 years. During the 2-year follow-up period, 30 patients died resulting in an overall survival probability of 95%. Further analysis of survival showed that age group, menopausal status, AJCC stage, distant metastasis at diagnosis, histologic grade, Ki-67 proliferation index and tumor size are significant predictors of overall survival ($p < 0.001$ for each). In contrast, the histologic type (ductal versus lobular carcinomas) does not indicate any significant differences in overall survival. However, the molecular subtypes differ significantly in overall survival ($P = 0.0446$). Of these TNBC and HER2/neu subtypes show a reduced survival probability at 2 years after diagnosis (89.4% and 91.7%, respectively) compared with luminal A and B cases (96.5% and 96.7%, respectively). In another large study with 1198 patients, Vallejos et al (2010) analysed their 5-year survival based on the 4 molecular breast cancer subtypes. During the 5-year follow-up period, 307 patients died, giving an overall survival rate of 73.5%. Further survival analysis with stratification according to clinicopathological characteristics show that clinical stage, distant metastasis at diagnosis; histologic grade, axillary lymph nodes involvement, and tumor size are significant factors of prognosis for the overall survival ($P < .0001$ for each factor). Additionally, a significant difference in overall survival is seen according to the age group ($P = .002$). In contrast, there are no significant differences in overall survival with respect to menopausal status, and AJCC tumour stage. However, they found significant differences in overall survival according to breast cancer molecular subtypes, with the highest probability of 5-year survival seen in luminal A subtype tumors (81.9%), followed by luminal B (72.8%), and then TNBC

(67.1%). HER2/neu subtype had the worst probability of survival at 62.4% (Vallejos et al, 2010).

5.3 CONCLUSION

The proportion of hormonal receptor positivity (ER, PR, and HER2/neu) is 50.6%, 30%, and 14.3% respectively, in the present study. Luminal A subtype is the most common. The majority of patients presented with advanced stage III and IV. Although this study does not demonstrate statistically significant association between most of the clinicopathological features and the breast cancer molecular subtypes, the pattern in the findings are consistent with those of larger studies that reached statistical significance. The findings in this study are also consistent with other studies as far as the pattern of lymph node involvement across the molecular types are concerned. Luminal B has the highest rate of axillary lymph node involvement and luminal A the least involvement at the time of diagnosis. There is however no statistically significant association between the molecular subtypes and axillary lymph node involvement. Molecular subtypes predominantly associated with the highest percentage of high grade, cancer is TNBC, while Luminal A had the least percentage of grade 3 ($p=0.587$), however it is not statistically significant.

5.4 LIMITATION OF THE STUDY

The main limitation of this study is that there is missing data in some patients, especially immunohistochemical results such as oestrogen receptor, progesterone receptor, human epidermal growth factor 2 receptor, tumour grade, axillary lymph node status, and Ki-67 proliferative index. The researcher did not include Ki-67 in re-classifying the molecular subtypes as this indicator was not routinely measured in most patients in the period of the study. Only 254 patients out of the 329 are evaluable in the final molecular subtype reclassification and analysis due to missing data. Seventy-five patients are excluded from final evaluation because at least one critical data was missing in their clinical records.

Another limitation involved the evaluation of overall survival. Pietersburg Hospital does not have a policy of following-up patients who do not return to the hospital by themselves on the appointment date for review. Although the state provided planned patient transport from the district or provincial hospital to Pietersburg Hospital, the patients still have to travel from their home to the nearest hospital to catch transport to the follow-up clinic at the tertiary hospital. It is possible that non-ambulatory patient who is too sick to travel from home, or who does not have the means to reach the pick-up point would miss their scheduled appointment. Additionally, there is no strict requirement for the caregivers of a patient who dies at home or in another hospital to inform the oncology clinic at Pietersburg Hospital. For these reasons most of the patients who are lost to follow-up could not be accurately accounted for hence calculation of overall survival is incomplete. McCormack et al (2020) underscored the significance of survival gap in mainly African women with breast cancer. They suggest that cancer survival estimates ideally need to be population-based, have few losses to follow-up, and quantify heterogeneity by clinical and epidemiological factors (McCormack et al, 2020). Although the health care social barriers that influence presentation at advanced stage are not the focus of this study, strengthening measures that will faciliate early diagnosis and treatment of breast cancer patients at the primary care level and tertairy level might improve the survival rate of breast cancer patients.

5.5 RECOMMENDATIONS

A significant proportion of the patients with breast cancer in this cohort could not be classified according to molecular sub-types due to missing immunohistochemical marker results. This signifies that they may have missed receiving the correct treatment for their specific type of cancer considering the heterogeneity of breast cancer. The following recommendations are therefore made based on the findings of this study in light of the need to personalize the treatment of a breast cancer patient:

- Firstly, that the minimum IHC testing for any suspected breast cancer biopsy specimen should include ER, PR, HER2 (with FISH or CISH if equivocal), and Ki67 markers. Secondly, the doctors concerned with primary treatment of breast cancer pateints , especially the surgical team, should employ a protocol whereby the basic IHC tests are requested for each biopsy sample submitted

to the laboratory.

- Thirdly, the histopathology laboratory should also ensure that the basic IHC test are conducted on any speciment submitted.
- Fourthly, that the treating oncology team must classify each breast cancer patient based on the IHC result and select the appropriate treatment for her.
- Lastly, but not least in importance, that measures should be taken to ensure that appropriate treatment is available to facilitate personalized breast cancer treatment, such as availability of endocrine therapy (tamoxifen, anastrozole, exemestane etc) for luminal A and B patients, and trastuzumab for HER2/neu enriched breast cancer patients.

The majority of patients in this study presented at advanced stage of the disease (stage III and IV). Researchers in the Soweto reported a delay of more than three months from first presentation of breast cancer symptoms to acess to a health care facility (Joffe et al., 2018). Even though factors related to late presentation of breast cancer patients was not a focus of this study, it is noteworthy that late presentation may have contributed to the low rates of 5-year survival. Therefore it is recommended that measures are explored to facilitate early diagnosis and treatment of breast cancer patients. Additionally, it is recommended that follow-up of patients should be strengthened as a significant proportion of the patients who were first seen at the medical oncology clinic could not be accounted for by the end of the study period

5.6 CONTRIBUTIONS OF THIS STUDY

This study retrospectively re-classified breast cancer patients treated at Pietersburg Hospital using their immunohistochemical test results and categorizes them into different breast cancer sub-types according to their clinicopathologic features in order to determine if a different treatment approach would have been recommended for each patient. It further assess the 5 year overall survival which was found to be low compared to the reported survival in other middle income countries. This study has further shown that it is possible to classify the patients according to immunohistochemical marker results into breast cancer molecular sub-types to facilitate a personalized treatment approach. To the knowledge of the research team this study is the first one that has investigated the prevalence of the breast cancer

molecular sub-types among patients in a hospital setting in Limpopo and the survival of patients according to their molecular sub-types. Further studies should be conducted with close monitoring of patients to reduce those lost to follow-up and the number of those not accounted for.

5.7 CONCLUDING REMARKS

This retrospective study reports difference in survival based on molecular subtypes. However, there was no significant association between molecular subtypes and survival. This study might assist in guiding therapeutic management of breast cancer in Pietersburg hospital. Future prospective studies should be undertaken in which patients are closely monitored to avoid missing data and loss to follow-up.

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7. ANNEXURES

ANNEXURE A: Data collection sheet

DATA COLLECTION SHEET: QUESTIONNAIRE

TITLE: Breast cancer classification according to immunohistochemical markers: clinicopathologic features in women treated at Pietersburg Hospital, Limpopo

Study ID:_____

1. Age

<20	20-29	30-39	40-49	50-59	>60

2. Menopausal status

Pre menopause (<55)	Post menopause (55+)

3. Immunohistochemistry

	positive	negative
ER		
PR		
HER2		
Ki 67		

4. Breast cancer subtype

Luminal A	Luminal B	HER2 enriched	Triple negative

5. Tumour grade

Grade 1	Grade 2	Grade3	Unknown

6. Tumour

T1 (<2cm)	T2 (2 cm <5cm)	T3 (> 5cm)	T4. Chest wall invasion and/or skin involvement and/or inflammatory disease	Unknown

7. Node

N0	N1	N2	N3	Unknown

8. Metastasis

No Metastasis	Metastasis present

9. AJCC group stage

Stage 1	Stage 2	Stage 3	Stage 4	Unknown

10. Treatment (Select all that apply)

Surgery	Chemotherapy	Hormonal therapy	Radiotherapy	No treatment

11. Survival time (from diagnosis to last available contact)

<12 months	12-24	25-36	37-48	49-60+	No follow up

ANNEXURE B: Permission to conduct the study in Pietersburg Hospital

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA
DEPARTMENT OF HEALTH

REQUEST FOR PERMISSION TO CONDUCT THE STUDY

Title: Breast Cancer Classification According to Immunohistochemical markers Clinicopathologic Features in Women Treated at Pietersburg Hospital, Limpopo Province.....TREC PROJECT NUMBER TREC/127/2021%PG

I am currently a final student in the Department of Radiation Oncology at Pietersburg hospital and would like to carry out a study as part of the fulfilment requiment for Fellow of College of Radiation Oncology of South Africa (Fe Rad Onc,SA).

This study: Breast Cancer Classification According to Immunohistochemical markers
Clinicopathologic Features in Women Treated at Pietersbtirg Hospitåi, Limpopo Province

This study is a retrospective study utilizing patients data obtained from Radiation Oncology. The patients' identity and other sensitive information will be kept confidential and may only be revealed to authorised personnel. Ethics approval has been granted from the University of Limpopo TREC.

I therefore request permission to conduct the study at Pietersburg hospital. Conducting the study will not interrupt normal workflow,

Dr RJ Mphahlele date.... 23/08/2021
Ramadimetje Joyce Mphahlele
Degree; M Med
Radiation oncology
Student Number [redacted]

Su ervisor:DrF.Ooko.. . ,me.date...,. 3/08/2021.....

.....date 24/08/2021

A proved	

Clinical
Director. . ...

A roved	Not A roved

CNR DORP AND HOSPITAL STREET, PRIVATE BAG X 9316, POLOKWA, 0700 TEL: (015) 287 5000, FAX: (015) 297 2604

ANNEXURE C: Turfloop Research committee Clearance certificate

University of Limpopo
Department of Research Administration and Development
Private Bag Sovenga, 0727, South Africa
Tel: (015) 268 3935, Fax: (015) 268 2306, Email:anastasia.ngobe@ul.ac.za

TURFLOOP RESEARCH ETHICS COMMITTEE

ETHICS CLEARANCE CERTIFICATE

PROJECT NUMBER: TREC/127/2021: PG

PROJECT:

Title:	Breast Cancer Classification According to *Immunohistochemical* Markers: *Clinicopathologic* Features in Women Treated At Pietersburg Hospital, Limpopo Province
Researcher:	RJ Mphahlele
Supervisor:	Dr F Ooko
Co-Supervisor/s:	N/A
School:	Medicine
Degree:	Master of Medicine in Radiation Oncology

MEETING: 17 August 2021

PROJECT NUMBER: TREC/127/2021: PG

PROF P MASOKO

CHAIRPERSON: TURFLOOP RESEARCH ETHICS COMMITTEE

The Turfloop Research Ethics Committee (TREC) is registered with the National Health Research Ethics Council, Registration Number: REC-0310111-031

Note:

i) This Ethics Clearance Certificate will be valid for one (1) year, as from the abovementioned date. Application for annual renewal (or annual review) need to be received by TREC one month before lapse of this period.

ii) Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee, together with the Application for Amendment form.

iii PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

ANNEXURE D: Permission to conduct the study in Limpopo Province

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

Department of Health

Ref : LP_2021-08-020
Enquires : Ms PF Mahlokwane
Tel : 015-293 6028
Email : Phoebe.Mahlokwane@dhsd.limpopo.gov.za

Ramadimetje Mphahlele

PERMISSION TO CONDUCT RESEARCH IN DEPARTMENTAL FACILITIES

Your Study Topic as indicated below**;**

Breast cancer classification according to Immunohistochemical Markers: Clinicopathologic features in women treated at Pietersburg hospital, Limpopo province

1.Permission to conduct research study as per your research proposal is hereby Granted.

1. Kindly note the following:
    a. Present this letter of permission to the institution supervisor/s a week before the study is conducted.
    b. In the course of your study, there should be no action that disrupts the routine services, or incur any cost on the Department.
    c. After completion of study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
    d. The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
    e. The approval is only valid for a 1-year period.
    f. If the proposal has been amended, a new approval should be sought from the
    Department of Health
    g. Kindly note that, the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated

_____
_____13/09/2021

PP **Head of Department** **Date**

ANNEXURE F: Copyright permission letter

DISSERTATION COPYRIGHT PERMISSION FORM

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Dissertation title: Breast Cancer Classification According to Immunohistochemical Markers: Clinicopathologic features In Women Treated at Pietersburg Hospital, Limpopo

University: University of Limpopo

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Student Number:
Signature gpA,6Uuta-tke . *Date 01/12/2021*
Ramadimetje Joyce Mphahlele

Signature_____ Date
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