UTILITY OF FINE NEEDLE ASPIRATION CYTOLOGY IN DISCRIMINATION OF BREAST CANCER SUBTYPES BASED ON BIOMARKER EXPRESSION PATTERNS COMPARED TO CORE NEEDLE BIOPSY

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Utility of Fine Needle Aspiration Cytology in Discrimination of Breast Cancer Subtypes Based on Biomarker Expression Patterns Compared to Core Needle Biopsy

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DECLARATION

This thesis is my original work and has not been presented foe a degree in any other university.

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This thesis has been submitted for examination with our approval as university supervisors

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DEDICATION

This piece of work is dedicated to my two lovely sons Ayden Dylan Kipkorir and Raynor Kiplagat and to my loving husband Peter Kibet Kiptim

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ABBREVIATIONS AND ACRONYMS

ASCO	American Society of Clinical Oncology
BRCA-1	Breast cancer suspectibility gene 1
BRCA-2	Breast cancer suspectibility gene 2
CAP	College of American Pathologists
DAB	3’3’ diaminobenzidine
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
EA	Eosin Azure
ER	Estrogen receptor
FFPE	Formalin fixed paraffin embedded
FNAC	Fine-needle aspiration cytology
HER2	Human epidermal growth factor receptor-2
HER3	Human epidermal growth factor receptor-3
HER4	Human epidermal growth factor receptor-4
HRP	Horse raddish peroxidase
ICC	Immunocytochemistry
IHC	Immunohistochemistry

KNH	Kenyatta national hospital
LCIS	Lobular carcinoma in situ
NBF	Neutral buffered formalin
NPI	Nottingham Prognostic Index
NR3C3	Nuclear receptor subfamily 3, group C, member 3
OG	Orange Green 6
PR	Progesterone receptor
PTEN	Phosphatase and *tensin* homologue
SOP	Standard operating procedure
TBS	Tris-buffered saline
TNBC	Triple negative breast cancer
WHO	World Health Organization

OPERATIONAL DEFINITIONS

Adjuvant therapy Treatment given after the primary treatment to increase the chances of a cure.

Biomarker A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to a therapeutic intervention.

Targeted therapy In cancer treatment, it is a substance that kills cancer cells by targeting key molecules involved in cancer cell growth.

Neoadjuvant therapy Treatment given as a first step to shrink a tumor before the main treatment, usually surgery is given.

Predictive biomarker A biomarker, which can be used to identify subpopulation of patients who are most likely to respond to a given therapy.

Prognostic biomarker A biomarker that provides information on the likely course of cancer disease in untreated individuals.

Luminal A breast cancer It is a breast cancer subtype that is positive for estrogen, progesterone receptors and negative for HER-2/neu receptor.

Luminal B breast cancer It is a breast cancer subtype that is positive for estrogen, progesterone and HER-2/neu receptors.

HER2 positive breast cancer It is a breast cancer subtype that is positive for HER-2/neu receptor and negative for estrogen and

progesterone receptors.

Triple negative breast cancer It is the subtype of breast cancer that is negative for estrogen, progesterone and HER-2/neu receptors.

ABSTRACT

Breast cancer is defined as a group of malignant neoplasms and accounts for up to 23% of all female cancers. It rarely occurs in men. In breast cancer, the level of human epidermal growth factor receptor-2 (HER2) overexpression is said to be a prognostic molecular marker that is used for selection of patients for targeted HER2- therapy. Estrogen receptor (ER) and progesterone receptor (PR) are both prognostic and predictive markers for response to hormonal therapy. Fine-needle aspiration (FNA) provides highly cellular sample. Assessment of HER2, ER and PR status in FNA samples is very important clinically. The study was aimed to validate that fine needle aspiration cytology can be used to assess HER2, ER and PR expression patterns in patients with breast cancer. Cell blocks were prepared from FNA and core needle biopsy material collected from 39 newly diagnosed breast cancer patients and immunocytochemistry (ICC) for HER2 and the hormonal receptors, ER and PR was done. Immunohistochemistry (IHC) for HER2, ER and PR was also done on the corresponding biopsy sections. Both positive and negative quality controls were included in the experiments. The Allred scoring system was used to determine the positivity for PR and ER. The overexpression of HER2 was assessed using a scale of 0-3+ for both proportion and intensity whereby 3+ and above was considered positive. The FNAC cell block results were compared with core biopsy results and breast cancer classified into various types as luminal A, luminal B, HER2 over expression and the triple negative. The results were then compared with those of core biopsy immunohistochemistry using ANOVA. Kappa statistics was done to check the level of agreement. Cell block and biopsy results were compared, for ER there was a concordance of 32/35(91.4%) r=0.842 Sensitivity of 83.3% and specificity of 85.0%. For PR the concordance was 32/35(91.4%) r=0.842 with sensitivity of 84.2% and specificity of 84.2%. For HER2 the concordance was 34/35 (97.1%) r=0.925 with a sensitivity of 88.9% and a specificity of 96.3%. There was moderate agreement between the two methods, k=0.719, $p<0.001$ .The results obtained from FNA cell blocks are reliable when compared with paired paraffin embedded tissue blocks. Therefore, HER2, ER and PR can be adequately assessed using cell blocks prepared from FNA material.

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Breast cancer is a malignant tumor of the breast and comprises of a diverse group of neoplasms accounting for about 23% of all female cancers. Over 1 million new cases are diagnosed annually globally resulting to about 400,000 breast cancer related deaths (Veronesi *et al* 2005, Bray *et al* 2013). It is the most prevalent cancer among women (WHO 2010). It rarely occurs in men accounting for approximately 1%. The overexpression of HER2/*neu* in breast cancer is a prognostic marker used to select patients for HER2 therapies and has been reported in approximately 20–30% of breast cancer (Blackwell *et al* 2010). The up regulation of HER2 is associated with poor prognosis as well as an aggressive disease course (Slamon *et al* 1987, Yokota et al 1986). The steroid hormonal receptors for estrogen receptor (ER) and progesterone receptor (PR) are both prognostic and predictive markers for response to hormonal therapies (Hamond *et al* 2010). The clinical-pathological parameters have been used for a long time to determine therapeutic management of patients with breast cancer. Studies indicate that breast cancer HER-2, ER and PR positive are difficult to treat and it is therefore believed that careful patients' stratification using these biomarkers would improve therapeutic outcomes (Strasser *et al* 2005). Tissue biopsy blocks have been the sample of choice for the assessment of HER2, ER and PR status. However, the cost of processing biopsies is very high especially in developing countries. Sometimes it might be impractical to obtain a surgical excision for HER2, ER and PR testing especially, in patients receiving neoadjuvant therapy or those with inoperable metastases. In such cases, it will be wise to assess HER2, ER and PR status using fine-needle aspiration cytology (FNAC) (Wolf *et al* 2014). Breast cancer is diagnosed by the triple assessment (clinical assessment, imaging and FNA and/ biopsy) (Senkus *et al* 2013). Breast cancers are classified into different subtypes based on their HER2 and hormonal receptor status as

obtained by immunochemistry results as luminal A (ER+,PR+, HER2-), luminal B (ER+,PR+, HER2+), HER2 over-expression (ER-, PR-, HER2+) and the triple negative type. Studies performed in Kenya have often utilized formalin fixed paraffin embedded tissues (FFPE) despite their limitations in preoperative treatment (Bird et al 2008). The assessment of HER2, ER and PR expression patterns using FNAC has not been adequately explored especially the stratification of breast cancer subtypes based on ER/PR and HER2 expression patterns. In developing countries where resources are limited, determination of these bio-markers on FNAC samples of breast cancer is of clinical significance. FNAC being minimally invasive has been proven to be safe, cheap and fast as compared to core needle biopsies (CNB) and can quickly characterize ER, PR and HER-2/neu status hence providing both diagnostic and prognostic applications. FNAC is accepted worldwide for the screening and diagnosis of breast cancer (Nizzoli *et al* 2000). Studies show that the evaluation of ER and PR status by immunocytochemistry on cell blocks obtained by FNAC highly correlates with results from tissue blocks as well as biochemistry results (Chang *et al* 2001). The purpose of this study was to determine the utility of fine needle aspiration cytology (FNAC) in the evaluation of HER2, ER and PR expression patterns compared to core needle biopsies (CNB) in breast cancer patients.

Men too, just like women also do get the same types of breast cancers as they have a small amount of breast tissue. The breasts of an adult man are similar to that of a girl before puberty. Breast cancer is uncommon in men accounting to about 1% of all breast cancer cases. Some of the risk factors in men include; old age, exposure to estrogen, family history of breast cancer, klinefelter's syndrome, liver disease, obesity and testicle disease or surgery (Mayo 2020)

1.2 Statement of the Problem

Being a group of neoplasms, breast cancer exhibits a wide range of genotypes, phenotypes and tumor physiology (Simpson *et al* 2005). Patients care is a challenge since it is not easy to determine the best regimen according to disease

characteristics. Due to poor prognosis of breast cancer diagnosed at advanced stages, stratification of patients for targeted therapy remains the most appropriate way for improving on the prognosis of breast cancer patients in Kenya (Goldhirsh *et al* 2013).

Advances in therapy have achieved some success, however, studies show that breast cancer HER-2, ER and PR positive are difficult to manage. It is therefore believed that more careful patients' stratification using these biomarkers will improve therapeutic outcomes. Tissue biopsy is always the sample of choice used for the assessment of HER2, ER and PR status. However, when it is impossible to obtain a biopsy for HER2, ER and PR testing especially, in patients on neoadjuvant therapy or those with inoperable metastases, it is often desirable to assess HER2, ER and PR status using fine-needle aspiration cytology since it provides highly cellular material representative of the lesions (Pinder *et al* 1995). FNAC is minimally invasive, fast, and cost effective as compared to core needle biopsies (CNB) especially for aged or weak patients with co-morbidities as well as those on follow up of neoadjuvant chemotherapy.

1.3 Study justification

Breast cancer, presents a clinical, diagnostic and therapeutic challenge. This is because the treatment decision is based on clinicopathological variables that are prognostic in nature, such as size of the tumor, metastasis to lymph node, and histological grade that are not sufficient for implementation of personalized therapy. Studies performed in Kenya have often utilized formalin fixed paraffin embedded tissues (FFPE) despite having limitations in preoperative treatment (Bird *et al* 2008). The use of FNAC for assessing HER2, ER and PR expression patterns has not been adequately explored in Kenya especially the stratification of breast cancer subtypes based on ER/PR and HER2 expression patterns. Therefore, in limited resource setting, the determination of ER, PR and HER-2/neu status on FNAC samples of breast cancer is of clinical importance especially due to cost implication. .A cheap and fast method to quickly

characterize PR, ER and HER-2/neu status will provide both diagnostic and prognostic applications. Immunocytochemistry (ICC) will be used to assess HER2, ER and PR protein expression. (Muchiri 1993, Nyagol *et al* 2013).

1.4 Alternate hypothesis

The utility of fine needle aspiration cytology in the evaluation of HER2, PR and ER expression patterns is comparable to core needle biopsies (CNB) among breast cancer patients.

1.5 General Objective

To determine the utility of fine needle aspiration cytology in the discrimination of HER2, ER and PR expression patterns compared to core needle biopsies (CNB) in patients with breast cancer at Kenyatta national hospital.

1.5.1 Specific objectives

i.) To identify breast cancer cases using fine needle aspirate and core needle biopsies (CNB).

ii.) To determine HER2, ER and PR protein expression by immunocytochemistry (ICC) and immunohistochemistry (IHC)

iii.) To compare HER2, ER and PR protein expression patterns in fine needle aspirate and core needle biopsy (CNB).

CHAPTER TWO
LITERATURE REVIEW

2.1 Epidemiology, risk factors and etiology

Breast cancer is ranked the most common carcinoma in women accounting for 23% of all female cancers globally. Over 1 million new cases are diagnosed annually, resulting in about 400,000 breast cancer related deaths yearly. Breast cancer is the second leading cause of mortality and Morbidity that affects the female gender (Manral *et al* 2016)It accounts for approximately 3-5% deaths in the developed world and 1-3% in the developing countries (Dumitrescu and Cotarla,2005). In Kenya, the incidence rate of breast cancer is 13.7%. It is therefore a major cause of mortality and morbidity. It is the most prevalent cancer among women (WHO 2010).

The etiology of breast cancer is not well understood as it is influenced by several factors including gender, age, genetic predisposition, alcohol, ionizing radiation, parity, atypical hyperplasia history, nulliparity, old age at first childbirth, use of oral contraceptives, obesity, lack of physical activity, consumption of alcohol and tobacco, high body mass index and endocrine factors (both endogenous and exogenous) (Ali and Coobes 2002, McTiernan 2003, Abdulkareem 2013). The most significant risk factors are gender and age. Breast cancer rarely affects persons below the age of 20 years, with incidence gradually increasing with age. (Ferlat *et al* 2010). The molecular feature of breast cancer can be well described by either the absence or presence of HER2, ER and PR (Asegaonkar *et al* 2015)

2.2 The pathogenesis of breast cancer

The pathogenesis of breast cancer is attributed to germ line gene mutation of high-penetrance susceptibility genes (p53, PTEN, BRCA-1 and BRCA-2), which contribute 5% to 10% of all breast cancer although a majority of breast cancer cases are not hereditary (Hynes 2005. Senkus *et al* 2013). BRCA-1 and BRCA-2 gene positive premenopausal women have risk of 80% of developing breast

cancer.(Dumitrescu and Cotarla 2005).

2.2.1 The role of human epidermal growth factor receptor 2 (HER2)

Breast cancer is associated with signal transduction of the receptor tyrosine kinases that are located on the epithelial cells on the breast surface, which plays a role in the disease pathogenesis. (Taneja *et al* 2010).The human epidermal growth factor receptor-2 (HER2) oncogene is located on chromosome 17(17q11.2- q12) and encodes for 185-kDa membrane receptor-like protein with tyrosine kinase activity (Pegram et al 2000). HER2/neu (also known as ErbB-2) is one of the four trans-membrane receptor tyrosine kinases, which are; epidermal growth factor receptor, HER-3, and HER-4 that are involved in signal transduction pathways which regulate cell growth and proliferation (Huang *et al* 2005. Roskoski, 2004)

HER2/neu is known for its role in the pathogenesis of breast cancer and is targeted for treatment. The HER2 gene is overexpressed in approximately 20–30% of breast cancer and up regulation of the HER2 is associated with aggressive disease course and poor prognosis. Clinically, HER2/neu is important as the target of the monoclonal antibody trastuzumab (Herceptin). Herceptin is only effective in breast cancer where the HER2/neu receptor is over-expressed (Verma 2010)

2.2.2 The role of estrogen receptor (ER)

The spontaneous development of disease could be driven by the activation of steroid hormones such as estrogen, by binding to their specific receptors in breast epithelial cells in order to promote cell growth and survival (Bjornstrom *et al* 2005)

ER being a member of the nuclear hormone family of intracellular receptors is activated by the hormone 17β-estradiol. Its main function is DNA-binding transcription factor that regulates gene expression. Each of the two forms of ER,

(α and β) is encoded by a separate gene. The α isoform is encoded by the ESR1 and the β isoform is encoded by the ESR2 gene. Its binding to estrogen receptor stimulates mammary cells proliferation, whith then increase cell division and DNA replication hence increasing mutation rate. This causes a disruption in the cell cycle, apoptosis and DNA repair processes leading to tumor formation (Dahlman *et al* 2006). Estrogen metabolism also leads to the production of genotoxic by-products that could directly damage DNA, resulting in point mutations. In developed countries ERs are over-expressed in about 70% of breast cancer cases, and are referred to as estrogen positive tumors (Eniu *et al* 2006). Interestingly studies have reported the overexpression of ER/PR among African breast cancer cases to be relatively low with estimates ranging between 18% and 33% (Bird *et al* 2008, Nyagol 2006)

2.2.3 The role of progesterone receptor (PR)

The progesterone receptor (PR) also nuclear receptor subfamily 3, group C, member 3 (NR3C3) is an intracellular steroid receptor binding progesterone. Progesterone receptor is encoded by the PGR gene, found on chromosome 11 (11q22). After the binding, conformation and dimerization follows then complex entry into the nucleus and binding to DNA (Edwards *et al* 1995). Up to 65% of ER-positive breast cancers are also PR-positive and about 5% of breast cancers are ER-negative and PR-positive. When cells possess receptors for both ER and PR or receptors for one of the two hormones, the cancer is considered hormone-receptor positive (Eniu *et al* 2006).

2.2.4 Triple negative breast cancer (TNBC)

Breast cancer in this category is negative for estrogen (ER), progesterone (PR) and HER-2/neu receptors. It is mostly associated with patients of low socio-economic status (O'Toole et al 2013, Fan *et al* 2006). It accounts up to 20% of all breast cancer cases and has been associated with high rates of recurrence, faster growth and poor prognosis as compared with other types of breast cancers (Huo

et al 2009). Basal-like and/or triple-negative breast cancer is common among African Americans than the white Americans (Carey *et al* 2006).

2.3 Breast cancer screening and diagnosis

Diagnosis is by triple assessment (clinical assessment, mammography and/or ultrasound imaging with core biopsy and/or excision/incision biopsy and/or fine needle aspiration cytology) (Senkus *et al* 2013)

2.3.1 Clinical Examination

Initial stage of breast cancer is painless with no symptoms. The commonest feature is a painless lump in the breast. A systematic breast examination procedure involves palpation on all the breast quadrants, the nipple-areola, the axillary tail and the axilla (Senkus *et al* 2013).

2.3.2 Imaging techniques

Mammography, ultrasonography and **magnetic resonance imaging (MRI)** are imaging techniques used in the screening and diagnosis of breast cancer (Giordano *et al* 2012). **Mammography** is recommended for breast cancer screening. A solid mass with or without stellate features, asymmetric thickening of breast tissues and clustered micro calcifications are suggestive of breast cancer.

Ultrasonography is a method of resolving equivocal mammography findings, defining cystic masses among others (Gotzsche and Nielsen 2011). It is used to guide fine-needle aspiration cytology with high reproducibility and high patient acceptance rate, but not reliable for lesions of 1 cm or less in diameter and when used alone it is a poor screening test (Fine *et al* 2014).

Magnetic resonance imaging on the other hand is a non-invasive, non-radiating imaging technique, used in characterizing mammography abnormalities (Warner

2008, Lalonde *et al* 2005).

2.3.3 Breast biopsy

Pathological diagnosis of breast lesion can be achieved with core needle biopsy and also excision/incision biopsies. The core needle biopsy is performed under local anesthesia and tissue samples obtained allows the diagnosis of invasive versus in situ cancer. It enables the pathologist to classify breast cancers under histopathological type, grade and stage of tumor (Gonzalez 2009, Lakhani 2012).

2.3.3.1 Classification by Histopathology

Breast cancer is classified primarily by its histological characteristics. Majority of breast cancers are derived from the epithelia that lines the ducts or lobules, and classified as ductal or lobular carcinoma. Carcinoma in situ is growth of low-grade cancerous or precancerous cells in a particular tissue compartment such as the mammary duct without invasion into surrounding tissue (Herman 2009).

2.3.3.2 Grade

Grading compares the appearance of the breast cancer cells versus normal breast tissue. Normal cells differentiate and take on specific shapes and forms that reflect their function. Cancerous cells lose differentiation. Cells are described as well differentiated (low grade), moderately differentiated (intermediate grade), and poorly differentiated (high grade) as the cells progressively lose features of normal cells. Poorly differentiated cancers have the worst prognosis (Elston 1991).

2.3.3.3 Stage

The TNM staging system is based on the tumor size (T), lymph node involvement (N) in the armpits, and metastases (M) spread to a more distant part of the body.. The main stages includes:

- Stage 0 is a pre-cancerous or marker condition, either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS),
- Stages 1–3 are within the breast or regional lymph nodes,
- Stage 4 is 'metastatic' cancer that has poor prognosis (Arpino 2004).

2.3.4 Fine needle aspiration cytology

Fine needle aspiration is achieved by making 6-8 passes within the lump using a needle to obtain cell sample (Mardanpour *et al* 2011). It is an effective tool in evaluating and diagnosing suspect lumps or masses. A quick diagnosis is that cancer is detected early, giving more options for interventions, or that benign lumps are diagnosed without the need for surgery. It is less invasive and only slightly uncomfortable as compared to a surgical biopsy (Mardanpour *et al* 2011). FNA-C is useful for pre-therapeutic diagnosis of breast cancer and often provides high cellular material representative of the lesions. FNA-C samples may be used as cellular smear for immediate diagnosis, and residual cells (needle washings) can be obtained by rinsing the needle and preserved for subsequent detection of biomarker by immunocytochemistry.

2.3.5 Comparison of FNAC and Core Needle Biopsy (CNB)

FNAC is a safe, simple, cost effective and a quick procedure. It has a high sensitivity and allows multidirectional passes in the lesion allowing broader sampling. FNAC can be reported within a short time. CNB compared to FNAC is more reliable and more invasive but less invasive as compared to surgical biopsy. CNB allows clinicians to plan therapeutic treatment but has complication that include but not limited to bruising, vasovagal reactions, severe pain, bleeding that require treatment, infections and hematoma.

2.3.6 Immunocytochemistry

FNA-C is accepted for the diagnosis of breast carcinoma (Nizzoli 2000). Previous studies have shown that the simultaneous evaluation of estrogen

receptor (ER) and progesterone receptor (PR) status by immunocytochemistry on cellular material obtained by FNAC has high correlation with results from paraffin sections and biochemical assays (Chang, Elledge 2001).The use of immunocytochemistry evaluation of ER on FNAC and on paired paraffin embedded tissues, showed a good correlation, with a rate of concordance ranging from 61% to 92% (Schmitt *et al* 1995). Although ER, PR and HER2 are critical prognostic and predictive markers in breast cancer diagnosis and treatment, there is lack of routine use for patient management in Kenya.

The use of FNAC samples to assess HER-2, ER or PR status using a cell block is recommended, especially in metastatic disease where a biopsy may not be possible. The advantages of cell blocks are that multiple sections can be obtained, long-term storage is possible and the same antigenic concentrations can be used as that of formalin-fixed histological sections. The use of FNAC for assessing HER2, ER and PR expression patterns has not been adequately explored in Kenya (Domanski *et al* 2013, Brifford *et al* 2000, Tafjord *et al* 2002).

2.4 Patient stratification of breast cancer subtypes using receptor status

Studies on gene expression profiles have identified at least four breast cancer subtypes and demonstrated the ability to predict clinical outcomes independent of other prognostic factors (Guiu et al 2012). Breast cancer, subtypes are classified by assessing the HER2, ER and PR status and classified as luminal A (ER+, PR+, HER-2-, and/or low proliferation index), luminal B (ER+, PR+, HER-2+, and/or high proliferation index) and have favorable clinical outcomes (Onitio *et al* 2009, Kumar *et al* 2012).

Luminal A and luminal B can be differentiated using Ki67, which is a proliferative marker. HER2–positive/ER-negative PR-negative subtype is characterized by overexpression of HER2, and basal-like subtype is negative for ER, PR, and HER2 also known as TNBC. These molecular differences have a strong correlation with clinical features and survival (Zaha *et al* 2010).

2.5 Breast cancer treatment and management

The management of breast cancers has changed over the last 20 years. Conservative and reconstructive surgery today is more popular than mastectomy with increased use of systemic, hormonal and cytotoxic drugs following hormone receptor testing (Shetet al 2009).ER, PR and HER-2/neu analysis have been accepted as established procedures in routine management of breast cancer patients. The expression of the three hormone receptors is informative in the molecular classification of breast tumors and their clinical assessment for treatment and further outcome (Hwang *et al* 2013)

2.5.1 Treatment

Hormone positive cancers are treated with long term hormone blocking therapy. Hormonal therapy is confirmed to render a benefit of 47% risk reduction for recurrence and 26% for mortality (Hwang et al 2013). The treatments are given with aggressiveness according to the prognosis and risk of recurrence. The Nottingham Prognostic Index (NPI) is a useful tool in assessing the prognosis. Stage 1 cancers have an excellent prognosis and are generally treated with lumpectomy and sometimes radiation. HER2+ cancers should be treated with thetrastuzumab (Herceptin) regime (Blackwell et al 2010). Stage 2 and 3 cancers with a progressively poor prognosis and greater risk of recurrence are treated with surgery (lumpectomy or mastectomy with or without lymph node removal), chemotherapy (plus trastuzumab for HER2+ cancers)and radiation (particularly following large cancers, multiple positive nodes or lumpectomy).Stage 4, metastatic cancer, has poor prognosis and is managed by various combination of all treatments from surgery, radiation, chemotherapy and targeted therapies.

CHAPTER THREE
METHODOLOGY

3.1 Study area

The clinical and experimental phases of the study were carried out at Kenyatta National Hospital.

3.2 Study Population

Individual aged 18 years and above already diagnosed with breast cancer through any of the triple assessment methods i.e. clinical assessment, imaging or FNAC.

3.2.1 Inclusion criteria:

Patients recruited in this prospective cross-sectional study were men and women of 18 years and above who had been diagnosed with breast cancer either through triple assessment (clinical assessment, Imaging techniques (mammography or ultrasonography) and biopsy/fine needle aspiration cytology or through referred cases of confirmed breast cancer and had completed an informed consent form and accepted to participate (Appendix I).

3.2.2 Exclusion criteria:

Women and men with other type of malignancies, or who declined to complete an informed consent form were excluded from the study. Patients on treatment for breast cancer or those who had undergone mastectomy were not eligible.

3.3 Study Design

It was a prospective cross-sectional study of breast cancer patients.

3.4 Variables

3.4.1 Independent variables:

Clinical and demographic data (age, gender, family planning use, family breast cancer history, level of education, cigarette smoking).

3.4.2 Dependent variables:

i.) Histopathological/Cytopathological parameters (tumor type, size, tumor invasion),

ii.) 14ER, PR and HER2 expression patterns

3.5 Sample Size Determination

Using a formula developed by Liao 2009, k individual discordant pairs allowed (kX1). If up to k pairs of the n samples are allowed to be discordant to imply agreement between the two measurement methods, then the probability of having at least k11 discordant pairs for a fixed α and β, the sample size n increases as k increases. As seen in the table 45 sample pairs are required to claim agreement at a discordance rate 0.05 and a tolerance probability of 90% when there is no discordant pair.

Sample size when all individual pairs agree with each other						
Tolerance probability β						
		80%	85%	90%	95%	99%
Discordance Rate x	0.01	161	189	230	299	459
	0.05	32	37	45	59	90
	0.10	16	19	22	29	44
	0.15	10	12	15	19	29
	0.20	8	9	11	14	21

N = 45

3.6 Sampling Method

The study recruited participants using convenienct sampling method.

3.7 Research design and methods

3.7.1 Data collection

A structured questionnaire formulated by the principal investigator was used to interview the participants attending the KNH surgical outpatient clinic (SOPC) and the FNA clinic in order to obtain data (Appendix II). Interviews were conducted to obtain both clinical and demographic data which was recorded for later analysis.

3.7.2 Recruitment and Counselling

Patients aged 18 years and above diagnosed with breast cancer by clinical and radiological examination or biopsy were enrolled into the study. Counselling was done by a pathologist at the clinic, qualified physician or nurse in the breast clinic and the patients were requested to give signed informed consent. The benefits of the study were explained to all the participants and after signing the consent form (appendix I) a structured questionnaire was used by the principal investigator to collect the demographic data after which sample collection procedures and processing proceeded for both FNA and CNB.

3.7.3 Specimen Collection for laboratory analysis

After obtaining a signed consent from the patient the pathologist then explained the procedure, assured and placed the patient in a comfortable and convenient position for sample collection. The pathologist then palpated and localized the lesion and by making several passes within the lesion using a 21 or 22 gauge needle, FNA material was obtained placed on the microscopic slide (Fig.1). Another slide was used to spread the material thus obtaining a thin conventional smear, which was fixed with 95% alcohol immediately. On arrival in the

laboratory the FNAC smear was stained using both papanicolaou staining (appendix V) and haematoxylin and eosin (appendix VI) methods for cytomorphological diagnosis of breast cancer and was reported using the national cancer institute (NCI) system (appendix III).The portion of the remaining aspirate in the needle (Fig. 1) was used for cell block (appendix VII) preparation and subsequent immunocytochemical staining to assess HER2-Clone A0485 (DAKO, Glastrup, Denmark), ER-clone ID5 (DAKO, Glastrup, Denmark) and PR-clone PgR636 (DAKO, Glastrup, Denmark) protein expression using heat induced epitope retrieval (appendix IV).

3.7.4 Reporting for immunocytochemistry

The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommendation guidelines were followed while reporting the ICC (appendix VIII) results for HER-2, ER and PR. The current recommendation by the ASCO/CAP is to report ER/PR staining results as the percentage of positive nuclei because response to hormonal therapy correlates with the level of ER/PR expression, and it is important that the pathology report documents the actual percentage of cells expressing ER/PR as opposed to simply listing a result as "positive" or "negative (Hammond et al 2010)

The current recommendation by the ASCO/CAP on the testing criteria to define HER2-positive status is when (on observing within an area of tumor that amounts to ›10% of contiguous Aw21nd homogeneous tumor cells) there is evidence of protein overexpression or gene amplification (HER2 copy number or HER2/CEP17 ratio by ISH based on counting at least 20 cells within the area). For cell block adequacy at least 100 tumor cells will be counted for assessing the biomarker status

3.8 Quality Assurance

The study was conducted by qualified personnel in all the laboratories during

collection of samples, processing and analysis of the results. Standard operating procedures (SOPs) were used during FNAC procedure, specimen preparation, staining and reporting.

Pre-analytic standardization: FNAC sample was fixed immediately in 95% alcohol for conventional smear and 10% Neutral buffered formalin (NBF) for FNAC washing.

Analytical standardization: Staining with Pap stain and haematoxylin and eosin was done by the Principal Investigator (PI) while following SOPs. Immunocytochemistry was performed by the PI following procedures written as per manufacturer's instructions SOPs. Clinically validated antibodies were used. Positive and negative controls were included in every batch run. Positive controls of known positive breast cancer tissues and negative controls of normal breast tissues or negative control of a section without primary antibody were run with the patient/study slides in all procedures. A validation phase of the study was carried out using FNAC cellblocks and histopathological biopsies for assay standardization. Immunohistochemistry was carried out on the histopathological biopsies of the same study patient population as the gold standard and compared level of agreement with the ICC results.

Post-Analytical standardization: The FNAC conventional smears were screened by PI, then reported and signed out by a board certified consultant anatomic Pathologist. The turnaround time for reporting and signing out smear was 3 days and the results were submitted to clinician. All ICC results of the study patients were blindly and independently re-evaluated by the supervisors who are board certified consultant anatomic pathologist. Internal quality control for interpretation/reporting of ICC and interobserver reproducibility of the results was independently re-evaluated by a cytopathologist /histopathologist experienced in reporting these biomarkers of breast cancer. After the completion of interpreting all ICC and results, a report was sent to the clinician.

3.9 Ethical Considerations:

The study protocol was submitted for approval by the institutional research and ethics committee of Kenyatta National Hospital in collaboration with the University of Nairobi (KNH/UON ERC). Inclusion in the study and collection of biological specimens were carried out only after obtaining a written informed consent from each participant. Participation was voluntary and no payment or incentives were offered to the study participants. Interviews were conducted ensuring confidentially to all participants. The results of the study were held in total confidentiality and were only revealed to the participant through the clinician.

3.10 Data Analysis and Presentation

Data was entered into SPSS version 21 and analyzed. The results were expressed as a mean ± standard deviation (parametric) or as median and range (nonparametric).

Percentages of the tumor cells stained positive for ER, PR and HER-2 immunoreactivity were calculated. Kappa statistics was used to check the level of agreement between ICC and IHC. Results were presented in bar charts, bar graphs, tables, histograms and pie-charts as appropriate

3.10.1 Dummy tables

Table 0.1: Socio-demographic characteristics of patients with breast cancer

Age Range (years)	Frequency (n)	Percent (%)
20 and below≤		
21 – 30		
31 – 40		
41 – 50		
51 – 60		
61 – 70		
71 – above		

Table 0.2: Comparison of ER, PR and HER2 staining on cellblock versus histopathology (n =45)

Cell Block	Histopathology	Number of cases	Correlation
ER+		ER+	Concordant
ER-		ER-	
ER+		ER-	Discordant
ER-		ER+	
PR+		PR+	Concordant
PR-		PR-	
PR+		PR-	Discordant
PR-		PR+	
HER2+	HER2+		Concordant
HER2-		HER2-	
HER2+	HER2-		Discordant
HER2-		HER2+	

ER**, estrogen receptor;** PR**, progesterone receptor,** HER2**, human epithelial growth factor receptor 2**

Table 0.3: Classification of breast cancer cases on the basis of their HER2/ER/PR profile on cellblocks and the corresponding histological section (n=45)

Classification of Breast cancer Cases	**Cell block**		**Histopathology**	
	Number of cases (n)	**Percent (%)**	**Number of cases (n)**	**Percent (%)**
Luminal A (ER+, PR+, HER2-)				
Luminal B (ER+, PR+, HER2+)				
HER2 Positive (HER2+, ER-, PR-)				
Triple Negative (HER2-, ER-, PR-)				

ER, estrogen receptor; **PR**, progesterone receptor, **HER2**, human epithelial growth factor receptor 2

3.11 Expected Outputs of the research and impact of the project to public health

The project was expected to assess the clinical utility of HER2, ER and PR that would improve the stratification of breast cancer patients in Kenya. It was expected that the detection of HER2, ER and PR positive breast cancer using the FNAC material would facilitate an early and better patient selection for adjuvant therapy and therefore improve treatment outcome for patients with breast cancer. These might increase the survival of breast cancer patients and thus improve their health condition and quality of life in Kenya.

3.12 Research finding dissemination

This research generated interesting and valuable findings that were published our findings in reputable international peer reviewed scientific journal named Journal of medical science and clinical research Vol 07/Issue01/Page1079-1085/January 2019. The findings were also presented to the university's board of post graduate studies.

CHAPTER FOUR
RESULTS

4.1 Introduction

The study was aimed to compare fine needle aspiration cytology to core needle biopsy in the evaluation of breast cancer subtypes based on prognostic marker expression patterns. It was a prospective cross-sectional study. A total of 45 newly diagnosed breast cancer patients were recruited in the study. One patient declined sampling after signing the consent form. Sample material from five of the subjects were inadequate. After processing and staining the remaining 39 samples for both FNAC and CNB, four of the FNAC slides has no adequate tumor cells. The remaining slides for both FNAC and CNB from 35 patients were compared for HER2, ER and PR. The analysis results were displayed in this chapter. For ease of visibility and understanding, the results were in absolute numbers and percentages that were displayed in form of tables, pie and bar charts. The results constituted demographic characteristics, identification of breast cancer cases using cytomorphological features of fine needle aspirate and core needle biopsies, determination of HER2, ER and PR protein expression by immunocytochemistry (ICC) and immunohistochemistry (IHC) and comparison of the HER2, ER and PR protein expression patterns in fine needle aspirate and core needle biopsy (CNB).

4.2 Demographic characteristics

The demographic information that was collected from the study respondents included age, gender, occupation, family planning, laterality and level of education.

The study findings showed that the mean age of the study respondents was 48.26 (SD 12.33) years with median age of 46 years (inter quartile range 17). The majority of the respondents 14 (35.9%) were aged between 41 to 50 years. (Figure 4.1, Table 4.1).

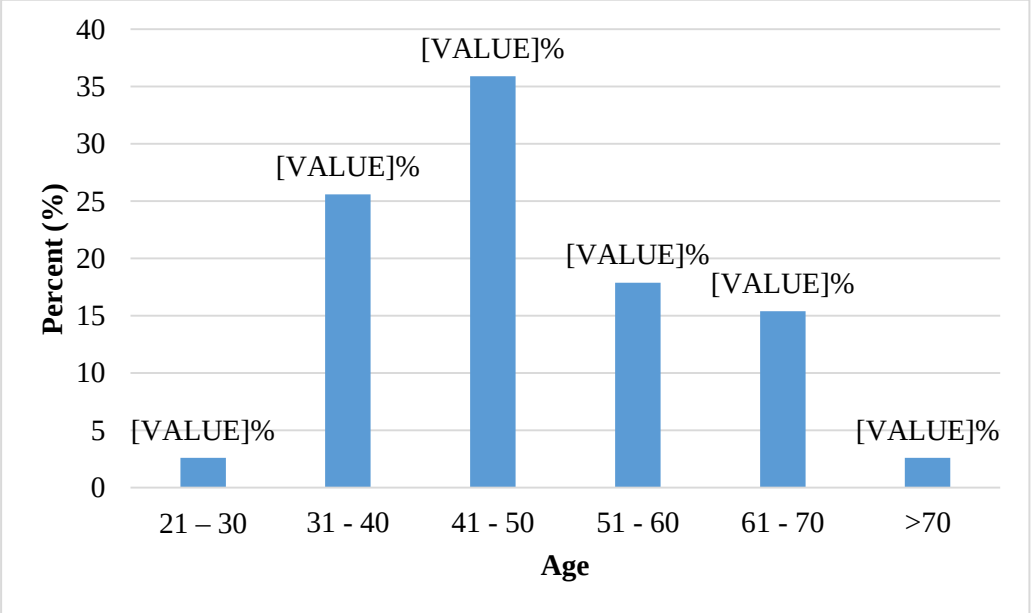


Figure 0.1: Age of respondents

Majority of the study respondents 38 (97.4%) were female (Figure 4.2, Table 4.1).

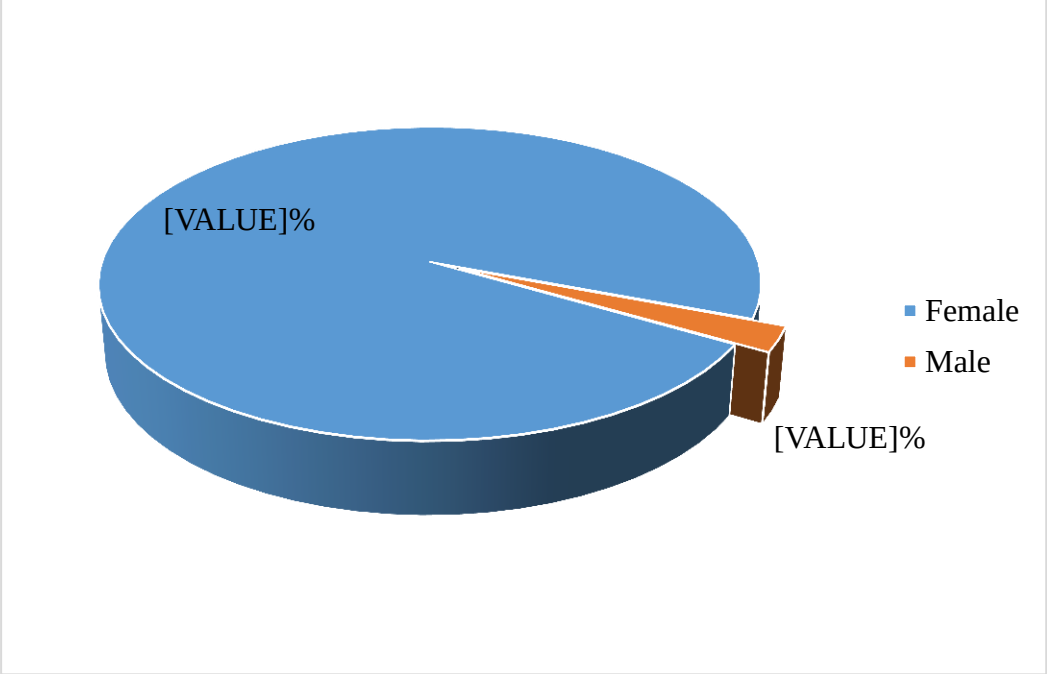


Figure 0.2: Gender

Majority of the study respondents 23 (59%) did not use family planning (Figure 4.3)

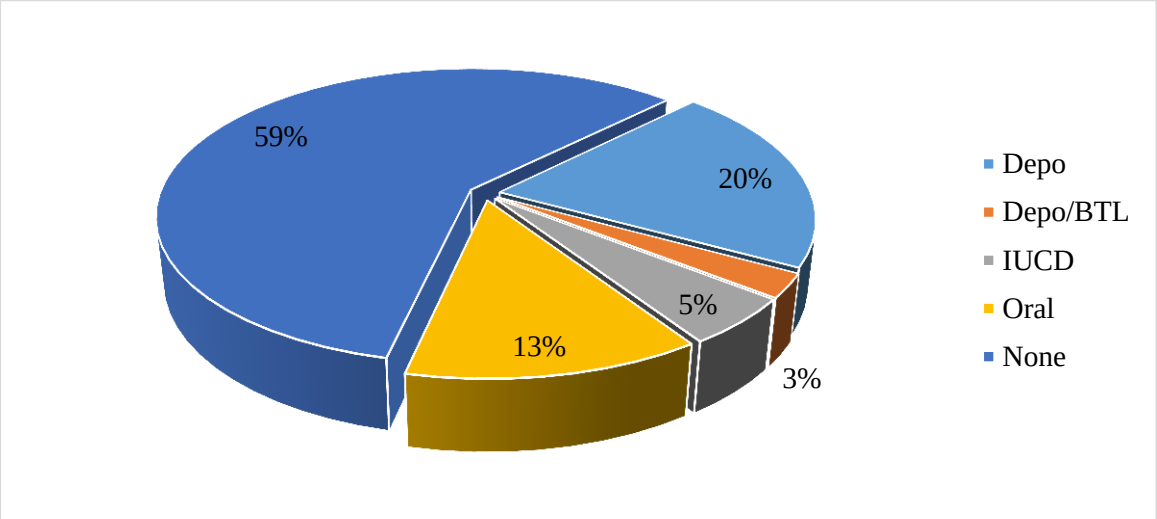


Figure 0.3: Family planning

Majority of the participants 26 (66.7%) had primary school education (Figure 4.4, Table 4.1).

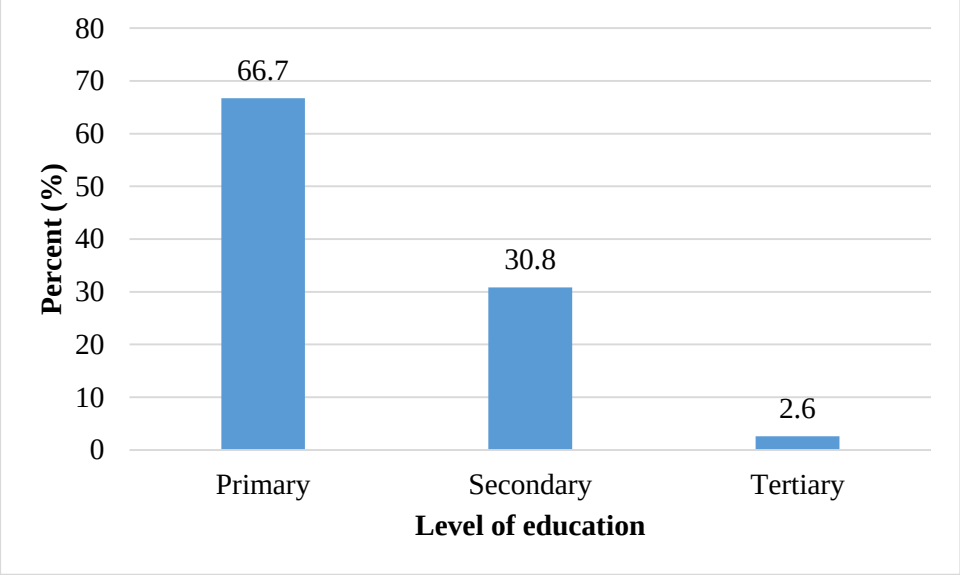


Figure 0.4: Level of education

Majority of the participants 15 (38.5%) were housewives (Figure 4.5, Table 4.1).

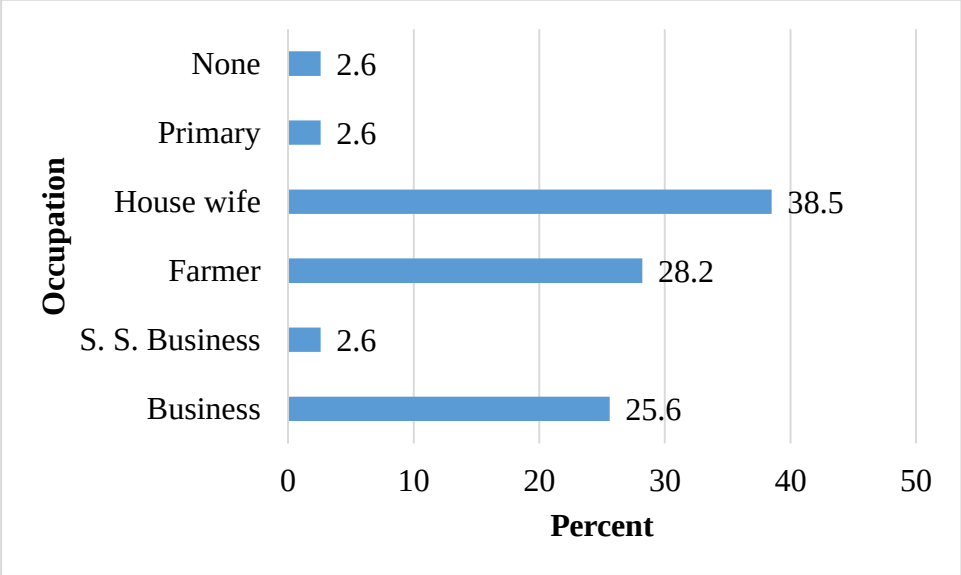


Figure 0.5: occupation

(Primary in the above chart represents Primary school teacher).

Majority of the participants 29 (74.4%) had cancer on their left breast (Figure 4.6).

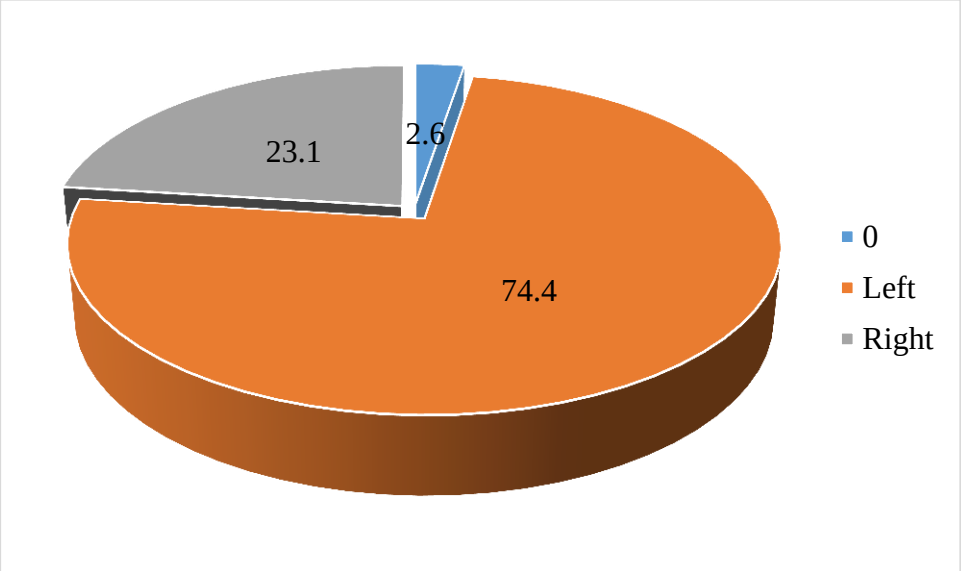


Figure 0.6: Laterality

The left breast was the most affected.

4.3 Cytomorphological features of breast cancer cases

Breast cancer cases were identified using cytomorphological features of fine needle aspirate (FNA) and core needle biopsies (CNB).

4.3.1 Fine needle aspirate

Breast cancer identification by the use of fine needle aspirate showed that majority of the study participants 31 (79.5%) had ductal carcinoma (Figure 4.3, Table 4.2)

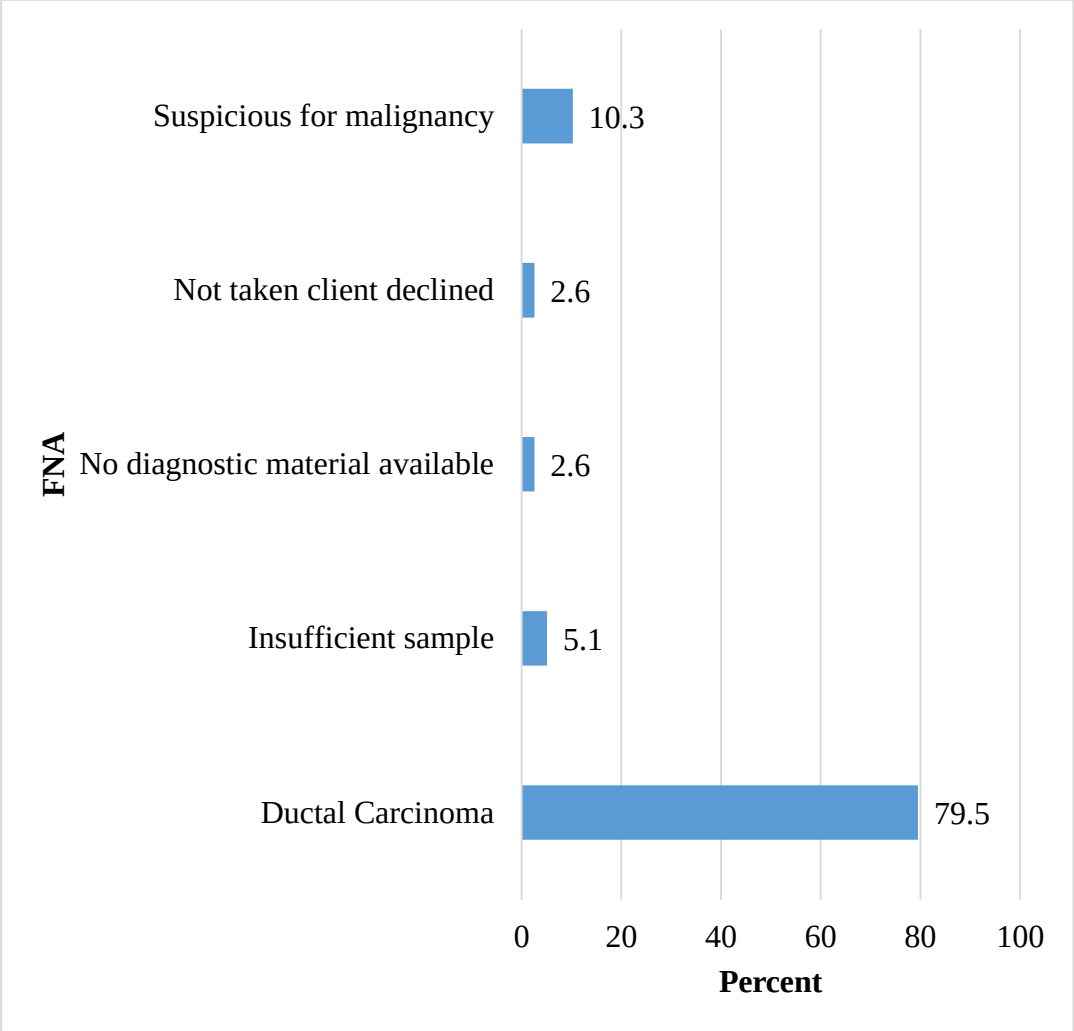


Figure 0.1: Fine needle aspirate (FNA) report

4.3.2 Core needle biopsies

Breast cancer identification by the use of core needle biopsies showed that majority of the study participants 17 (43.6%) had ductal carcinoma grade II (Figure 4.4, Table 4.2)

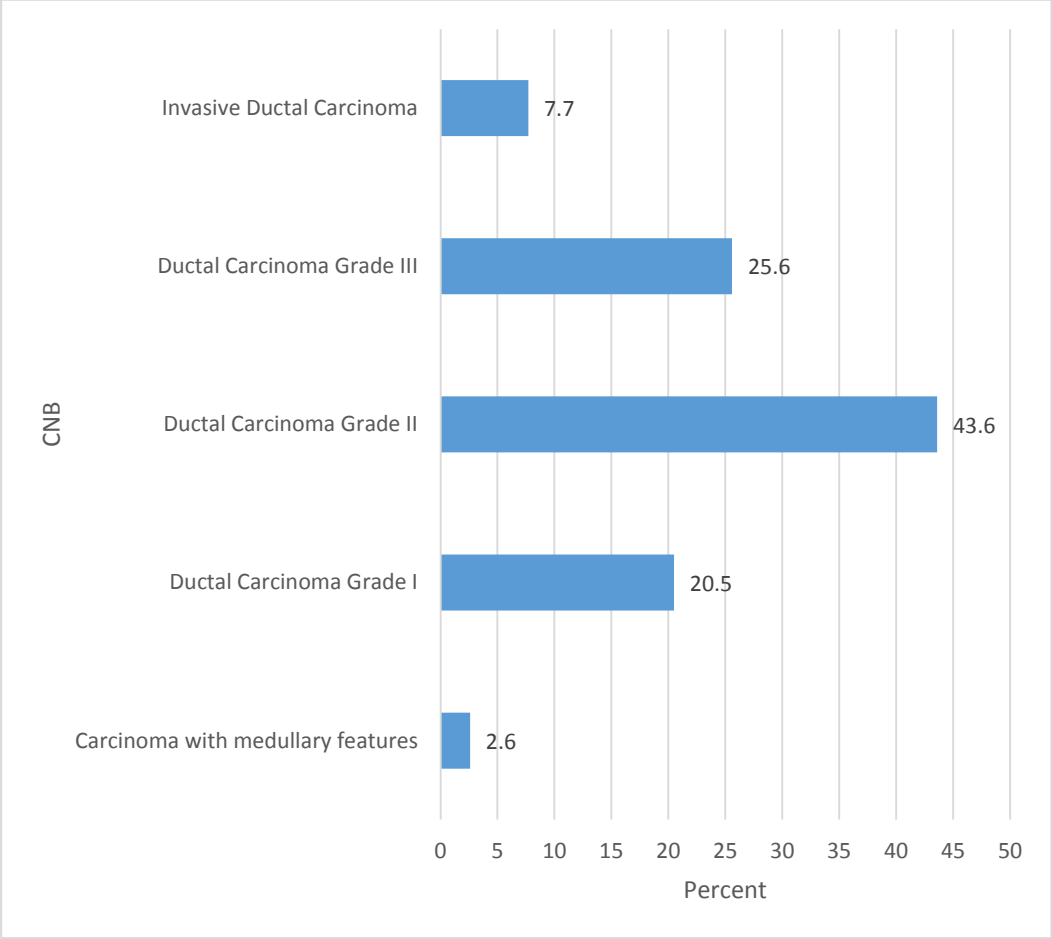


Figure 0.2: Core needle biopsies report

4.4 Protein expression

The Human epithelial growth factor receptor (HER2), Estrogen receptor (ER) and Progesterone receptor (PR) proteins were expressed by immunocytochemistry (ICC) and immunohistochemistry (IHC). The study findings showed that immunohistochemistry was more effective in identifying estrogen receptor, 20 (51.3) and progesterone receptor, 21 (53.8), and human epithelial growth factor receptor, 11 (28.2) (Table 4.3)

Table 0.1: Observed counts

Proteins	Immunocytochemistry (ICC)		Immunohistochemistry (IHC)	
	Frequency (n)	**Percent (%)**	**Frequency (n)**	**Percent (%)**
Estrogen receptor (ER)				
ER+	15	42.9	20	51.3
ER-	20	57.1	19	48.7
Progesterone receptor (PR)				
PR+	16	45.7	21	53.8
PR-	19	54.3	18	46.2
Human epithelial growth factor receptor (HER2)				
HER2+	8	22.9	11	28.2
HER2-	27	77.1	28	71.8

n **- Observed count;** % **- Percentage**

4.4.1 Staining on FNAC cell block vs CNB

Table 0.2: Comparison of ER, PR and HER2 staining on FNAC cellblock versus CNB (n = 35)

FNA Cell Block	CNB	Number of cases	Correlation	
ER+	ER+	15	Concordant 32/35 (91.4%)	*r*=0.842 Sensitivity 83.3%
ER-	ER-	17		
ER+	ER-	0	Discordant 3/35 (8.6%)	Specificity 85.0%
ER-	ER+	3		
PR+	PR+	16	Concordant 32/35 (91.4%)	*r*=0.842 Sensitivity 84.2%
PR-	PR-	16		
PR+	PR-	0	Discordant 3/35 (8.6%)	Specificity 84.2%
PR-	PR+	3		
HER2+	HER 2+	8	Concordant 34/35 (97.1%)	*r*=0.925 Sensitivity 88.9%
HER2-	HER 2-	26		
HER2+	HER 2-	0	Discordant 1/35 (2.9%)	Specificity 96.3%
HER2-	HER 2+	1		

ER, estrogen receptor; **PR**, progesterone receptor; **HER2**, human epithelial growth factor receptor 2; ***r***, correlation coefficient

4.4.2 Breast cancer subtypes

The study findings showed that majority of the study participants, 17 (43.6) for cell block and 16 (41.0) for histopathology, had triple negative breast cancer subtype (Table 4.4)

Table 0.3: Classification of Breast cancer subtypes

Breast cancer Cases subtypes	Cell block		Histopathology	
	N	%	n	%
Luminal A (ER+, PR+, HER2-)	7	17.9	10	25.6
Luminal B (ER+, PR+, HER2+)	8	20.5	9	23.1
HER2 Positive (HER2+, ER-, PR-)	1	2.6	2	5.1
Triple Negative (HER2-, ER-, PR-)	17	43.6	16	41.0

ER - estrogen receptor; **PR -** progesterone receptor, **HER2 -** human epithelial growth factor receptor 2; **n** - Observed count; **%** - Percentage

4.4.3 Classification of Breast cancer Cases

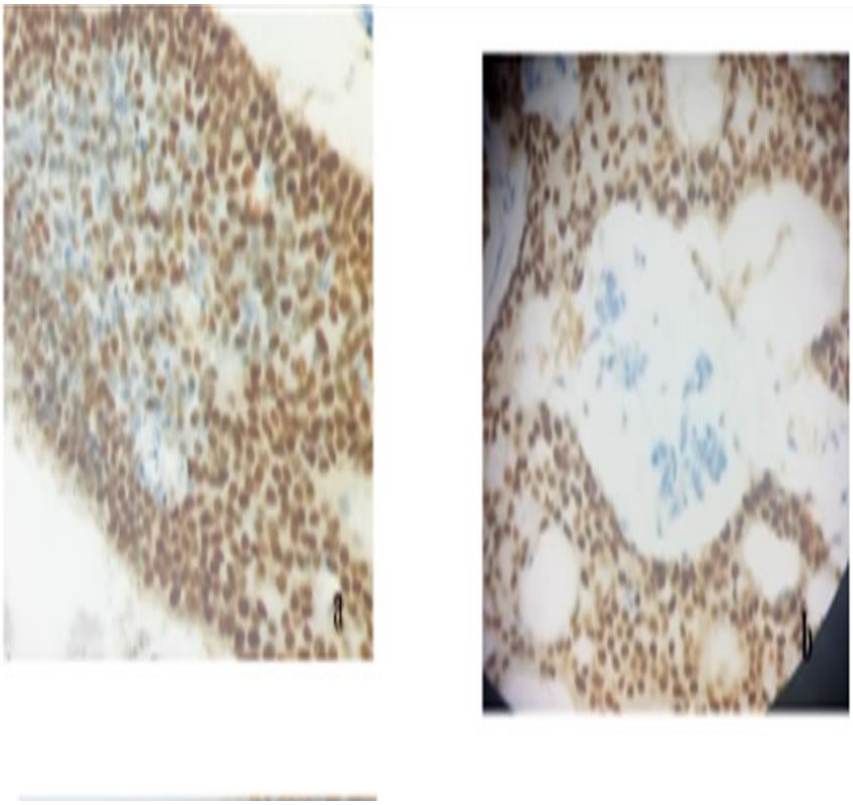
Cohen's κ was run to determine if there was agreement between fine needle aspirate and core needle biopsy. There was moderate agreement between the two methods, $\kappa = 0.719$, $p < .0001$ (Table 4.5).

Table 0.4: Classification of Breast cancer Cases

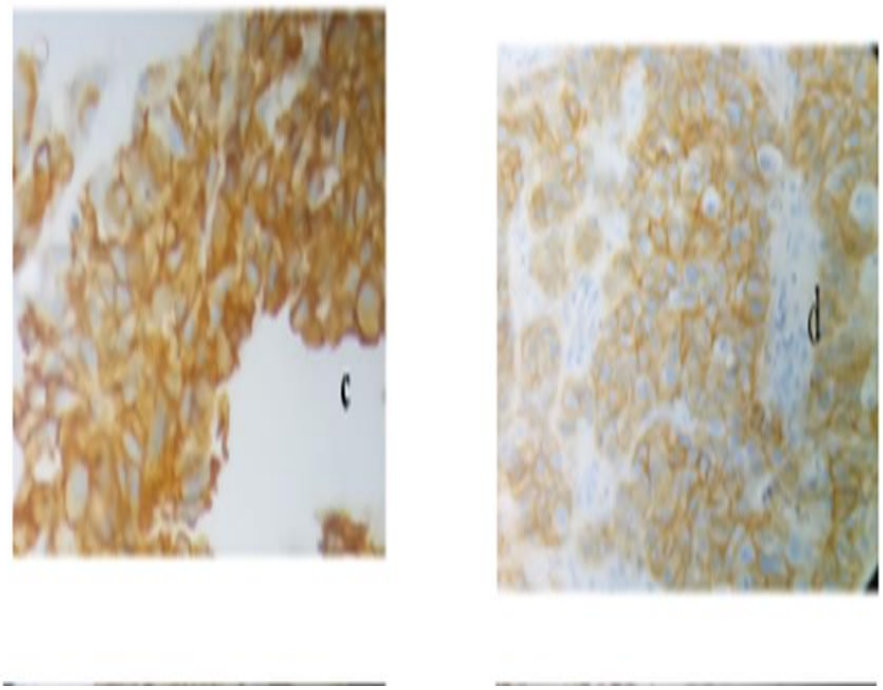
Classification of Breast cancer Cases	FNA/ICC		CNB/IHC			
	Number of cases (n)	Percent (%)	Number of cases (n)	Percent (%)	*K*	P
ER+, PR+, HER2+	7	17.9	9	23.1		
ER+, PR+, HER2-	7	17.9	10	25.6		
ER+, PR-, HER2-	1	2.3	1	2.6		
ER-, PR-, HER2-	17	43.6	16	41.0	0.719	<0.001
ER-, PR-, HER2+	1	2.6	2	5.1		
ER-, PR+, HER2-	2	5.1	1	2.6		
No tumor cells seen	4	10.3	0	0		

ER - estrogen receptor; **PR** - progesterone receptor, **HER2** - human epithelial growth factor receptor 2; **FNA** - fine needle aspirate; **CNB** - core needle biopsies; **ICC** - immunocytochemistry; **ICC** - immunocytochemistry;
n - Observed count; **%** - Percentage; ***K*** - Cohen's kappa test; **P** - P value (level of significance <0.05)

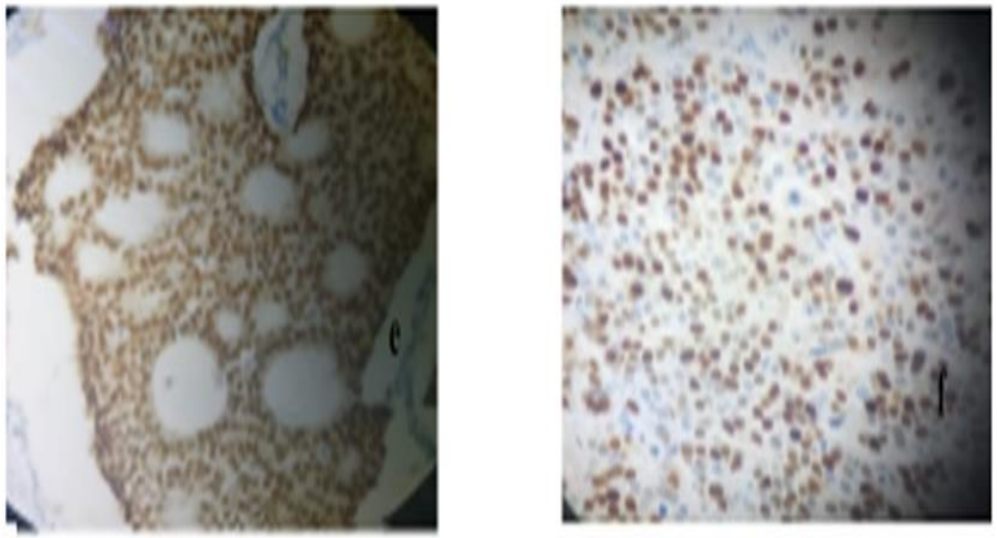
FNAC cell block and CNB figures on immunocytochemistry and histocytochemistry



(a) FNA cell block ER strongly positive with a proportion score of 5 and intensity score of 3 total score of 8.(b) CNB ER strongly positive with a proportion score of 5 and intensity score of 3 total score of 8.



(c) FNA cell block HER2 strongly positive with a score of 3+ (d) CNB HER2 positive with a score of 3+



e) CNB tissue block PR strongly positive with a proportion score of 5 and intensity score of 3 total score of 8. (f) FNA cell block PR strongly positive with a proportion score of 5 and intensity score of 3 total score of 8.

CHAPTER FIVE
DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

FNAC has widely been used as part of the assessment for breast cancer in patients with palpable breast masses suspected to be cancerous. When an FNA report is positive for breast cancer, the patients are normally subjected to core biopsy procedure for histology to confirm the breast cancer and subsequent immunohistochemistry for hormonal receptors ER and PR as well as for human epidermal growth factor receptor 2 HER2/neu (Hammond *et al* 2010). The core biopsy procedure has some disadvantages in that it is very expensive compared to FNA and exposes the patient to a more invasive painful procedure that has to be done under local anesthesia (Mardanpour *et al* 2012). It would be therefore sensible if both cancer diagnosis and immunostaining to determine HER2, ER and PR status would be done using FNA material. The current study has demonstrated that FNAC can be used to determine PR, ER and HER2 expression patterns. The study had ER positivity of 42.9% on cellblocks and 51.3% on histology which highly agrees with many Indian studies that have reported an ER positivity ranging from 40% to 45%. PR positivity was 45.7% on cell blocks and 53.8% on histology. This was very close to results obtained by Kyama *et al* 2018 on the positivity of PR on cell blocks which was 47%. HER2 positivity was 22.9% on cell blocks and 28.2% on histology and this highly compared to Bird et al report of HER2/neu over-expression in 26% of breast cancer cases among Kenyan women. When a correlation coefficient was done for cell block immunochemistry and tissue block immunochemistry, the results had a concordance of 32/35(91.4%) r=0.842 Sensitivity of 83.3% and specificity of 85.0% for ER. For PR the concordance was 32/35(91.4%) r=0.842 with sensitivity of 84.2% and specificity of 84.2%. For HER2 the concordance was 34/35 (97.1%) r=0.925 with a sensitivity of 88.9% and a specificity of 96.3%. Kumar et al 2012 did a similar study in 2012 and reported a concordance

between cell blocks and tissue blocks of 90% for ER, 94% for PR and 90% for HER2. Their results were very similar to our study findings and this means that the two methods highly correlates with each other. Nishimura *et al* 2016 did a study on HER2 immunochemistry on cell blocks and histology sections and reported a concordance of 77% between the two methods. That was slightly lower compared to the results obtained in the present study of HER2 concordance of 97.1%. The Cohen's kappa test was 0.719 indicating that there was an agreement between the two methods ICC and IHC. Most of the IHC and ICC results were concordant and many reasons would have attributed to the discordant results including but not limited to; low FNA cellularity, poor or inadequate fixation, inadequate antigen retrieval and errors in methodology. In the present study, the Allred scoring system was used to determine the ER/PR status whereby positivity in more than 10% of the tumor cells was considered to be positive. For HER2/neu, the grading for positivity was based on both proportion score and intensity in staining and a score of more than 3+ was considered positive. Previously paraffin embedded tissue sections IHC has been the method of choice for assessing ER, PR and HER2/neu status in patients with breast carcinoma. The present study perfectly agrees with the recent studies on assessment of HER2/neu, PR and ER expression patterns on cell blocks (Kyama *et al* 2018, Kumar *et al* 2012, Nyagol *et al* 2013).

5.2 Conclusion and recommendations

Based on the study outcome, we concluded that cell blocks prepared from FNA can be adequately used to assess HER2/neu and hormonal receptor expression patterns in patients with breast cancer provided that the FNA contains enough tumor cells (high cellularity). This would be a very affordable method especially in developing countries like Kenya with limited resources. Most of the study respondents were unemployed house wives from rural areas and had primary school education indicating they were either not informed on breast cancer awareness programs or they had limited resources to access breast cancer

screening. For most of them, the breast carcinoma had already advanced at the time of screening. It's therefore recommended that routine breast cancer screening and assessment of protein expression patterns using FNAC be made available, accessible and affordable to all for better management of breast cancer cases. Programs on breast cancer awareness should be made accessible to all and especially to individuals with little or no education to make them understand the risk factors of breast cancer and the initial symptoms of breast cancer to facilitate early diagnosis and proper management of breast cancer cases. The triple negative subtype was found to be the most common (43.6%) on cell block and (41.0%) on CNB. In as much as it seems to be the most difficult type to manage, the healthcare providers should be prepared on how to manage this subtype of cancer effectively. The study concluded that assessment of HER2, ER and PR on FNA cell blocks is a valid method since it highly correlates with results obtained from histology blocks. Therefore there is no point of taking tissue biopsy from cancer patients for the purpose of assessing HER2, ER and PR protein expression patterns.

5.3 Study limitations

The major challenge in the present study was getting the study subjects. The process of client recruitment took quite some time since the process encountered several challenges from Striking doctors at the study institution, non-consenting patients and few new breast cancer cases being diagnosed. Some FNA samples did not have enough material for evaluation.

5.4 Dissemination of study findings

The study findings were published in the journal of medical science and clinical research. A copy of the research was submitted to JKUAT board of post graduate and another copy submitted to JKUAT department of medical laboratory sciences. A soft copy of the work was submitted to KNH research committee and a copy submitted to KHN-UON ethics and research committee.

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1 APPENDICES

Appendix I: Informed consent

Introduction and objectives of the study

My name is Christine Osale, a postgraduate student pursuing a master of science in Medical laboratory Science Cytology/Histopathology at Jomo Kenyatta University of Agriculture and Technology. I am carrying out a study to determine breast cancer biomarkers (human epidermal growth factor receptor-2 (HER2), estrogen receptor (ER), and progesterone receptor (PR)) expression patterns in fine needle aspiration cytology samples of breast cancer patients using facilities at Kenyatta National Hospital. The testing of these biomarkers will help the doctor in decision regarding the best treatment regimen for your condition.

The objectives of the study are as follows:

The objective of the study is to determine breast cancer biomarkers expression pattern in fine needle aspiration cytology samples of selected patients with breast cancer in KNH.

Specific objective(s) of the research:

1. To identify breast cancer cases using cytomorphological features of fine needle aspirate.
2. To determine HER2, ER and PR protein expression by immunocytochemistry (ICC)
3. To compare HER2, ER and PR protein expression by immunocytochemistry (ICC) and immunohistochemistry

Benefits and Risks of the study to you:

POTENTIAL BENEFITS:

There may be direct benefit to the participant since the results will guide the clinician on the best treatment strategy of managing your breast cancer subtype.

Also assessment of the clinical utility of hormonal receptors and human epidermal growth factor receptor 2 using fine needle aspiration cytology samples to stratify breast cancer subtypes may be used for better management of your condition by the policy makers.

POTENTIAL RISKS

Some of the information required from the study participant may be personal.

There is minimal pain during sample collection and the procedure is minimally invasive.

I kindly request you to join the study and allow us to use the specimen to determine breast cancer and also determine breast cancer biomarkers expression pattern.

Confidentially

Names will not be required in the study, since you will be identified by study number.

Questionnaire will be kept under key and lock and only the principal investigator will access it.

Questionnaires will be kept for one year then destroyed. Any information given to us will remain confidential and will be for your benefit. You will get your results for conventional smear in the usual manner during your next visit.

Participation in this study will be voluntary and it is part of your routine evaluation and you are free to withdraw any time without losing the benefits to which you are entitled in this institution.

Contact information:

If you have any question regarding the study please contact me Christine Osaleon mobile number 0723104518

Supervisors:

Dr.MutundaKyama Mobile:0711169526

Dr. Mary Mungania Mobile: 0722820155

CONSENT FORM:

I ……. after reading and being explained the study purpose do hereby give informed consent to participate in the in the study fully aware of the benefits and risks. I have not been pressurized to participate in this study in any way. I understand that participation in this study is completely voluntary and that I may withdraw from it at any time and without loss of any benefit or quality of management to which I am entitled. I am fully aware that the results of this study will be used for scientific purposes and may be published.

Participant Signature: ……..……..Date……..

Doctor/Nurse Signature:…….……Date ……..

Principal investigator Signature:……Date ……..

Appendix II: Fomu ya idhini

KICHWA CHA UTAFITI:

Jina langu ni Christine Osale mwanafunzi wa chuo kikuu cha Jomo Kenyatta. Ningependa kubaini kipimo cha chembechembe za human epidermal growth factor receptor-2 na hormonal receptors kutoka kwa sampuli ya wagonjwa wanaougua saratani ya matiti katika hospitali kuu ya Kenyatta. Tafadhali soma ujumbe ufuatao kwa makini. Ujumbe huu utaelezwa kwa lugha ya kingereza na kiswahili. Una uhuru wa kuchagua lugha ambayo utaelewa vyema.

MALENGO YA UTAFITI HUU NI:

Kubaini kipimo cha HER-2, ER and PR kutoka kwa sampuli yawagonjwa wanaougua saratani ya matiti katika hospitali kuu ya Kenyatta.

MALENGO MAALUM YA UTAFITI HUU NI:

1. Kutambua kesi za saratani ya matiti ukitumia kipengele cha cytomorphology kutoka kwa fine needle aspirate.
2. Kubaini kiwango cha HER2, ER and PR protini tukitumia immunocytochemistry (ICC)
3. Kulinganisha HER-2, ER na PR tukitumia immunocytochemistry (ICC) na immunohistochemistry (IHC)

FAIDA NA MADHARA YA UTAFITI HUU KWAKO

Kunaweza kuwa hakuna faida ya moja kwa moja kwa mshiriki lakini habari inayotokana na matokeo inaweza kutumika kwa ajili ya usimamizi bora wa hali yako na watunga sera.

Tathmini ya uchunguzi huu ni kuweza kuwatambua wagonjwa wa saratani ya

matiti walio na kiwango cha juu cha chembechembe za homoni receptor na human epidermal growth factor receptor 2, jambo ambalo litaweza kuongoza daktari kuchangua matibabu bora ya wagonjwa walio na saratani ya matiti.

Kujitoa kutoka kwa utafiti

Kushiriki katika utafiti huu itakuwa ni kwa hiari yako na nisehemu ya tathmini yako mara kwa mara na unaweza kujiondoa wakati wowote bila kupoteza faida ambayo una haki katika taasisi hii.

MAWASILIANO YA HABARI

Kama una swali lolote kuhusu utafiti tafadhali wasiliana na mimi; Christine Osale Chuo Kikuu cha Jomo Kenyatta kupitia nambari ya simu 0723104518

FOMU YA IDHINI

Mimi ..... baada

Ya kusoma na kuelezewa madhumuni ya utafiti huu ili kutoa ridhaa ya kushiriki katika uchunguzi kikamilifu na ufahamu wa faida na hatari. Si kushinikizwa kushiriki katika utafiti huu kwa njia yoyote. Mimi naelewa ya kwamba ushiriki katika utafiti huu ni kwa hiari yangu kabisa na kwamba naweza kujiondoa wakati wowote bila hasara ya faida yoyote au ubora wa usimamizi. Mimi nimefahamu kikamilifu kwamba matokeo ya utafiti huu yatatumika kwa ajili ya madhumuni ya kisayansi na yanaweza kuchapishwa.

Sahihi ya Mshiriki..... ....Date ....

Sahihi ya Daktari / Muuguzi..... .....Tarehe..... ..

Sahihi ya Mkuu wa uchunguzi .....Tarehe..... ..

MUCHUNGUZI, Christine Osale Chuo Kikuu Cha Jomo Kenyatta

Nambari ya simu 0723104518

Wasimamizi

1. Dkt. Mutunda Kyama Mobile:0711169526
2. Dkt. Mary Mungania Mobile: 0722820155

Appendix III: Study questionnaire

Project title: Determination of human epidermal growth factor receptor-2 and hormonal receptor expression in fine needle aspiration cytology samples of breast cancer patients within Nairobi County.

Study number----------

Demographic data

1) Age ----------
2) Age at onset of menarche----------
3) Age at first full-term pregnancy----------
4) Breast feeding Yes ☐ No ☐
5) Age at onset of menopause----------
6) Parity----------
7) Physical activity Yes ☐ No ☐
8) History of breast cancer Yes ☐ No ☐
9) Family history of cancer Yes ☐ No ☐
10) Tobacco/cigarette smoking Yes ☐ No ☐
11) Oral contraceptive use? Yes ☐ No ☐
12) (a) Any other hormonal use Yes ☐ No ☐

(b) Type used ----------

13) Education level None ☐ primary ☐
Secondary ☐ Post secondary ☐

14) Marital status single ☐ Married ☐
Widowed ☐ other (specify)

15) What is your occupation? Housewife ☐ Farmer ☐

Business ☐ other specify

Appendix IV: Reporting results

FNAC smear results will be reported using the National Cancer Institute[78]

Results report form

Clinical Staging

Tumour size	< 2cm	☐	> 2cm	☐
Node	Yes	☐	No	☐
Metastasis	Yes	☐	No	☐

Breast Mass Location	Upper outer quandrant	☐
	Upper inner quadrant	☐
	Lower Outer quadrant	☐
	Lower Inner quadrant	☐
	Periariolar	☐

Fine needle aspiration cytology (FNAC) results (tick appropriately)

1. Unsatisfactory (C1) ☐
2. Cells present all benign; no suspicious features (C2) ☐
3. Cells suspicious but probably benign (C3) ☐
4. Cells suspicious but probably malignant (C4) ☐
5. Malignant (C5) ☐

Histopathological classification results (tick appropriately)

1. Ductal Carcinoma in situ (DCIS) ☐
2. Infiltrating or invasive ductal carcinoma (IDC) ☐
3. Medullary carcinoma ☐
4. Lobular Carcinoma in situ (LCIS) ☐
5. Infiltrating Lobular Carcinoma (ILC) ☐
6. Tubular carcinoma ☐
7. Mucinous carcinoma or colloid ☐
8. Paget's disease ☐
9. Inflammatory breast cancer ☐
10. Metastatic breast cancer ☐

Grading

1. Well differentiate ☐
2. Moderately differentiated ☐
3. Poorly differentiated ☐

Appendix IV: Immunochemistry

Immunocytochemical staining for ER, PR and HER2 will be performed on 4 μm paraffin sections cellblocks. Immunostains for each marker will be performed after the antigen retrieval according to established protocols.

ESTROGEN AND PROGESTERONE IMMUNOCYTOCHEMICAL STAINING

The cellblock sections mounted on slides will be deparaffinised by use of xylene followed by alcohol washes. The slides will then be exposed to 3% hydrogen peroxide for 5 minutes. They will then be incubated with primary antibody for 30 minutes and with labelled polymer for 30 minutes, 3,3′-diaminobenzidine (DAB) as a chromogen for 5 minutes, and hematoxylin as counterstain for 5 minutes at room temperature. Between incubations sections will be washed with Tris-buffered saline (TBS). Cover-slipping will be performed using the Tissue-Tek SCA coverslipper. **HER2 IMMUNOCYTOCHEMICAL STAINING**

After deparafinization the slides will be exposed to 200 μL peroxidase-blocking reagent for 5 minutes, followed by rinsing tris buffer and then placed in 200 μL primary anti-HER2 protein for 30 minutes. They will then be rinsed twice before being immersed in 200μL substrate chromogen solution for 10 minutes. The slides will be counterstained with hematoxylin, and finally coverslipped. HER2 results will be determined based on the maximum area of staining intensity, according to the package insert and ASCO/CAP guidelines

NB

Positive controls of known positive breast cancer tissues and negative controls of normal breast tissues and/or negative control of breast cancer tissues incubated without primary antibody will be run with the patient/study slides in all procedures.

Appendix V: Papanicoloau staining method

A conventional smear will be stained using PAP stain and examined microscopically by PI and the pathologists to report and sign out.

PAPANICOLOAU STAINING METHOD

Principle of the stain

Haematoxylin stains the nuclei blue by dye lake formation. The eosin azure solution being acidic stains the cytoplasm. The eosin stains the mature cells while light green stains the young cells. Orange G stains the cytoplasm and stains keratin.

Staining technique

1. Fix the smear in 95%ethanol

2. Hydrate smears through ethanol grades of 80%70%and then 50%

3. Rinse in distilled water	10 dips
4. Stain in Harris haematoxylin for	4 minutes
5. Rinse in tap water.	
6. Differentiate in 0.05% acid water	10 dips
7. Rinse in tap water and blue in Scott's tap water	10 dips
8. Rinse in 95% ethanol	10 dips
9. Stain in O.G 6 for	2 minutes
10. Rinse in 95% ethanol	10dips

11. Stain in E.A.50 for	4 minutes
12. Rinse in 95% ethanol	10 dips
13. Dehydrate in changes of absolute ethanol	10 dips each
14. Clear in 3 changes of xylene	10 dips each
15. Mount in D.P.X	cover-slip

Appendix VI: Haematoxylin and Eosin (H&E) staining method

A conventional smear and /or cellblock section of about four microns will be cut and labelled with patient's identification number. It will be stained using H/E stain and examined microscopically by PI and the pathologists.Any available tissue section of about four microns will be cut used in the histological classification breast cancer.

Haematoxylin and Eosin (H&E) staining method.

1. Bring sections to distilled water
2. Stain nuclei with the alum haematoxylin
3. Rinse in running tap water
4. Differentiate with 0.3% acid alcohol
5. Rinse in running tap water
6. Rinse in Scott's tap water substitute
7. Rinse in tap water
8. Stain with eosin 2 mins
9. Dehydrate, clear and mount.

Appendix VII: Cell block preparation

In brief, 10–20 ml of the aspirate will be centrifuged and supernatant discarded.

Two drops of pooled plasma will be added, followed with gentle shaking.

Thereafter, two drops of thromboplastin will be added and mixed well to activate clotting factor, followed with two drops of calcium ion.

The mixture will be left to stand for 5 min, followed with transfer of the clot to moistened filter paper.

The clot will be wrapped well, put in a cassette, and then fixed in 10% neutral buffered formalin for at least 6 hours.

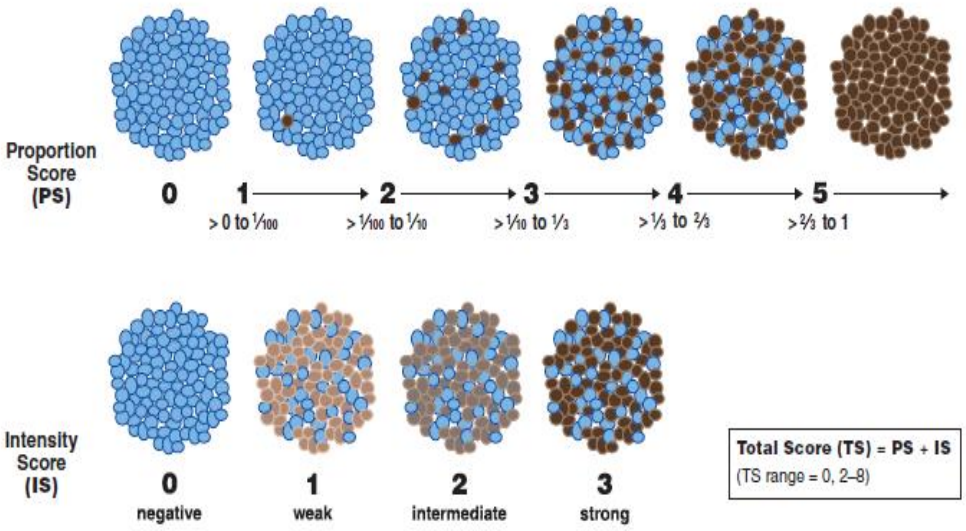
The sample will be then processed using the routine tissue processor.

Appendix VIII; ER/PR Allred Scoring System

Allred scoring stratifies a breast cancer patient's ER/PR status into cancers that are likely to respond to hormone therapy with tamoxifen.

Proportion Score (PS)	Observation	Intensity Score (IS)	Observation
0	None	0	None
1	>1%	1	Weak
2	1 – 10%	2	Moderate (Intermediate)
3	10 – 33%	3	Strong
4	33 – 66%		
5	66 – 100%		

Total Score	Interpretation
Sum of proportion score and intensity score	
0 – 2	Negative
3 – 8	Positive



- A Proportion Score (PS) is assigned representing the proportion of tumor cells with positive nuclear staining.
- An Intensity Score (IS) is assigned representing the AVERAGE staining intensity of all positive tumor cells.

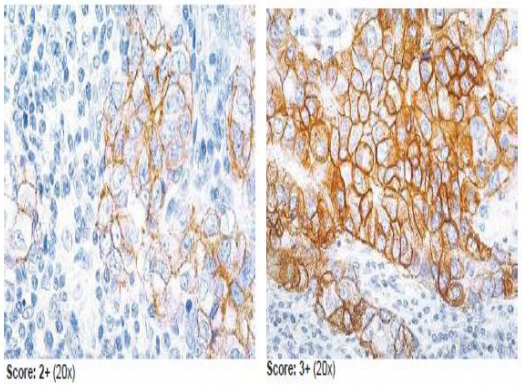
Appendix IX; Scoring system for HER2

Guidelines for Scoring HercepTest™ - Breast

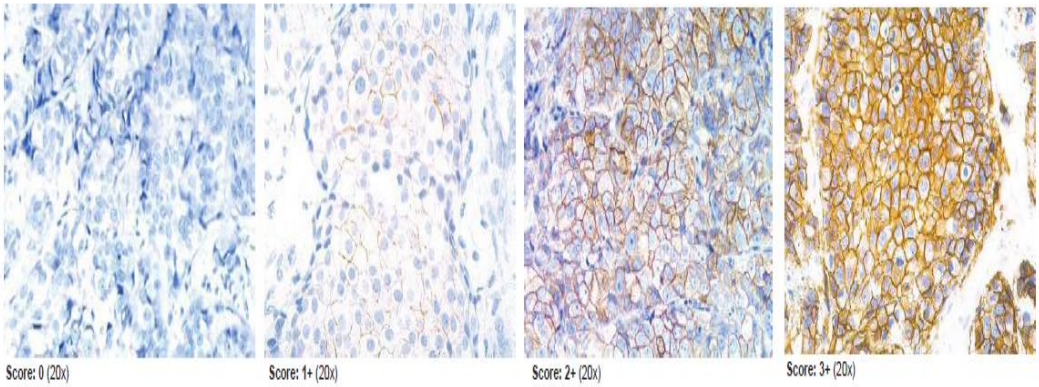
Score to Report	HER2 Protein Overexpression Assessment	Staining Pattern
0	Negative	No staining is observed or membrane staining is observed in less than10% of the tumor cells.
1+	Negative	A faint/barely perceptible membrane staining is detected in more than10% of the tumor cells. The cells are only stained in port of their membrane.
2+	Weakly Positive* (Equivocal)	A weak to moderate complete membrane staining is observed in more than10% of the tumor cells.
3+	Strongly Positive**	A strong complete membrane staining is observed in more than 10% of the tumor cells.

* Weakly positive cases (2+): May be considered equivocal and reflexed to FISH testing.

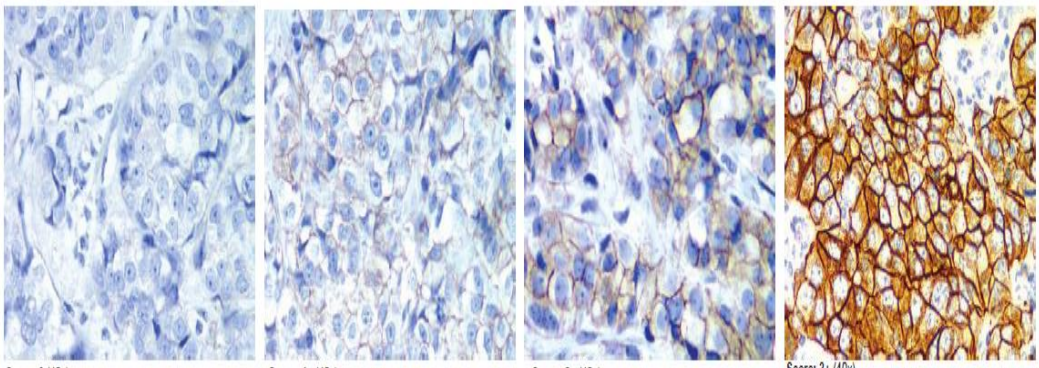
** Strongly positive cases (3+): Based on recent testing guidelines a 30 percent cut-off for reporting positivity is recommended. FDA-approved scoring guidelines recommend a 10 percent cut-off for reporting positivity. Patient outcome for cases resulting between 10 percent and 30 percent positivity has not been defined. FISH may be used as a complementary test in these instances.



Score: 2+ (20x) Score: 3+ (20x)



Score: 0 (20x) Score: 1+ (20x) Score: 2+ (20x) Score: 3+ (20x)



Score: 0 (40x) Score: 1+ (40x) Score: 2+ (40x) Score: 3+ (40x)