CHARACTERIZATION OF BREAST CANCER INTRINSIC SUBTYPES IN RELATION TO RISK FACTORS, TUMOR GRADE AND TUMOR INFILTRATING LEUKOCYTES IN WESTERN KENYA

BY

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DECLARATION

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ABSTRACT

Breast cancer contributes to 23% of all female cancers and is mostly developed among women aged 40-49 in Kenya. There is paucity of data explaining why breast cancer in Kenya and other African countries occurs at a younger age, although many risk factors were identified and studied in Western countries, there is a lack of research on the consistency of these risk factors among developing countries. Breast cancer seen in Africans is likely to be high-grade and hormone receptor negative, however profiling of breast cancer by hormonal receptor status is not documented because this is not routinely done in most Kenyan hospitals. Breast cancer in Kenya is uniquely aggressive and seem different for each individual. The explanation for this may be related to how an individual's immune system mounts a response to cancer antigens. There was need to determine the type and density of immune cell infiltration in breast tumors of Kenyan women. This study was conducted at Moi Teaching and Referral Hospital (MTRH) which is located in Uasin Gishu County. The study determined the risk factors of breast cancer; characterize breast cancer into intrinsic subtypes, determining the type and density of TILs in tumor microenvironment and correlated this across grades and subtypes. A comparative cross-sectional study design was used to collect data from 160 participants who consented. Sixty nine breast cancer cases and 91 non cancer controls were consecutively enrolled from May 2011 to May 2013. Structured pre-tested, interviewer administered questionnaire was used to collect data on demographics, family history, age at first menarche, and number of pregnancies, breast feeding, use of contraceptives, smoking, alcohol consumption and other environmental factors. Tissue micro assays (TMAs) were constructed from all breast tissues then stained with heamatoxin and eosin for histological typing and grading. Immunohistochemical technique was used to stain for a panel of primary antibodies ER, PR, HER2, Ki67, CD4, CD8, CD20, CD68, CD163, and CD25. Immunohistochemistry (IHC) images were quantified with Aperio Image Analysis Tools software, output results were exported as an Excel file. Data was summarized using frequencies for categorical variables and median (IQR) for continuous/discrete variables. Multiple binary logistic regression was used to identify risk factors of cancer controlling for confounders. Ki67 and TILs markers were compared across grade and molecular subtype by Kruskal-Wallis followed by Dunn's multiple comparison tests. Level of significance was set at $p < 0.05$. Marital status and environmental factors such as exposure to wood smoke are high level risk factors to breast. Alcohol consumption was a significant risk factors of breast cancer (p=0.029). The Kalenjin tribe were more likely to be cases compared to other tribes (OR; 95%CI: 3.192(0.661-15.404) though not statistically significant. Similarly, those using injection for contraceptive are more likely to be cases (OR; 95%CI: 4.499(0.735-27.545)). The mean age of the study population was 48.4 (SD 16.8). The tumors analyzed were heterogeneous by grade: grade I (5.8%), grade II (53.8%), and grade III (40.4%). Most patients presented with large tumors (>2.0cm) (80%). Invasive ductal carcinoma was the predominant (79%) histological type. Intrinsic subtypes were; luminal B (30.2%), basal/triple negative (TN) (34%), luminal A (26.4%) and HER2 (2%). There was a significant increase in percentage of tissue and alternative macrophages ($CD68^+$, $CD163^+$/M2 respectively) ($p \leq 0.0001$) in cancer and non-cancer individuals. Cancer tissues showed an increase infiltration of $CD4^+$ (helper) and $CD25^+$ (inducible regulatory) T cells ($p = 0.03$; $p=0.0001$ respectively), $CD8^+$ and $CD20^+$ showed no significance. TNBC subtype had a much higher proliferative index ($Ki67^+$) than the other intrinsic subtypes, there was no significant correlation between TIL type and density across subtype and tumor grade. Findings of the current study suggest sporadic genetic changes triggered by environmental, social and cultural changes are associated to the early onset of breast cancer. Routine staining for IHC4 clinical markers in breast cancer tissues will enable the identification of patient subgroups with different treatment requirements.

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ABBREVIATIONS

AMPATH	Academic Model Providing Access to Healthcare
BBC	Bilateral Breast Cancers
BRCA	Breast Cancer Antigen
CD	Cluster of Differentiation
CGHFBC	Collaborative Group on Hormone Factors in Breast Cancer
CMF	Cyclosphosphamide Methotexate and Fluorouracil
DAB	Diaminobenzidine
DCIS	Ductal Carcinoma *in Situ*
DCs	Dendritic Cells
DHEA	Dehydroepiandroepiadrosterone
ECD	Extracellular Domain
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
ER	Estrogen Receptor
ERBB2	Receptor Tyrosine-Protein Kinase erb-2
FFTP	First Full Term Pregnancy
FNA	Fine Needle Aspirate
GMCSF	Granulocyte Monocyte Colony Stimulating Factor

GPR	G Protein Receptor
GPR30	G-Protein-Coupled Receptor 03
HER2	Human Epidermal Growth Factor Receptor 2
HIER	Heat Induced Epitope Retrieval
HRT	Hormone Receptor Treatment
HSP	Heat Shock Protein
HSP90	Heat Shock Protein 90
IARC	International Agency for Research on Cancer
IBC	Inflammatory Breast Cancer
IDC	Invasive Ductal Carcinoma
IDC-NOS	Invasive Ductal Carcinoma Not Otherwise Specified
IDC-NST	Invasive Ductal Carcinoma of no Specific Type
IFN-γ	Interferon Gamma
IHC	Immunohistochemistry
IL	Interleukin
ILC	Invasive Lobular Carcinoma
IREC	Institutional Review Ethics Committee
KBHP	Kenya Breast Health Program

KEMRI Kenya Medical Research Institute

KI-67 Kiel Clone 67

LA Luminal A

LB Luminal B

LCIS Lobular Carcinoma *in Situ*

LMIC Low and Middle Income Countries

M2 Non- Classical Activated Macrophages

MCP Macrophage Chemotactic Protein

MHC Major Histocompatibility Complex

MI Classical Activated Macrophages

MIF Macrophage Inhibitory Protein

MMP Matrix Metalloproteinase

MMTV Mouse Mammary Tumor Virus

MTRH Moi Teaching and Referral Hospital

NCD Non Communicable Disease

NCI National Cancer Institute

NIH National Institute of Health

NK Natural Killer

OS	Overall Survival
pCR	Pathological Complete Response
PR	Progesterone Receptor
PT	Pre-Treatment
SEER	Surveillance, Epidemiology and End Results
SPSS	Statistical Package for Social Scientists
TAMs	Tumor Associated Macrophages
T_{FH}	T Follicular Helper
TGF	Tumor Growth Factor
Th	T Helper
TILs	Tumor Infiltrating Leukocytes
TMAs	Tissue Microarrays
TN	Triple Negative
TNBC	Triple Negative Breast Cancer
WHI	Women's Health Initiative
WHO	World Health Organization

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DEFINITION OF OPERATIONAL TERMS

Risk factor is anything affecting chance of getting a disease, risk factors don't tell us everything.

Age at diagnosis referred to the age at which a patient was diagnosed with breast cancer.

History of breast cancer referred to family of breast cancer, thus whether a woman had a relative with the disease, either mother, father, sister, brother or aunt

Menstrual history referred to age at menarche and age at menopause.

Age at first menarche meant the age at which an individual started menstruating.

Menstruation is the "shedding of the outer two-thirds of the endometrium with accompanying bleeding as a result of a lowering of estrogen secretion by ovaries at the end of the monthly cycle"

Age at menopause meant the age at which a woman went through menopause.

Menopause referred to the cessation of menstruation.

Women were classified as menopausal, if they had not menstruated during the past year before the date of data collection and the reason for their menstrual period stopping was "natural menopause."

Ethnicity is Someone's cultural background or where one comes from.

Tribe is used interchangeably with ethnicity

Passive smoking is breathing second hand smoke

Environment is the living and working conditions as well as physical, biological, social and cultural responses to these condition

Participants are People who consented to participate in the study

Non-modifiable risk factors are risk factors that cannot be changed

Modifiable risk factors are the risk factors that can be changed because they represent lifestyle choices.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Breast cancer is the second most commonly diagnosed cancer among all populations after lung cancer. It causes the greatest number of cancer-related deaths in women worldwide. According to World Health Organization (WHO) latest global estimates, 522,000 women died of breast cancer in 2012 (IARC, 2013). In the United States, about 12% of women develop invasive breast cancer in their lifetime (American Cancer Society, 2013). The mortality rate due to breast cancer in the developed world has dropped dramatically (Tsu, *et al.,* 2013; Forouzanfar *et al*., 2011). This is as a result of extensive research in cancer diagnosis during the last decade and innovation of advanced technologies for treatment. In contrast, developing countries are increasing in both cancer incidence and mortality rate (Tsu *et al.,* 2013;Forouzanfar *et al*., 2011).

In sub-Saharan Africa the burden of cancer is rising and the region is predicted to have greater than 85% increase in cancer burden by 2030 (Briton *et al*. 2014). According to Shulman *et al*., (2010), regional mortality rate among all breast cancers was as follows: 48% in low-income, 40% in low-middle income, 38% in high-middle income and 24% high-income countries. Globally, 70% of cancer burden is in low and middle-income countries (Kenya cancer statistics & national initiatives, 2013). In Kenya, cancer ranks third as one of the most common disease causing death in the country. The common types of cancer are breast and cervical cancer for women with age adjusted death rate of 15.76 per 100,000 of population placing Kenya at rank number 102 in the world (Kenya cancer network, 2011).

Estimations made by Kenya cancer network indicate that cancer is the third highest cause of morbidity after infectious diseases and cardiovascular diseases and accounted for about 7% of

deaths per year (Kenya cancer network, 2011). Breast cancer contributed to 23% of all female cancers and is mostly developed among women aged 40-49 (Ministry of Public Health, 2012). Seventy-five percent of Kenyan women die within 5 years of their breast cancer diagnosis. In Nairobi alone, 1,000 new cases are reported every year (Nyagol *et al*., 2006). The regional cancer registry at Kenya medical research institute (KEMRI) in Nairobi found about 80% of reported cancer cases were diagnosed at a late stage and were not treatable by the time the patient sought care (Kenya cancer network, 2011).

In Moi Teaching and Referral Hospital (MTRH) breast cancer is the second most common cancer in women after cervical cancer (Tenge, *et al*., 2009; Kadhel and Multigner, 2014). Being the second national referral center in Kenya, MTRH through AMPATH-Oncology department provide care and treatment for cancer patients from all over Western Kenya.

Although age frequency distributions have revealed that African women were younger at diagnosis compared to European women (Kadhel and Multigner, 2014), little is known about the natural history, demographics, incidence and molecular variations of breast cancer in Kenya (Alterman *et al*. 2008, Hayanga and Newman, 2007). Breast cancer in Africans appears in low prevalence rate, however, it is more aggressive than in women from European origin (Fregene and Newman, 2005; Easton, 2005) and is likely to kill by the age of 40 years (Easton, 2005). It is not clear if risk factors associated with development of breast cancer in African women are similar to those recorded in breast cancer patients from outside Africa. Risk factors accounting for differences in prevalence rates include non-modifiable (gender, age, genetic susceptibility, history of breast cancer, ethnicity and menstrual history) and modifiable (socio- demographic profiles, lifestyle behaviors and reproductive factors) (Kluttig and Schmidt-Pokrzywniak, 2009).

Women breast cells are constantly exposed to growth–promoting effect of estrogen and progesterone. This alone predisposes women to cancer more than men. Age increases the risk of breast cancer in women (Fregene and Newman, 2005). Family history of both first and second degree relatives increases the risk of developing breast cancer (Chen *et al*., 1999). Early menarche (<12 years) and late menopause (>55 years) increases the duration of estradiol and progesterone that increases the risk of cancer (Grant, 2008) while late menarche leads to lower endogenous estrogen levels thereby reducing breast cancer risk (Fregene and Newman, 2005). Long term use of oral contraceptive may have an increased risk of breast cancer (Fox 2006). Multiple parity has a protective role for it lowers endogenous estrogen levels over a life time therefore reduces the risk of breast cancer (Fregene and Newman, 2005). Prolonged lactation has been reported to reduce breast cancer risk since breast feeding for a longer period decreases cumulative number of ovulation menstrual cycles which in turn reduces the risk of breast cancer (Fregene and Newman, 2005).

Although there is incomplete cancer registration in African, it has been estimated by GLOBOCAN that by 2050 the incidences of breast cancer will double majorly in women under the age of 65 years (Sighoko *et al*., 2013). The young average age at diagnosis of breast cancer in African women is partly due to shorter life expectancy. Development of breast cancer at a younger age in Africans compared to Caucasians (Brinton, *et al*., 2008a), suggest that there could be additional risk factors involved including genetic and environmental factors or an interplay of the two. This study will identify risk factors associated with development of breast cancer in Kenyan women with the intension of providing information that will help prevent this upcoming pandemic.

Breast cancer molecular subtypes have been categorized according to the immunohistochemistry results for ER, PR, HER2 and Ki-67 termed IHC4 score, as recommended by the 12th International

Breast Conference (Zhang *et al*., 2015) as follows: Luminal A type (LA): ER or/and PR positive, HER2 negative and Ki-67 < 14%, Luminal B type (LB): ER or/and PR positive, HER2 negative and Ki-67 ≥ 14%, HER2 amplified type (HER2): ER and PR negative and HER2 overexpressed or/and amplified; Triple-Negative type (TN): ER, PR and HER2 negative. These intrinsic subtypes have varied survival rates and also respond differently to treatment (Cadoo, *et al.*, 2013). Routine classification of breast cancer (based on hormonal receptor status) is practiced in most developed countries (Galukande, *et al*., 2014). In countries where severe resource constraints exist, the practice of profiling breast cancer by hormonal receptor status is not routinely done and therefore characterization of breast cancers into intrinsic subtypes is not well documented (Galukande *et al.*, 2014). Therefore this study characterized breast cancer into four intrinsic subtype by staining for ER, PR, HER and Ki67 in breast tissues from Kenyan women.

Most standard care and treatments used globally for treating breast cancer are derived from research on patient populations from North America and Europe (Bird, *et al*., 2008). However, the disease etiology, progression, and response to treatments can be quite heterogeneous across patient populations. For example, in the U.S., African American women develop breast cancer that has early-onset, high-grade, node-positive, and hormone receptor-negative. Also, in contrast to global increases in breast cancer incidence and mortality, the U.S. breast cancer mortality declined as much as 34% since 1990 (Fregene and Newman, 2005; DeSantis, *et al*., 2014). However, the decline is not consistent across patient groups and varies significantly by race/ethnicity. Non-Hispanic white women have the highest incidence of breast cancer, while African American women have the highest mortality rate associated with breast cancer (DeSantis, *et al*., 2011). From 2006-2010, African American women had the highest mortality rates that are cancer related (30.8 deaths per 100,000 females) compared to non-Hispanic whites (22.7 deaths per 100,000 females)

and had the lowest 5-year cause-specific survival (78.9%) compared to non-Hispanic whites (88.6%) (DeSantis *et al.,* 2014; Fang *et al.,* 1996). In Kenyan death rate is even higher (34 deaths per 100,000) according to records from Kenya Cancer Statistics & National Initiatives (2013) and less than 30% of these patients remain alive for 5 years following their breast cancer diagnosis.

Ideally the immune system destroys and inhibits tumor growth. However, down regulation of the immune system can instead promote tumor progression, given the proper context. Classifying the ability of an individual immune cells to promote or inhibit cancer progression can be difficult because the immune cell function can be influenced by the tumor microenvironment that is made up of other immune cells. Such that tumor infiltrating leukocytes (TILs) can be activated, regulatory, or anergic (Krell *et al*. 2012).

Recent research has led to increased development and application of immunotherapy as cancer treatment (Denkert *et al*., 2013; Loi *et al*., 2014a). Both the type and density of TILs contributes to the host immune response, however the role of these cells in malignancy (Krell, *et al*., 2012) is not clear. In breast cancer, infiltration of breast tumors by effector T cells correlates with better prognosis (DeNardo *et al*., 2011; Galon *et al*., 2012; Loi *et al.*, 2014b), and infiltration of $CD8^+$ T cells positively correlates with tumor grade (Mahmoud *et al*., 2011). In contrast, infiltration of $CD25^+$ regulatory T cells promotes tumor growth and progression by suppressing the effector function of cytotoxic cells (Nedergaard, *et al.,* 2007). Also, $CD4^+$ T cells in tumors have plasticity and have both anti- and pro-tumor roles (Sharma *et al*., 2009).

In African women, breast cancer is diagnosed at a young age and is aggressive, with tumors that are of advanced grade, large in size, and triple negative (TN; ER-, PR-, HER2-) (Adebamowo *et al.,* 2003; Huo *et al*., 2009; Galukande *et al*., 2014). The cause of this aggressive cancer has not been elucidated but may be related to the immune response of these patients. The heterogeneity of

recruited immune cells to the tumors might vary between cancer subtypes, suggesting that different immune cell populations may have different roles to the tumor suppression or progression that are specific to a given subtype (Salgado *et al*., 2015). The variable density and type of immune cells within breast tumors may trigger the aberrant immune responses in these patients. The current study determined if the immune cells that infiltrate the tumors of breast tumors from Kenyan patients might be related to breast cancer subtype and/or tumor grade. Since a significant percentage of breast cancer patients in Africa present with aggressive breast cancer with poor prognosis, including many patients with TNBC breast cancer, these patients may not benefit from standard therapies and may require alternative therapies, such as immune therapy. Understanding the pattern and function of immune cell infiltration in aggressive tumors of African women is a step towards unfolding potential therapeutic targets to this population.

1.2 Statement of the Problem

There is paucity of data to explain why breast cancer in Kenya and other African countries occurs at a younger age and is more aggressive than the western type age-for-age and stage-for-stage. Although many risk factors were identified and studied in Western countries, there is a lack of research on the consistency of these risk factors among developing countries. No data on the risk of breast cancer have been reported in Kenya. Furthermore, little is known of the natural history, demographics and incidence breast cancer in Kenya. It was therefore necessary to find out if risk factors associated with development of breast cancer in Kenyan women are similar to those recorded in breast cancer patients from Western countries. High mortality rate experience by breast cancer patients in the Kenya is due in part to lack of access to health care for both early detection and treatment of disease, these mortality rates also reflect a differential incidence of intrinsic subtypes of breast cancer with poor prognosis across patient populations. A country like Kenya

where severe resource constraints exist, the practice of profiling breast cancer by hormonal receptor status is not routinely done and therefore characterization of breast cancers by intrinsic subtypes is not well documented. Studies have indicated that characterizing breast cancer into intrinsic subtypes helps in identifying cancer with poor prognosis across patient populations. There was need therefore to characterize breast cancer into intrinsic subtype by staining for ER, PR, HER and Ki67 in breast tissues from Kenyan women. The biological behavior of breast cancer seen in most patients in Kenya is uniquely aggressive and seem different for each individual. The explanation for this scenario has not been elucidated but may be related to how an individual's immune system mounts a response to cancer antigens. The heterogeneity of recruited immune cells to the tumors might vary between cancer subtypes, suggesting that different immune cell populations may have different roles to tumor suppression or progression that are specific to a given subtype. There was need to determine the type and density of immune cell infiltration in breast tumors of Kenyan women.

1.3 Research Objectives

1.3.1 General objective

To examine breast cancer risk factors, histological types, grades, molecular subtypes and role of tumor infiltrating lymphocytes in breast cancer in Western Kenya.

1.3.2 Specific objectives

i. To identify modifiable and non- modifiable risk factors associated with breast cancer.
ii. To determine the Histological and molecular subtypes of breast cancer seen in patients from western Kenya.
iii. To identify type and density of tumor infiltrating leukocytes ($CD4^+$, $CD8^+$, $CD20^+$, $CD25^+$, $CD68^+$, $CD163^+$) in breast cancer microenvironment.
iv. To correlate type and density of TILs across breast cancer grades and intrinsic subtypes

1.4 Research Questions

i. What are the modifiable and non-modifiable risk factors associated with breast cancer?
ii. What are the histological and molecular subtypes of breast cancer seen in patients of Western Kenya?
iii. What type and density of tumor infiltrating lymphocytes is seen in breast cancer microenvironment?
iv. What is the relationship between the TILs and breast cancer grades and subtypes?

1.5 Significance of the Study

Breast cancer, unlike other type of cancers, is often preventable or highly treatable if diagnosed early and different therapies targeting breast cancer subtypes have proven effective. But this will continue being an illusion in Kenya if factors that can lead to early diagnosis of breast cancer are not determined. Profiling of molecular subtypes is a step to the primary prevention strategies demonstrated by many cancer associations. Determining expressed patterns of clinical markers in a western Kenyan patient population, will identify appropriate early detection and therapeutic strategies that will reduce cancer mortality rates in these patients. A better characterization of the regional differences in breast cancer will guide the development of early detection programs and effective treatment strategies designed to reduce the cancer mortality rates in both Kenya and related patient populations.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Cancer is a broad term used for identifying a large number of diseases characterized by a common feature ability of uncontrolled cell proliferation that cannot be checked by the normal cell kinetics regulators. A normal cell suddenly turns into a rogue cell and start dividing continuously without check, leading to the development of solid lumps (tumors) or an abnormal rise in the number of dispersed cells like the blood corpuscles. Cancer can occur in any part of the body and in any organ or tissue. Even though most of the cancers are generally associated with old age, no age group is immune to this disease.

Cancer development is a multi-stage process that involves initiation, promotion and progression as was proposed by Berenblum and Shubik in 1949. Initiation is a process of spontaneously stable cellular changes as a result of mutation of cellular genome. This stable change gave rise to a neoplastic development predisposing the affected cell and its progeny to subsequent neoplastic transformation (UNSCEAR 1993, Cox 1994). The human DNA sequences that are responsible for transformation are termed oncogenes. Normal cells evolve progressively, this process is described by a new generation approach that termed this complex signaling and characteristics cancer hallmark (Hanahan and Weinberg 2013). This is a multistep developmental capabilities acquired by normal self-cells when they are transformed to form neoplastic (Hanahan and Weinberg 2013). Six hallmarks of cancer have been proposed; ability to sustain proliferative signal, evasion of growth suppression, resisting cell death, enabling replicative immortality, inducing angiogenesis and activation of invasion and metastasis. This capabilities make tumors to be complexes of tissues that are made up of distinct cells that interact in a heterotopous manner with their microenvironment. Tumor-associated stroma consist of normal recruited cells that actively

participate in tumor genesis by contributing to certain hallmark capabilities (Hanahan and Weinberg, 2013).

2.2 Tumorigenesis of Breast Cancer

Molecular underpinnings of breast cancer tumorigenesis have given many ideas that form the basis of cancer pathogenesis (Place *et al.*, 2011). Detectable changes in the breast cancer tumorigenesis process is loss of regulation of cell number. This often results in epithelial hyperplasia or sclerosing adenosis. Subsequent genetic instability occurs in multiple small clonal populations of cells. This is recognizable histologically as atypical hyperplasia. After progression to carcinoma, numerous cellular aberrations can be identified, including increased expression of oncogenes such as c-ras, c-myc and c-erb-B2, decreased expression or function of tumor suppressor genes such as p53 and alterations in cell structure. These alterations in cell structure can result in a loss of cell adhesion, increased expression of cellular proteins such as cyclins and Ki-67), increased expression of angiogenic factors such as vascular endothelial growth factor (VEGF), and increased expression of proteases like cathepsin-D. However no combination of the above changes is consistently seen in any one breast cancer subtype suggesting that the malignant phenotype is due to an accumulation of multiple changes, rather than a predictable and orderly progression. It should also be noted that many of these genetic and cellular alterations can be found in both invasive and in-situ breast tumors.

2.3.0 Breast Cancer Epidemiology

2.3.1 Global breast cancer epidemiology

The global burden of cancer continues to increase largely because of growth of aged world's population alongside increase adoption of lifestyle behaviors associated with development of

cancer. According to GLOBOCAN estimates of 2012, 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) were recorded in 2012 worldwide. Fifty seven percent (8 million) of the new cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions (Ferlay *et. al.,* 2015)

Breast cancer is the second most common cancer in the world and, by far the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers) (Ferlay *et al*. 2015). The number of women diagnosed with breast cancer in the developing world is increasing. Incidence rates of breast cancer vary across the world regions, with rates ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 96 in Western Europe. Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) and while it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer (Ferlay *et al*. 2015).

In the United States and Canada, breast cancer incidence rates have stabilized since the early 2000s, after increasing for several decades. Mortality rates have decreased since early 1990s, with a faster decrease in whites than blacks and in affluent than in poor women (Autier P. 2012)

In Asia, breast cancer mortality trends are expected to maintain the secular trend for the next decade mainly as the prevalence of risk factors changes and population ages in Japan, Korea, and Taiwan. Early detection and treatment improvement will continue to reduce mortality rates in Hong Kong and Singapore as in the Western countries (Shin and Varghese 2012).

2.3.2 Breast cancer epidemiology in Africa

African continent is facing a new epidemic of non-communicable (NCD) together with the persisting old and new communicable diseases (Dalal *et. al*., 2010; Holmes *et. al.,* 2010). The developing countries have in the past enjoyed a lower incidence of cancer, currently the incidence of cancer is rising at an alarming rate that is frustrating if considered that already this countries are faced with a challenge of beleaguered health care systems that barely meet the needs of their population (Farmer *et al*. 2010). This rising cancer incidence in the developing countries portends huge economic costs and is matched with a glaring lack of preparedness in most low and middle income countries (LMIC) (World Economic Forum, 2011).

Forouzanfar *et al*. (2011) reported that there is an increase in breast cancer incidence by 3.1% over a 20 year with a heterogeneous increase in the cumulative probability of breast cancer incidence in women aged 15-70 years. Registries records show that the age standardized incidence rate of breast cancer in Africa between 1960 and 1969 was 13.7 per 100,000, this doubled in four decades and to 24.7 per 100,000 by 1988- 1999. The incidence in 2009-2010 was at 53 per 100,000 persons, this representing 100% increase in the recent decades (Sacco *et al*. 2011).

The incidence of breast cancer is rising in many countries, however the reasons are not completely understood but is likely to reflect changing reproductive patterns, increasing obesity, decreasing physical activity and some breast cancer screening activity (Parkin *et al.,* 2005; Colditz *et al*., 2006). Similarly mortality rates are also increasing, most likely due to lifestyle changes associated with westernization compounded by the delayed introduction of effective breast cancer screening programs and, in some cases, limited access to treatment (Jermal *et al*., 2010; Ito *et al*., 2009).

In Kenya cancer of the breast accounted form 5% of all malignancies and is second to cancer of the cervix (Alterman *et al*. 2008). Over 90% of patients present late to the clinics and thus have advance stage disease, a contributing factor to high mortality rates (Busakhala and Torrorey 2012).

2.4.0 Breast Cancer Risk Factors

A risk factor is anything that alters the chances of an individual to develop the disease such as breast cancer. However, having a risk factor does not mean that one will necessarily acquire the disease because some individuals have risk factors but never develop the disease and some do not have the risk factors, yet acquire the disease (American Cancer Society, 2007). There are numerous risk factors associated with breast cancer. The known risk factors associated with breast cancer may be classified as non-modifiable and modifiable (Figure 1).

2.4.1.0 Non Modifiable Breast Cancer Risk Factors

2.4.1.1 Age at diagnosis

Risk of breast cancer increases with age, however, this differs among racial groups (Fregene and Newman, 2005). In the whites older women are at a higher risk where approximately 77% of the breast cancer cases occur in women over 50 years (American Cancer society, 2013). In African population less than 40% of women who develop breast cancer are above 50 years (Rambau *et. al*., 2011; Ogundiran *et al*., 2010). However, in African, breast cancer is common at much younger age of between 35 and 45 years (Rambau *et al*., 2011; Akarolo-Anthony *et al*., 2010; Kruger and Apffelstaedt, 2007). Age at diagnosis determines risk since the earlier a woman develops a first primary breast cancer, there is a greater risk of developing a secondary primary breast cancer (Chen *et al*., 1999). Akarolo-Anthony *et al*., (2010) reported that early onset breast cancer in African women is a demographic phenomenon that is justified by the fact that most African countries have

a cone-shaped population pyramid with majority of their citizens being children and young adults with very little elderly population at the top. There was need to determine which risk factor are associated with the early onset of breast cancer in Western Kenyan population.

2.4.1.2 Tribe/ cultural practices

Initially, association of breast cancer prevalence with respect to different ethnic origins was lacking, but this space is now largely filled by extensive research on polymorphism and genetic mutations. So far wide ranges of founder mutations on various genes have been observed in different populations. Hayat *et al*., (2007) reported that increased trends regarding high incidence and mortality rates for all cancer sites were observed more frequent in black people. Although Africans being the minority have low incidence of breast cancer, the mortality rate remain the highest (Chlebowski *et al*., 2005).

Studies of ethnicity-related variation in breast cancer burden within the USA have also demonstrated that African-American women are more likely to be diagnosed with estrogen receptor-negative, high-grade tumors that are node-positive (Fregene and Newman 2005). These disease patterns also characterize the tumors that occur in women who harbor mutations in breast cancer susceptibility genes, prompting speculation that hereditary factors may explain some ethnicity-related issues. Ethnicity has been associated with poor outcome in African American, with the mortality rates higher than their white counterparts (Newman *et al*., 2006). Since breast cancer subtypes can determines prognosis, there was need to determine which tribes in Kenya tend to develop the breast types associated with poor outcomes.

2.4.1.3 Family history

It has been observed in several studies that around 5-10% of women suffering from breast cancer already have a history of mammary tumor in maternal or parental lineage (Center *et al*., 2015; Pluchinott *et al*., 2015; Balmana *et al*., 2009; Hoffman and Johnson, 1995). An estimated relative risk (RR) of breast cancer among females having a familial history cancer in first degree relative was observed as 2.1% (Pharoah *et al*., 1997). Risk estimation may vary with age at diagnosis of the affected relative, number of relatives involved and closeness to affected personnel on pedigree basis. Family history of other types of cancers like ovarian cancer also poses a threat for breast cancer (Antoniou *et al*., 2003). It has been observed that first degree relatives of ovarian cancer patients had a modest risk of breast cancer of around 1.27% in Utah Cancer Registry (Kerber and Slattery, 1997). Familial history of either breast or ovarian cancer alone or together increase likelihood of presence of a cancer predisposing mutation (Couch *et al*., 1997; Shattuck- Eiders *et al*., 1997).

A woman that has a close relative diagnosed with breast cancer puts her at a higher risk of developing the disease (Pakseresht *et al*., 2009). One is at double risk of developing breast cancer if one first- degree female relative (sister, mother, and daughter) is diagnosed with the disease (Bevier *et al*., 2012; Pakseresht *et al*., 2009). If two first- degree relatives have been diagnosed the risk could be up to 5 times higher than average (Bevier *et al*., 2012; American Cancer Society 2008). Currently there is no data on the family history as a risk factor for developing breast cancer in Kenya. Briton *et al*., (2014) reported that the diagnostic information is not widely discussed among breast cancer patients and or their relatives leading to underestimation of the true prevalence of familial history. There was need to determine the prevalence of family history as risk factor to breast cancer in this study.

2.4.1.4 Menstrual and reproductive history

Early menarche and late menopause have been shown to increase the chances of breast tumors (Sprague *et al*., 2008; Friedenreich, 2001; Sasco, 2001). These risk factors are also largely reduced by early full-term pregnancy (Friedenreich, 2001). Women having an early puberty (menstrual cycle start) before the age of 12 years are twice at high risk as compared to those who mature after 13 years of age (Key, 2003; Sprague *et al*., 2008; Friedenreich, 2001; Sasco 2001). Attaining menopause after 55 years of age doubles the risk of developing breast cancer compared with women having an early menopause around the age of 40 years (Key, 2003; Handerson, *et al*., 1992). Age at menarche in an African women varies although generally these women experience menarche at older ages compared to non-Africans (Fregene and Newman 2005). Reason for this difference in age at menarche has been reported to depend on the interaction between genetic and environmental factors (Karapanou and Papadimitriou 2010).

Although it has been indicated that long menstrual history increases life time exposure to sex hormones predisposing to breast and ovarian cancers. It is not documented if age at menarche and or menopause is a risk factors of developing certain subtypes of breast tumors in Kenya.

2.4.2.0 Modifiable Breast Cancer Risk Factors

2.4.2.1 Socio-demographic profiles

2.4.2.1.1 Marital status

Marital status has been associated with breast cancer (Abbasis *et al*., 2009; Ebrahim *et al*., 2002). Furthermore it has reported that single and nulliparous married women do have a similar increased risk for breast cancer when compared to women of the same age who have children (Abbasis *et*

al., 2009). In another study lone mother have been recognized as vulnerable group, have fewer children, higher unemployment (Hemminki and Li, 2003).

Shaikh *et al*. (2014) in their study reported that marital status remains a risk factor for breast cancer development and unmarried, delayed marriage, delayed first child birth are strong cofactors for development of breast cancer. Furthermore it has been documented that unmarried patients were likely to present with metastatic cancer and have high chances of under treatment hence results to increased mortality rates (Aizer *et al*. 2013). There is no data on the relationship of marital status and breast cancer risk in Kenya, there this study determined the association of marital status and risk of developing breast cancer.

2.4.2.1.2 Place of residence

There is a doubling risk of breast cancer in women living in urban areas compared to those living in rural areas because, urban areas are frequently characterized by westernized behaviors and lifestyles (Fregene and Newman, 2005). Place of residence may also affect breast cancer patients with their decision to obtain early medical help. People from the rural areas tend to refrain from the modern therapeutic methods for they prefer to seek medical help from traditional healers (Vorobiof *et al*., 2001).

2.4.2.2 Reproductive factors

2.4.2.2.1 Contraceptive use

Prolonged uses of oral contraceptives increases breast cancer risk (Marchbanks *et al*., 2002; Kumle *et al*., 2002). This risk is not different in among current use and prolonged use of 10 or more years (Cancer *et al*., 1997). However, use of oral contraceptive in BRCA mutation carriers as prevention against ovarian cancer may increase breast cancer risk up to 28% as observed in Jewish population

(Ursin *et al.,* 1997). In another study, no statistical significant value has been observed in BRCA1 mutation carriers after using oral contraceptives for one year (Haile *et al*., 1996). It has been shown that BRCA mutation carriers as well as women with strong familial history are more prone to exogenous hormones present in oral contraceptives (Pasanisi *et al*., 2009). A meta- analysis of 51 studies concluded a positive correlation of relative risk of breast cancer with postmenopausal hormone replacement therapy (HRT). Relative risk value calculated was 1.35% for women who had used HRT for 5 or more years after menopause (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). Similar findings were also noted by Women's Health Initiative (WHI) controlled on postmenopausal women with RR value of 1.24% (Chlebowski *et al*., 2003). There is no documented data on the use of hormonal drugs as contraceptive and or replacement therapy and risk of developing breast in Kenya. Therefore there was need to determine if the use of oral contraceptive and hormone replacement therapy is associated with risk of developing breast cancer in Kenya.

2.4.2.2.2 Parity

Multiple pregnancies reduces the risk of breast cancer, especially if the first full term pregnancy occurs at a young age (Fregene and Newman, 2005). However multiparity following a late age of first full term pregnancy and absence of breast feeding is associated with an increased risk of breast (Lord *et al*., 2008).

Breast cancer is related to increased number of regular cycles and lifetime exposure of ovarian hormones (Kotsopoulos *et al.,* 2005; Travis and Key, 2003). Multiparity causes endogenous oestrogen levels to be low over time decreasing cumulative risk of breast cancer (Fregene and

Newman, 2005). Nulliparous women have higher concentrations of prolactin than porous women, this explains why nulliparous women have a higher risk for breast cancer (Travis and Key, 2003).

There is evidence that there was up to 7% reduction in the relative risk of breast cancer for each birth independently from other pregnancy related factors (Collaborative Group on Hormone Factors in Breast Cancer, 1996). Yang *et al*., (2008) in their study estimated that there was 30% decrease in risk of breast cancer in multiparous women of 5 or more births. Although it has been shown that there is a long term protective effect of parity, and specifically multiparty on breast cancer risk (Talamini *et al*., 1996; Yang *et al*., 2007), however, long spacing (that is 5 to 10 years after each birth) is associated with a transient increase breast cancer risk (Bruzzi *et al*., 1988).

The dual effect of parity on the risk of breast cancer may be due to differences in reproductive patterns explaining variations in primary tumor biology and tumor aggressiveness, however, in women of African origin the concept is still not well understood (Fregene and Newman, 2005). There was need to determine whether parity predisposes women to breast cancer in Kenya.

2.4.2.2.3 Breast feeding

Studies have suggested that breastfeeding protects women against both pre- and post-menopausal breast cancer (Nagata *et al*., 2012; Glade, 2008; AICR, 2008). The protection of breast feeding is associated with hormonal changes in the body, that manifest as delayed ovulation, increased breast separation, change in hormonal environment of the breast and excretion of carcinogenic agents (Lord *et al*., 2008; Clemons and Goss, 2001). Delay of the menstrual periods by breast feeding is protective because it reduces the woman's lifetime exposure to hormones such as estrogen (AICR, 2008; Clemons and Goss, 2001). Breast reaches the final stages of maturity during pregnancy and breast feeding when milk making cells grow and reproduce. During lactation there is shedding of

breast tissue and programmed cell death that has been documented that it decreases the risk of developing cancer by damaging oncogenic cells (AICR, 2008).

The period of breast feeding has been associated with breast cancer development where long periods (≥16 months) of breast feeding is known to decrease the risk for breast cancer. This is because breast feeding for long separates ductal epithelial cells giving it protection against carcinogens and increase prolactin levels that may contribute to separation of ductal epithelial cells (Fregene and Newman, 2005). Women who breast feed for more than 12 months have reduced life time risk and that every birth reduces risk of developing breast cancer by 7% (Lord *et al*., 2008). Breastfed babies are protected from cancer because breast feeding reduces the likelihood of that child becoming overweight (Araujo, *et al.*, 2006). Protection from weight gain is important because childhood overweight have tendency to continue to adulthood overweight (Samaras, 2010; Araujo, *et al.*, 2006 ;) and adults with excess body fat are at increased risk of postmenopausal breast cancer (AICR, 2008). It has been reported that the developing world tend to copy the western lifestyle including shorter duration of breast feeding, longer spacing of birth, less number of children (Vecchia and Pelucchi, 2012) it is not clear whether breastfeeding is associated with the increased incidence of breast cancer seen in Kenya today.

2.4.2.3.0 Life style behaviors

2.4.2.3.1 Alcohol use

There is evidence that alcohol consumption increases the risk of cancer of the colorectal, breast, larynx, liver, esophagus, oral cavity and pharynx (Bagnardi *et al*., 2012). Alcohol use is associated with both pre- and post-menopausal breast cancer (AICR, 2008; Wrensch *et al*., 2003). Studies have linked recent alcohol intake with increased risk of breast cancer (Key *et al*., 2003; Wrensch

et al., 2003). Alcohol drinking is also linked to increased risk of death from breast cancer (Grant, 2008; Tan *et al*., 2006). Number of years in which alcohol was used influence the risk of developing breast cancer (Tan *et al*, 2006; Parodi, 2005).

It has been further shown that women who started drinking before their first full-term pregnancy have a higher risk than women who started afterwards. These effects were observed in hormone-receptor positive and negative tumors pointing to non-hormonal pathways that need to be further investigated (Romieu *et al*, 2015)

A recently published meta-analysis selected and analyzed 56 out of 2,785 studies and concluded prevalence of alcohol use was 52% in Eastern Africa. University students and sex workers have the highest prevalence of alcohol use. The studied countries include Ethiopia, Tanzania, Uganda, Kenya, Seychelles and Rwanda. However, no specific number is listed for Kenya (Francis, 2014). McCormack and Boffetta (2011) suggested that a 1g ethanol increase per day would translate to a 0.8% increase in risk of breast cancer in women in low- and middle-income counties. Association between alcohol and breast cancer is linked to increased estrogen and androgen or increased levels of plasma insulin like growth factors that are produced by liver following alcohol consumption (Sarkar *et al*., 2001; Xue, 2009) . However there is no evidence showing whether alcohol intake is a risk factor for breast cancer in Kenya.

2.4.2.3.2 Smoking

Most epidemiological studies associated heavy smoking, long term smoking, smoking before a first full term pregnancy (FFTP) and passive smoking with increased risk of breast cancer in women with high levels of estrogen (Catsburg *et a*l., 2015; Dossus *et al*., 2014a; Manjer *et al*., 2001; Xue *et a*l., 2011). Furthermore, studies have reported that initiation of smoking before

menopause and particularly before first full-term pregnancy was most strongly associated with an increased risk of breast cancer (Johnson *et al*., 2010; Xue *et al*., 2011) this is because of estrogenic effect of smoking among premenopausal women which may increase their high endogenous estrogen levels further (Xue *et a*l., 2011).

Passive smoking increases breast cancer risk by 70% in younger, primarily pre-menopausal women (Gray, *et.al*., 2009). The California Environmental Protection Agency concluded that passive smoking causes breast cancer (Miller *et al*., 2007). The shift of tobacco consumption from developed world to the more vulnerable low-resource countries like Kenya could be associated with the rise of cancer incidences. However data documented is not available, therefore there was need to determine if cigarrete smoking is associated with the rising incidences of breast cancer witnessed in Kenya.

2.4.2.4.0 Environmental risk factors

Environment is the living and working conditions as well as physical, biological, social and cultural responses to these condition and environmental exposure that involve activities which subject people to agents that they, as individuals, cannot control, such as pesticides, dioxins, passive tobacco smoke, and other chemicals and ionizing radiation (Laden and Hunter, 1998). Some of these agents may be present in air, food, water, and soil. Environmental exposure can occur at home, at school, in the work place, in the health care facilities and other setting at daily life activities (International Summit on Breast Cancer and Environment, 2002).

2.4.2.4.1 Shift work

It is thought that night work, and being exposed to artificial light, reduces the amount of melatonin in the body (Navara and Nelson, 2007). In women, melatonin reduces the amount of estrogen in

the body, and it may slow the growth of breast cancer cells (Mirick and Davis, 2008). Some studies have suggested that women who work shifts, particularly night shifts, have a slightly higher risk of developing breast cancer (Davis, *et al*., 2001; Haus and Smolensky, 2013; Menegaux *et al*., 2013; Schernhammer *et al*., 2001). Furthermore IARC (2010) reported that shift work that involves circadian disruption are thought to be potential carcinogens. This is so because the abnormal circadian rhythms happens whenever the body's circadian timing does not synchronizes with that of the environment. Studies have reported that light at night suppresses melatonin levels which will in turn lower anti-estrogen effect, increasing the risk of breast cancer(Menegaux *et al*., 2013; del Rio *et al.*, 2004). Another consequence of circadian disruption is down regulation of cell growth as well as suppression of immune surveillance especially when sleep is deprived (Costa *et al*., 2010).

2.4.2.4.2 Use of firewood

Exposure to wood smoke has previously been reported to increase the risk of developing esophageal cancer (Patel, *et al*., 2013). Similarly, Kayamba *et al*., (2015) reported that HIV infection and domestic smoke exposure are risk factors for esophageal squamous cell carcinoma in Zambia. It has been documented that exposure to air pollution at birth alters DNA methylation, which will in turn increase levels of E-cadherin, a protein that is known to play a role in maintaining a stable cellular environment (Michel *et al*., 2013). Women with breast cancer who lived in a region with more air pollution were more likely to have the alteration in the DNA in their tumor than those who live in a less-polluted regions (Michel *et al*., 2013). Since many people in Kenya use or get exposed to wood smoke, there is need to determine if exposure to wood smoke is associated with the rising incidences of breast cancer in Kenya.

2.4.2.4.3 Living in a house with mice

Mouse mammary tumor virus (MMTV) has been regarded as a potential model for human cancer since it was described as an agent involved in mouse mammary carcinogenesis (Moore *et al*., 1971). Immunoreactivity against the envelope protein (env) of MMTV was seen in breast cancer samples but not in normal tissues (Pogo *et al*., 2010) and that antibodies against Env were found in patients with breast cancer (Moore *et al*., 1971; Pogo *et al*., 2010; Day *et al*., 1984). Furthermore viral particles with morphological characteristics of a retrovirus were detected in 60% of milk from patients with a history of breast cancer but only in 5% of milk from normal individuals (Moore *et al*., 1971). Pogo *et al*., (2010) demonstrated that 38% of breast cancer samples from US patients contained env gene sequences 95% to 99% homologous to MMTV, whereas only 1% of normal breast samples were positive. Faedo *et al*., (2004) reported a correlation of mouse mammary tumor-like virus with p53 expression, and Ford *et al*., (2003) found that the sequences were more prevalent in invasive tumors than *in situ* carcinomas. The greatest prevalence of sequence positive breast cancer has been reported in Tunisia, which had the highest reported prevalence of rapidly progressing breast cancer similar to inflammatory breast cancer (IBC) (Pogo *et al*., 2010).

The amount of detectable oncogenic virus has been associated with the tumor aggressiveness in animal models, suggesting that the presence of viral sequences might be related to tumor aggressiveness in human patients (Levine *et al*., 2009). Viruses from mice can be easily passed to humans beings especially if they share habitats. In Africa almost all rural home have mice infestation, however no data on the passage of MMTV to humans. There was need to determine if there was a relationship between living in a house with mice and development of breast cancer subtypes seen in Kenya.

Table 2.1 Categories of non-modifiable and modifiable risk factors for breast cancer.

Risk factors	Categories	Level of risk for Breast cancer	Reference
Non-modifiable risk factors			
Age at diagnosis	*35-45 years	High	
	≥46 years	Medium	
	≤34 years	Low	(Adebamowo *et al*. 2003;Rambau *et al.* 2011)
	†>50 years	High	(Rambau *et al*. 2011)
History of breast cancer /Family history	First degree relative	High	(Chen *et al.* 1999)
	Second degree relative	Medium	
	No relative	Low	
Menstrual history			
Age at menarche	≤12 years	High	(Key *et al*.2003)
	>12 years	Low	
Age at menopause	≥55 years	High	(Key *et al*. 2003)
	≤54 years	Low	
Modifiable risk factors			
Socio-demographics profiles			
Marital status	Never married	High	(Abbasis et al. 2009)
	Ever married	Medium	
Place of residence	Urban	High	(Fregene & Newman 2005)
	Rural	Medium	
Lifestyle behaviors			
Alcohol use	Yes	High	(Grant 2008).
	No	Low	
Smoking	Yes	High	
	No	Low	
	Side stream	Medium	
Reproductive factors			
Oral contraceptive (OR) use	Yes	High	(Gammon *et al*. 1999)
	No	Low	
Duration of OR use	≥12 months	High	
	<12 months	Medium	
Parity	Not having children	High	(Travis and Key 2003)
	Having children	Low	
Number of children	1-5	Medium	
	≥5	Low	
Breast feeding	<6 months	High	(Ursin *et al*. 2005)
	6-11 months	Medium	
	≥12 months	Low	
Number of children breast fed	None	High	
	≤4	Medium	
	≥5	Low	
Environmental factors			
Shift work	Yes	High	
	No	Low	
Use of firewood	Yes	High	(Michel *et al*., 2013)
	No	Low	
Live house with mice			
	Yes	High	(Moore *et al*., 1971; Pogo *et al.*,2010;Day *et al*., 1984)
	No	Low	

* Level of risk for African women. † Level of risk for White women

2.5.0 Histological Breast Cancer Subtypes

Breast cancer is a collection of different diseases with varied biological and pathological features, they present in different ways, therefore need to be treated in different ways so as to manage their unique clinical behavior that have unpredicted clinical outcome (Dieci *et al.*, 2014). In the effort of organizing and standardizing this variation, pathologists have designed breast cancer classification systems. According to World Health Organization (WHO) classification, breast cancer can be divided into 21 distinct histological types on the basis of morphology, growth, and architectural patterns. Breast cancer can be broadly categorized into *in situ* carcinoma and invasive (infiltrating) carcinoma.

2.5.1 Breast cancer *in situ* (BCIS)

Breast cancer cells when circumscribed in their place of origin and do not spread, either in the surroundings or at distant organs, are termed in situ breast carcinomas. These types of cancers are further classified into two types on the basis of growth patterns and cytological features (Malhotra *et al*., 2010). Tumors arising in the milk ducts is termed as ductal carcinoma in situ (DCIS) while cancer originating and localizing in the lobules of the breast tissue are termed as lobular carcinoma in situ (LCIS). Both these types remain localized at their respective site of origin and show no invasion to the stromal tissue (Lishman and Likhani, 1999; Mai *et al*., 2000).

2.5.2 Ductal carcinoma *in situ* (DCIS)

Ductal carcinoma *in situ* is further subdivided into five subtype based on architectural features, these subtypes are; Papillary (noncomedo), and comedo (Cribiform, Micropapillary, and Solid). DCIS is more common than LCIS, in the US with approximately, 64,000 cases of breast cancer seen in a year being DCIS (Swart, 2013). In African population DCIS is the most common with a

frequency of over 80% (Ikpatt *et al*., 2002; Ebughe *et al.*, 2013). The reason for this is not documented.

Ductal carcinoma *in situ* is thought to be a precursor for invasive cancer of the breast (Leonard and Swain, 2004). Studies have unraveled the information on the pathogenesis and natural history of DCIS, this has led to the adoption of a variety of approaches in management of disease. DCIS is a benign tumor that can evolve to an invasive cancer, therefore lumpectomy is the recommended treatment (Wellings and Jensen, 1973).

The incidences of DCIS are on the rise because of better screening technique like mammography (Brinton, *et al*., 2008b). Ductal carcinoma *in situ* is less common compared to invasive breast cancer and is mostly diagnosed in postmenopausal age (Kerlikowske, 2010). The incidence of DCIS, like invasive breast cancer, is strongly related to age. DCIS is not common prior to ages 35–39 (2.5 per 100,000 for women aged 30–34). The incidence rises steadily to a peak of 96.7 per 100,000 at ages 65–69 and then declines slowly until age 79 and steeply after age 79. In contrast, invasive breast cancer peaks at ages 75–79, with incidence of 453.1 per 100, 00 (Virnig *et al*., 2010).

The age-adjusted incidence of DCIS was the highest among Caucasian women, followed by African-Americans and Asian-Pacific Islanders. Hispanic women had the lowest age-adjusted incidence of DCIS (Virnig *et al*., 2010). While in African women the age-adjusted incidence of DCIS is not known.

Menarche and menopause is thought to be another risk factor but no study found a statistically significant association between age at menarche and DCIS incidence. Age at menopause is challenging to examine in the context of DCIS because the risk of DCIS increases with age,

particularly around the age of menopause (ages 45–60). Thus, it can be challenging to separate the effects of aging with the hormonal changes associated with menopause.

Hormone replacement therapy (HRT) is thought to increased risk of invasive breast cancer. According to the Women's Health Initiative, a large randomized trial of HRT and breast cancer risk, there was no increased risk of DCIS associated with HRT (Chen *et al*., 2002; Chlebowski *et al*., 2003). However there was no consistent association between HRT and DCIS in five observational studies.

DCIS cases are increased among those who had their first child above 20 years of age compared to women who were less than 20 years of age at first live birth (Allegra *et al*., 2010). Several studies reported a decreased risk of DCIS associated with more children relative to no children or only one child (Meeske *et al*., 2004; Millikan *et al*., 2008; Yang and Jacobsen, 2008)

Gill *et al.*, (2006) found that women with higher breast density had increased risk of DCIS relative to those with lower breast density. For example, a nested case control study found approximately three times increased of DCIS among women with higher than 50% versus lower than 10% mean breast density (Gill *et al*., 2006)

In Kenya the association of development of DCIS and these risk factors is not known. This study therefore associated the development of DCIS with breast cancer risk factors.

2.5.2.1 Papillary carcinoma of breast

It is an extremely rare form of cancer which is observed in postmenopausal women (Pal *et al*., 2010). An invasive papillary carcinoma usually has a well- defined border and is made up of small, finger-like projections. Papillary cancer cells are generally graded as moderate grade or Grade 2

on a scale of 1 to 3, with Grade 1 describing cancer cells that look and behave somewhat like normal, healthy breast cells, and Grade 3 describing quite aggressive, fast-growing cancer cells (Koerner, 2010). In most cases of invasive papillary carcinoma, ductal DCIS is also present (Koerner, 2010).

2.5.2.2 Cribriform carcinoma of breast

In invasive cribriform carcinoma, the cancer cells invade the stromal tissues in nest like formations between the ducts and lobules. Invasive cribriform carcinoma is usually low grade, meaning that its cells look and behave somewhat like normal healthy breast cells. In about 5-6% of invasive breast cancers, some portion of the tumor can be considered cribriform. Excellent prognosis has been observed in this rare form of carcinoma (Venable *et al*. 1990).

2.5.3 Other histological subtypes

Lobular Carcinoma *in situ* arise from the terminal duct apparatus and it presents as a non-palpable mass in most cases because it distributes to the whole breast. The incidence of this tumor is doubled in the past 25 years and now is 2.8 per 100,000 Caucasian women especially those that age between 40-50 years of age (Swart 2013). Although this histological group is not common in most population, the incidence in the study population is not known.

Invasive (Infiltrating) breast cancer is a mammary tumor invading the surroundings or spreading into distant organs via blood or lymphatic channels. Like DCIS, invasive carcinomas are a heterogeneous group of tumors differentiated into histological subtypes. This form of cancer is further classified as invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), mucinous (colloid), tubular, mammary Paget disease and medullary carcinoma (Malhotra 2010).

Invasive Lobular Carcinoma (ILC) are cancers that originate from the lobules and later on extrapolate either in vicinity or in different organs of the body. These lobes are actually the exocrine glands responsible for milk production. ILC is less common compared to IDC observed in various populations, it constitute less than 15% of cases on invasive breast disease (Swart 2013). Invasive Lobular Carcinoma can metastasize to axillary lymph node first then more to different parts of the body.

Invasive Ductal Carcinoma (IDC) begins in duct cells and then after breaking from the ductal walls invades the surrounding tissues. Ellis et *al*. (2003) classified this type as invasive ductal carcinoma not otherwise specified (IDC-NOS) or of no specific type (IDC-NST). This form of cancer is the commonest and accounts for 75% of breast cancer cases and has a tenancy to metastasize via lymphatics. It is common at the age of 35-39 years (Virnig *et al*. 2010). A study by Bennis *et al*. (2012) reported a prevalence rate of 87% in north-east Morocco and another study in Tunisia reported that (83.7%) of their new cases were IDC (Missaoui 2011). The prognosis value varies according to the severity of disease progression. Usually lumpectomy, mastectomy, radiations or chemotherapy are the treatment tools used either alone or in combination to treat these types of breast cancer. Some alternate forms of this cancer in which cells behave slightly different are as follows; Tubular carcinoma of breast cancer; Tubular carcinoma is usually uncommon histological type involving 1-2% of all breast cancers. It affects women with an average age of 50 years (Berg and Hutter, 1995). It is less likely to metastasize and is therefore easily treated.

Medullary carcinoma of breast cancer is a rare subtype of IDC and accounts for about 3-5% of all breast cancer cases. It is termed “medullary” carcinoma because the tumor is soft, fleshy mass, resembling medulla of the brain. This type of carcinoma has frequently been observed in Japanese women and also in BRCA1 mutation carriers (Shousha, 2000; Armes and Venter, 2002). Tumor

cells are usually large, pleomorphic retaining large size nuclei they and have diffused cell growth pattern with minimal or no glandular differentiation (Armes and Venter, 2002). Medullary carcinoma does not grow quickly and usually does not spread outside the breast to the lymph nodes (Ridolfi *et al*., 1977). For this reason, it is typically easier to treat than other types of breast cancer. The biology of this type of breast cancer is not known in the current study population.

Mucinous carcinoma of breast accounts for about 2-3% of all breast cancer cases globally (Komaki *et al*., 1988). It is also termed as colloid carcinomas. In this type of cancer, the tumor is formed from abnormal breast cells producing mucus. Mucinous carcinoma can also be found in association with other forms of tumors (Komaki *et al*., 1988). A mucinous carcinoma may have some areas that contain IDC cells. If the IDC cells make up more than 10% of the tumor, the cancer is called a "mixed" mucinous carcinoma (Paramo *et al*., 2002). A pure mucinous carcinoma means that at least 90% of the cells are mucinous in nature. Mucinous carcinoma tends to affect women after they have gone through menopause (Paramo *et al*., 2002). It is less likely to metastasize and have a better prognosis (Di Saverio *et al*., 2008; Perkins *et al.,* 2009).

Inflammatory breast carcinoma (IBC) is a rare but very aggressive form of breast cancer (Woodward and Cristofanilli, 2009). Inflamed cells actually block the lymphatic channels in the skin of the breast. In this type of breast cancer the organ appears red, swollen hence is termed as inflammatory breast cancer (IBC). About 1-3% of breast cancers are IBC (Singletary, *et al*., 1994). High metastasizing tendency has been observed in IBC as compared to other forms. Inflammatory breast cancers are always staged as stage IIIB, unless it has spread to other organs (Singletary *et al.*, 1994). The disease affects approximately 2.5% of breast cancer patients in the United States typically with younger age of onset and higher incidence in African-Americans(Wiggins *et al*., 2012).

Paget's disease contributes to only 1% of all types of breast cancer and is generally associated with bleeding, or crusting and scaliness of the nipple or areola. The primary symptoms include eczema like rash accompanied by a burning sensation which may further lead to fluid discharge, crusting and a sore that does not heal. Prognosis is better when compared with other types of tumors (Noel *et al*., 2010).

Swart (2013) describe IDC as a cancer that can metastasize through the lymphatics resulting to poor prognosis. This tumor is frequently associated with DCIS (Sinn, 2013), in that they have similar risk factors that include age, breast density, family history, and history of benign breast disease (Virnig *et al*., 2010; Kerlikowske, 2010). It was reported that IDC increased at reducing rate in the US. Between 1980s and 1990s it increased by 4% followed by 3% between 1995 and 2004, this decline was associated with reduction in hormone therapy use. The general rise of breast cancer prevalence is expected to also increase the rate of IDC in Africa, there was no clear documentation associating the risk factors and histological breast cancer subtypes in Kenya. There was need to determine association between the breast cancer risk factors and histological types in breast cancer patients seen in western Kenya.

2.6.0 Grading of Breast Cancer

Histological tumor grade and tumor type are important characteristics that can be determined by any pathologist. Tumor grade is the assessment of the degree of differentiation (that is tubule formation and nuclear pleomorphism) and proliferative activity (that is mitotic index) of a tumor, it indicates tumor aggressiveness (Weigelt and Reis-Filho, 2009). Histological grading is a powerful indicator of prognosis in breast cancer and internationally done as per Etston and Ellis

(1991) semi quantitative method (Table 2.2). The final grade is the sum of what is got from Etston and Ellis and it is interpreted as per National Cancer Institute (2004) recommendation (Table 2.3)

Studies have shown that more women have grade III tumors in Africa than in Europe (Abdulrahman and Rahman, 2011). In Tanzania, 56.4% have tumors with histological grade III (Rambau *et al*., 2011), while, in Nigeria, 45.1% have grade III tumors (Ikpatt *et al*., 2002). On the contrary, only 15.8% of Finnish women have a grade III tumor. Most women in Europe present with a grade I or II tumor (Boder, 2011). Black British women have been shown to have higher rates of grade III tumors and lymph-node-positive disease than white British women (Bowen *et al*., 2008). This findings could explain why breast cancer seen in women of African origin tend to the more aggressive compared to the whites. However there is no data on risk factors and prognosis of different grades of breast cancer in Kenya. There is need therefore to determine the risk factors associated to each grade in our population.

Table 2.2 Summary of semi quantitative method for assessing histological grade in breast carcinoma

Feature	Score
Tubule formation	
Majority of tumor (>75%)	1
Moderate degree (10-75%)	2
Little of non (<10%)	3
Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic count	
Dependent on microscope field area	1-3

Histological breast cancer grading as per Etston and Ellis (1991) semi quantitative method

Table 2.3 Grading grid

Total feature score	Tumor Grade	Appearing of Cells
3-5	Grade I tumor	Well- differentiated (appear normal, growing, not aggressive)
6-7	Grade II tumor	Moderately-differentiated (semi-normal, growing moderately fast)
8-9	Grade III tumor	Poor-differentiated (abnormal, growing quickly, aggressive)

Source: National Cancer Institute, 2004.

2.7.0 Intrinsic Breast Cancer Subtypes

Breast cancer presents diverse clinical, molecular, and pathological features that need advance techniques of classification (Kuo *et al.,* 2011). Due to more biologic diversity among breast cancer types, use of histology alone may not classify breast cancer into the intrinsic subtypes that are currently used for treatment and management of this disease. Diagnostic oncologist in their current understanding of biology of breast cancer have established groupings or subtypes of breast cancer based on both biologic and clinical relevance (Kimberly and Allison, 2012). Gene expression microarrays have enabled classification of breast cancer in distinct molecular subtypes including two estrogen receptor negative (ER-) tumors: triple negative and human epidermal growth receptor (HER2) overexpressed, and two types of estrogen receptor positive (ER+) tumors: Luminal A and Luminal B (Sorlie *et al*., 2001). In addition, use of proliferation index (Ki67) has enabled separation of ER positive patients into two intrinsic different populations, Luminal A and Luminal B (Sorlie *et al*., 2001). Today, it is widely acceptable that a panel of ER, PR, HER2 and Ki67 can be used to classify breast cancer with specificity and sensitivity relevant to genetic subtyping (Cheang *et al*., 2009). Microarray technique is accurate but it is not practical to use it routinely especially in low resource countries Kenya included. Therefore, the surrogate immunohistochemistry (IHC) markers could be the better option to classify breast cancer subtypes.

2.7.1.0 Characterization of breast cancer

Immunohistochemical analysis of estrogen (ER) and progesterone receptors (PR) and HER2 has been used to characterize breast cancer into subtypes as well as in predicting the response of patients to routine management. Lack of expression of all three of these biomarkers predicts non-response to available endocrine (tomaxifen, aromatase inhibitors) and anti-HER2 (transtuzumab) targeted therapies, and has become known as a triple-negative phenotype (TNP). Approximately 70–90% of triple-negatives are reported to be basal-like breast carcinomas (Bertucci *et al*., 2008; Wang *et al.,* 2008), hence TNP has been used as a surrogate for the basal-like subtype.

It is generally accepted that estrogen receptor-positive (ER+) and ER-negative (ER−) breast cancers are two different disease entities. ER− tumors tend to be of high grade, have more frequent p53 mutations and worse prognosis compared with ER+ disease (Rakha *et al*., 2010). Both ER+ and ER− tumors can be either HER2 positive or negative. Low-grade tumors are typically ER positive, almost always HER2 nonamplified (Yoder *et al*., 2007). Stage II tumors are more likely to be either ER+ and or PR+ (Yoder *et al*., 2007). Other studies have reported that high grade (grade III) tumors tend to be ER negative and have frequent loss of P53 function and commonly over express HER2 (Rastelli and Crispino, 2008; Misek and Kim 2011) . In the high grade tumors, loss of p53 function is associated with 17p13 deletion, mutation or inactivation, while overexpression of HER2 is usually because of 17q12 amplification (Misek and Kim 2011).

Gene expression profiling can be used to separate breast cancers into distinct molecular subtypes with prognostic significance (Voduc *et al*., 2010) Commercially available assays based on gene expression profiling, including Oncotype DX (Genomic Health, Redwood City, CA) and MammaPrint (Agendia, Amsterdam, the Netherlands), may provide useful prognostic information

(Voduc *et al*., 2010). Other studies have found that using immunohistochemical surrogates for molecular subtyping can provide much of the prognostic information obtained by gene expression profiling (Nielsen *et al*., 2004; Cheang *et al*., 2009). Breast cancer molecular subtypes can be categorized according to immunohistochemistry results for ER, PR, HER2 and Ki-67, as recommended by the 12th International Breast Conference (Untchet *et al*., 2013; Gnaant *et al.*, 2011; Zhang *et al*., 2015), this will give the following breast cancer subtypes: Luminal A type (LA): ER or/and PR positive, HER2 negative and Ki-67 < 14%; Luminal B type (LB): ER or/and PR positive, HER2 negative and Ki-67 ≥ 14%, ER or/and PR positive and HER2 overexpressed or/and amplified; HER2 amplified type (HER2): ER and PR negative and HER2 overexpressed or/and amplified; Triple-Negative type (TN): ER, PR and HER2 negative (Table 2.4). The intrinsic breast cancer subtypes vary with respect to prognosis and treatment, however this is not done in most hospitals in Kenya. There was need to determine the intrinsic breast cancer subtypes seen in the study population.

2.7.1.1 Luminal A subtype

Luminal A also termed estrogen receptor positive (ER+) breast tumors, look like the cells of breast cancers that start in the inner (luminal) cells lining the mammary ducts. This subtype tend to be Estrogen receptor-positive (ER+) and/or progesterone receptor-positive (PR+), HER2/neu-negative (HER2-) and are likely to be tumor grade I or II (Carey *et al*., 2006; Koboldt *et al*., 2012). According to "IHC-4" score this subtype is ER+ and/or PR+, HER2-, low Ki67 (Table 2.3). Luminal A is the most common intrinsic breast cancer subtype (Blows *et al*., 2010) with prevalence rate of 40% in American population (Carey *et al*., 2006) and 33% in Indigenous African population (Huo *et al*., 2009).

Table 2.4 Characterization of four major breast cancer subtypes, population prevalence, and clinical characteristics

Subtype	Molecular/genetic characteristics	Prevalence†	Prevalence*	Clinical Characteristics
Luminal A	ER+ and/or PR+, HER2-, low Ki67	40%	33%	Slow-growing Less aggressive Low recurrence High survival Best prognosis of all types Respond to endocrine therapy
Luminal B	ER+ and/or PR+, HER2+ (or HER2- with high Ki67)	10-20%	17%	High proliferative rates Worse prognosis than Luminal A Respond to endocrine therapy
Triple negative	ER-,PR-,HER2- any Ki67	20%	23%	Young age at diagnosis High histological grade Higher rates of distant recurrence after surgery Poor short-term survival Lack targeted therapy
HER2 overexpressed	ER-,PR-, HER2+ any Ki67	10-15%	14%	Tend to grow and spread more aggressively More likely to be high grade and node positive Poor short term survival Targeted therapies exist

ER+/-, estrogen receptor positive or negative; PR+/-, progesterone receptor positive or negative; HER2 +/-, human epidermal growth factor positive or negative. †prevalence in American population (Carey *et al* 2006);* prevalence in Indigenous African population (Huo *et al* 2009)
Source: American Cancer Society. Breast Cancer Facts & Figures 2013-2014.

Tumors that are ER+ are more common than those that are ER- (Cadoo *et al*., 2013). However ER positivity tend to increase with age such that ER+ tumors are seen more in postmenopausal women (Hess *et al*., 2003). These tumor are likely to be smaller and common in Caucasians (Anderson *et a*l., 2002). Reproductive risk factors associated with development of hormone positive subtypes are null parity and late age at first child birth (Phipps *et al*., 2011; Millikan *et al*., 2008).

Metzger *et al*., (2013) reported that luminal A breast tumors have better prognosis and prolonged survival, this is because of their ability to response to tamoxifen a target drug to the estrogen receptor. This subtype is also known to have high level of tumor suppressor activity (The cancer genome atlas, 2012). Since tumor receptor status is not routinely ascertained in most African hospitals (Eng *et al*., 2014), patients who present with tumors that are known to have prospect of good survival may end having poor prognosis because, establishment of hormone receptor status help in choosing appropriate treatment. There was need to determine the level of Luminal A presentation in the study population with the aim improving prognosis of this disease by choosing the appropriate treatment.

2.7.1.2 Luminal B subtype

Luminal B are ER+ and/or PR+, HER2+ (or HER2- with high Ki67) (Voduc *et al*., 2010; Zhang *et al*., 2015). This subtype is more aggressive, treatment resistant with worse prognosis compared to luminal A (Metzger-Filho *et al*., 2013; Tran and Bernard *et al.,* 2011). Overall survival in untreated luminal B is similar for the aggressive subtypes (TN and HER2 overexpressed) (Tran and Bernard, 2011). Reduced low survival rate in this subtype is associated to the fact that this tumors are relatively insensitive and poorly responsive to both endocrine and chemotherapy (Tran and Bernard, 2011).

This subtype is also associated with clinical risk factors such as an earlier distant metastasis than luminal A and are more likely to lead to relapse in bone and pleura (Tran and Bernard, 2011). The prevalence of this subtype in Indigenous African population is 17% (Huo *et al*., 2009; Eng *et al*., 2014). While that of American population is 10-20% (Carey *et al*., 2006). A study by Cheang *et al*. (2009) reported that a Ki67 score ≥14% distinguishes luminal B from luminal A, ER positive

HER2 negative tumors. This approach termed "IHC-4" score was supported by Cuzick *et al*. (2011), and the St Gallen 2011 Expert panel, for it showed similar prognostic performance to the 21-gene sequence score in breast cancer.

Luminal B subtypes portray a transition between tumor suppressive immune response to one that is tumor tolerant, suggesting that the immune response in this subtype could be polarized towards tumor progression (Edin *et al.,* 2012). Determining the biology of luminal B in this study may give some information on the prognosis of this type of breast tumor.

2.7.1.3 HER2 overexpressed subtype

HER2 overexpressed subtype is hormone receptor negative but express the oncogene HER2 that belongs to the epidermal growth factor receptor family (Slamon *et al*., 2001). When classified using "IHC-4" score this subtype is ER-,PR-, HER2+ any Ki67 (Table 2.3) with a prevalence rate of 10-15% prevalence in American population (Carey *et al*., 2006) and up to14% in Indigenous African population (Huo *et al*., 2009). They tend to have high frequency of TP53 mutation (The cancer genome atlas, 2012). They also show a high risk of regional and local metastasis and are likely to be large tumors that have poor tumor differentiation and associated with young age (Yang *et al*., 2007). In the absence of treatment, HER2 overexpressed tumors are associated with poor overall survival compared with other breast cancer subtypes (Sjogren *et al*., 1998).

Management of HER2 overexpressed subtype has been revolutionized by the development of therapies targeting this receptor leading to improvement in patient outcome. Success of trastuzumab, a humanized monoclonal antibody, has led to the development of a series of other agents such as lapatinib, pretuzumab and ado-trastuzumab-emtansine that are further improving

patient outcomes (Verma *et al*., 2012). However these drugs are very expensive and may not be affordable to most patients in Africa.

Although HER2 overexpressed subtype is least represented in African population there is no data on the rate of HER2 subtype in Kenya. There was need therefore, to determine the level of expression rate of this disease in the study population.

2.7.1.4 Triple negative breast cancer (TNBC)

Triple negative breast cancer (TNBC) is heterogeneous group of tumors that do not express ER, PR or HER2. They are ER-,PR-,HER2- any Ki67 according to IHC-4 score (Table 2.3) with up to 20% prevalence in American population (Carey *et al*., 2006) and over 23% prevalence in Indigenous African population (Huo *et al*., 2009). Studies have shown that 23%-44% of breast cancer tissue samples collected from East African women in Kenya, Ethiopia, and Uganda are triple negative (Bird *et al*., 2008; Rody *et al*., 2011; Nalwoga *et al*., 2007; Trinkaus *et al*., 2011 ; Nyagol *et al*., 2006; Kantelhardt *et al*., 2014)

Triple negative breast cancers (TNBCs) are seen at a younger age of 53.0 years compared to 57.7 years in luminal subtype, they are larger (>3cm) and mostly (66%) of advance grade (grade III) resulting in an aggressive phenotype with poor overall prognosis (Kaplan *et al*., 2008; Dent *et al*., 2009). Furthermore patients with TNBCs have higher rate of recurrence in the first 4 years after diagnosis with an observation that distant metastasis sites are different from other breast cancers (Dent *et al*., 2009; Foulkes *et al*., 2010).

The risk factors that have been associated with TNBCs are BRCA1 mutation (75%), higher body mass index and waist-to-hip circumference ratio, higher parity and lower duration of breast feeding (Millikan *et al*., 2008; Yang *et al*., 2007). It has been noted that the immune response is responsible

for aggressiveness and hence poor prognosis of tumor that do not express hormone receptors (Desmedt *et al*., 2008).

Although it has been reported that high prevalence of TNBC in African population regardless of age contribute to poor prognosis of breast cancer, however there is scanty of data on this subtype in Kenya. This study determined the factors that are associated with the development of this type of disease.

2.8.0 Role of Immune Cells in Breast Cancer

Immune system is expected to naturally suppress tumors by killing tumor cells or inhibiting their growth, but that is not always the case for evidence have indicated that the immune system could also promote tumor progression in a yet to be understood manner (Krell *et al*., 2012). Some studies have explained that, a tumor can be promoted even in immune competent individual by two major ways; first is through a positive selection of viable seed tumor cells and second is by a negative selection by suppression and/or elimination of antitumor immune cells (Xioguo, 2013; Schreiber *et al*., 2011). This observation was actually exposed in the hallmarks of cancer that involved, the capability to modify or reprogrammed cellular metabolism of cells and allowing this cancer cells to evade killing by effector immune cells (Hanahan and Weinberg, 2011). Tumor progression is a result of complex immune mediated mechanisms in the tumor microenvironment. It is now clear that the immune microenvironment in breast cancer can predict not only overall survival and relapse free survival but also influence response to chemotherapy (DeNardo *et al*., 2011).

Innate antitumor immune response also referred to as natural immunity, is mediated by cells or soluble factors that naturally exist in tissues or body fluids. Immune cells that are known to mediate antitumor immunity include, macrophages, granulocytes, natural killer cells, non-MHC restricted

T cells (γδ T) cells. It has been shown that natural specific antibodies to the surface markers of tumor cells also play a role (Whiteside, 2010). Other serum factors including complement, C-reactive proteins, mannose-binding protein, and serum amyloid protein also form part of innate immunity (Pierce *et al*., 2009; Whiteside, 2010).

The other arm of immune response is termed adaptive and is mediated by $CD3^{+}$T-cell receptor (TCR^{+}) T cells, antigen presenting cells (APCs) expressing self-MHC molecules, other $CD45^{-}$ expressing leukocytes including B-cell receptor positive (BCR^{+}) $CD20^{+}$ cells B cells and multiple myeloid-lineage cells including $CD68^{+}$ tumor- associated macrophages (TAMs). Adaptive antitumor immune response begins with uptake of tumor associated antigens (TAs) by APCs at the tumor site. This will be followed by antigen processing, and cross-presentation to T cells in the tumor-draining lymph nodes. The first ligand, termed primary signal is made by a complex of peptide-TCR-MHC (Whiteside, 2013). The success of the primary signal up regulates two other subsequent ligands; co-stimulatory and co-activation lead to a full T cell activation, that will give rise to clones of effector repertoire ($CD45RA^{+}$) and memory cells ($CD45RO^{+}$) (Whiteside, 2013).

Tumor-infiltrating lymphocytes (TILs) are white blood cells that have left the bloodstream and moved into a tumor. White blood cells are immune cells made by bone marrow to help the body fight infection (Mantovani, 2006). Immune cells that are frequently found in the tumor microenvironment are lymphocytes, which are able to mediate both innate and adaptive immunity with the assistance of monocytes, TAMs, and DCs. All these cells are in an intimate contact with tumor cells, stromal fibroblast, extracellular matrix components and blood vessels (Whiteside, 2013).

Effector cells accumulate in solid tumors and there role is not conclusively uncovered. TILs are viewed by others as victims of tumor-microenvironment because their effector function is impaired

by tumor-derived factors, and their failure in eliminating tumor cells leads to tumor progression (Whiteside, 2013). Other studies have demonstrated that immune system can completely eliminate a tumor or keep it in a dormant state, through tumor inhibiting cytokines and infiltration of cells of both innate and adaptive immunity (Galon *et al*. 2006; DeNardo *et al*. 2011; Loi 2014; Schreiber *et al.* 2011).

Recent research has led to increased development and application of immunotherapy to cancer treatment (Loi *et al*., 2011; Denkert, 2013). Both the TIL type and density contribute to the host immune response (Krell *et al*. 2012). However, the association of type and density of TILs to aggressiveness of breast tumors in the study patient population has not been documented. This study will determine pattern and density of TILs in tumor microenvironment.

The concept of 'cancer immunoediting' describes how the immune system and tumor cells interact during the course of cancer development. It consists of three distinct phases, termed 'the three E's'; elimination, equilibrium and escape according to Kim *et al*., (2007). Elimination entails the complete obliteration of tumor cells by T lymphocytes. In equilibrium, a population of immune resistant tumor cells appears. Simultaneously, there is an unremitting immunologic pressure on nonresistant tumor cells. This phase can last for years (Kim *et al*., 2007). Finally, during escape, the tumor has developed strategies to evade immune detection or destruction. These may be due to loss of tumor antigens, secretion of inhibitory cytokines or down regulation of major histocompatibility complex (MHC) molecules (Stewart and Abrams, 2008).

2.8.1 Role of T helper ($CD4^{+)}$ cells in breast cancer

$CD4^+$ T cells form the integral part in immune response by activating and regulation immunity to antigens of pathogens and tumors. They are termed helper T cells since they prime activated

specific $CD8^+$ T cells to differentiate into cytotoxic T lymphocytes (CTLs) and long-term $CD8^+$ memory T cells (Dobrzanski, 2013). This type of response is termed Th1 or cell mediated immunity. $CD4^+$ T cells also have the ability to help B cells produce antitumor specific antibodies in a Th2 response that is classified as humoral immunity.

The roles of polarized $CD4^+$ T cell subsets in antitumor immune response are determined by the kind of cytokines released. Tumor $CD4^+$ T cells is plastic in nature, for one group can switch to another by the influence of underlining transcription factors (Dobrzanski, 2013).

Emerging evidence suggest that $CD4^+$ T cells have more sub lineages that can induce and maintain destructive immune responses to tumor cells (Kim and Cantor, 2014). $CD4^+$ CTL are a subpopulation of $CD4^+$ T cells that contribute to tumor eradication by directly killing MHC^+ class II tumors and or indirectly killing MHC II^- via macrophages or natural killer (NK) cells in the absence of $CD8^+$ T cells (Haabeth *et al.,* 2014). This group of $CD4^+$ T cells are termed helper 1 (Th1) that predominantly produce IFN-γ and are involved in priming and maturation of $CD8^+$ T cells through activation of DCs at the tumor microenvironment. This group further inducing elimination of tumor by activating NK cells and type 1 macrophages (Palucka and Banchereau, 2012). Furthermore IFN-γ can induce development of Th1 cell lineage that are tumor suppressing (Rotondi *et al*., 2003).

The other $CD4^+$ lineage cells are; T helper 2 (Th2) polarized $CD4^+$ T cells that are characterized by chronic inflammation that foster tumor progression and metastasis (Mantovani *et al.,* 2008). Th2 effector cells coordinate humoral immunity and allergic immune response by producing IL-4, IL-5 and IL-13 (Dobrzanski, 2013); Follicular helper T cells (T_{FH}) that are T cells with the ability to migrate to follicles in secondary lymphoid organs and interact with B cells. They are responsible for providing B cell help and supports B cell expansion and differentiation (Ma *et al.,*

2013). The role of T_{FH} in antitumor immunity is not clear, however some studies have associated humoral immune response with promotion of tumor growth (Kim and Cantor, 2014). In contrast Gu-Trantien (2013) reported that tumor- infiltrating T_{FH} cells play a role in promoting tumor immunity by enhancing chronic inflammation that is characterized by immune cell recruitment to the tumor and formation of intra-tumoral follicular structures called ectopic germinal centers. There was need find out if there were significant differences in the density and distribution of B cell infiltrate in the breast cancer subtypes seen in Kenya.

2.8.2 Role of T cytotoxic ($CD8^+$) cells in breast cancer.

$CD8^+$ T cells are the cytotoxic lymphocytes that mediate major antitumor effector function in breast cancer (Jiang and Shapiro, 2014). Many studies have reported the antitumor activity of $CD8^+$ T cells with the evidence of favorable survival rates in patients with high densities of these cells in all types of tumors (Mahmoud *et al*., 2011; Distel *et al*., 2009; Piersma *et al*., 2007). Cytotoxic $CD8^+$ T lymphocytes are crucial components of tumor-specific cellular adaptive immunity that attack tumor cells presenting tumor-associated antigen peptide with MHC class I on their surface. $CD8^+$ T cells produce IFN-γ following interaction with their tumor targets. The IFN-γ- dependent mechanisms of tumor cell cytostasis and killing consequently occurs by cell cycle inhibition, apoptosis, angiostasis, and induction of macrophage tumoricidal activity (Smyth *et al*., 2006; Dunn and Schreiber, 2004). Man *et al*., (2013) proposed that the presence of $CD8^+$ T lymphocytes in both normal and transformed epithelial microenvironment might contribute to the maintenance of tissue integrity and repair, while Matsumoto *et al*., (2015) indicated that increased $CD8^+$ T cells within a tumor is associated with favorable prognosis in cancers. Liu *et al.,* (2012) have shown a positive association with better prognosis in basal-like breast cancers but not in luminal and HER overexpressed subtypes. Baker *et al*. (2011) demonstrated significant association between

presence of $CD8^+$ TILs and better prognosis in ER negative and high grade breast tumors. In TNBC infiltration of $CD8^+$ T cells have been reported to demonstrate significant overall survival (OS) only if in association with low levels of macrophages and CD4+ T cells (DeNarrdo *et al*., 2011). Although TNBC subtype remains a challenge for it lacks therapeutic target yet up to 37% of breast cancer cases in this group fall in this category (Sawe *et al*., 2013), no data on the pattern of TILs in breast cancer subtypes in Kenya. There was need to determine the pattern and density of CD8+ T cells in breast tumors because it offers a great potential in developing immune based therapy.

2.8.3 Role of macrophages in breast cancer

Macrophages carry out a variety of functions, including clearing cellular debris (Mosser and Edwards, 2008), responding to pathogens (Mosser, 2003) and facilitating wound healing (Khanna *et al*., 2010; Lucas *et al*., 2010). Macrophages differentiate in the tissue from circulating monocytes, and just as other immune effector cells can have multiple subtypes, macrophages take on different phenotypes depending on their microenvironment (DeNardo *et al*., 2008).

Macrophage polarization states have been described in terms similar to the Th1/Th2 paradigm, where Th1-type responses promote cell-mediated immunity and cytotoxic responses important for dealing with intracellular pathogens while the Th2-type response promotes the humoral immunity important for dealing with extracellular pathogens, as well as mucosal immunity. Increased Th2 responses result in chronic inflammatory disease states, such as asthma and allergy (Wan, 2010).

When monocytes differentiate in a Th1 immune environment, the resulting macrophage develops an M1, or classical, activation state. This is in contrast to the M2 activation state, derived in a Th2 immune environment (Van *et al*., 2008). Overall, M1 macrophages (with relatively high IL-12 and low IL-10 levels) promote antitumor responses by activating the adaptive immune system, while

M2 macrophages (relatively low IL-12 and high IL-10 levels) enhance tumor growth by producing antigenic factors, stromal breakdown factors and down regulating antitumor immune responses. Additional support for the relationship between wound-healing macrophages and breast tumors with poor prognoses comes from Chang *et al.* (2013). This group identified a wound-response gene-expression signature, derived by identifying the genes that were activated when fibroblasts were cultured with serum, a normal initiator of wound healing (Chang *et al*., 2013). The 'activated' versus 'quiescent' wound-healing signature has been shown to be associated with particularly poor prognosis in a number of cancers, including breast cancer (Troester *et al*., 2009). These data suggest that aggressive breast tumors arise in an environment similar to that of a healing wound, to which M2 macrophages would normally be recruited.

Although the M1–M2 nomenclature has been challenged given the evidence that macrophage populations exist with features of both activation states (Mosser and Edward, 2008) much of the literature still describes macrophage phenotypes in these terms; this construct helps to conceptualize how TAMs and tumor cells influence one another, and how that interaction can be manipulated for therapeutic benefit.

Although TAMs make up the majority of infiltrating leukocytes, they are an integral part of a complex immune network and thus interact with other inflammatory cells. Tumor associated macrophages (TAMs) interact with myeloid-derived suppressor cells (MDSCs) and T cells in the tumor microenvironment, the end result of which is to promote a Th2-type polarized environment with high levels of $CD4^+$ T cells, and low levels of $CD8^+$ cytotoxic T cells, which promotes tumor growth (Sinha *et al*., 2005).

Certain breast cancers produce CSF-1, CCL2, STAT3 and STAT6, which promote macrophage infiltration and M2 differentiation. High Th2 $CD4^+$ T cells with low $CD8^+$ T cells results in a

protumoral environment with increased metastatic risk. Interactions between M2 macrophages and MDSCs lead to high levels of IL-10 and low levels of IL-12, further reinforcing the M2 phenotype and increasing levels of Th2-type $CD4^+$ T cells. These $CD4^+$ T cells produce IL-4, which also polarizes macrophages toward M2, creating a feedback loop. Meanwhile, $CD8^+$ T cells are suppressed, resulting in an overall immune-permissive environment for tumor growth and spread.

M2-polarized macrophages also increase the numbers of MDSCs, which then inhibit T cells, including cytotoxic $CD8^+$ T-cell responses (Sinha *et al*., 2005, Sinha *et al*., 2007). $CD8^+$ T-cell function is increased if M2 macrophages are blocked. One method of doing this is targeting legumain, a molecule overexpressed in M2 macrophages (Luo *et al.,* 2006).

Biswas *et al.* (2010), reported that B cells also have the capacity to polarize macrophages. The recruitment of inflammatory cells to a mouse model of skin cancer has been shown to depend on the presence of B cells, (de Visser *et al*., 2005) which have been found to produce IL-10 and drive macrophages toward the M2 phenotype (Wong *et al.,* 2010).

Additionally, the pattern of lymphocyte distribution may also affect their role in response to tumors, with studies suggesting that tumor-infiltrating lymphocytes have different prognostic significance than lymphocytes scattered in the stroma (Demaria *et al.,* 2001). All these findings suggest that macrophages act in concert with other inflammatory cells, with TAMs contributing to an overall pro-tumoral environment.

There is a suggestion that M2 macrophages are polarized not only by tumor cells, but also by other infiltrating leukocytes, implicating the immune environment in facilitating tumor survival. Many groups have noted alterations in immune function in breast cancer patients, and the involvement

of M2 macrophages in breast tumor progression may be consistent with these observations (Campbell, *et al.,* 2005).

The finding that particular patient populations with poor outcomes in breast cancer, such as minority groups, may have particularly elevated levels of TAMs raises intriguing questions about the risk factors for their presence. Our current understanding of TAM–tumor cell interactions does not permit determining whether certain patients are predisposed to immune dysfunction, perhaps because of genetic or environmental reasons, or whether certain tumor types are able to induce these immune alterations in the host. There in need to determine the pattern and distribution of TAMs in different subtypes of BC putting into consideration the risk factors.

2.8.4 Role of B cells in breast cancer

These are the $CD20^+$ cell that originate and differentiate in the bone marrow. They are rare in many tumors. The main function of these cells is production of tumor antigen specific antibodies. Infiltration of breast tumors with $CD20^+$ B cells have been associated with good prognosis (Schmidt *et al*., 2012). This is evident when anti-tumor immunoglobulin that are specific to tumor antigen eradicate early neoplasm cells (Stagg and Allard, 2013). Rody *et al*. (2011) reported that up to 32% of the TNBC with a ration of high B cell and low IL-8 had good prognosis. Determining the density and distribution of B cells in breast tumors across subtypes will give an insight on the tumor suppressing nonspecific B cells from the tumor favoring chronic activated B cells.

Biragyn and Lee-chang (2012) noted that $CD20^+$ B cells co-localized with activated $CD8^+$ T cells but the role of these cells in the aggressive forms of breast cancer is not clear and seems not suppressing but instead favors progression of tumor. Some studies suggests that the presents of both $CD20^+$ B cells and $CD8^+$ T cells correlated with increased patient's survival (Nielsen *et al*.,

2012; Whiteside and Ferrone, 2012). A subset of tumor-evoked B regs can induce conversion of resting $CD4^+$ T cells to immunosuppressive T regs (Olkhanud *et al.,* 2011). Similarly tumor promoting B cells secrete IL-10 polarizing macrophages from an M1 phenotype to a pro-tumoral M2 phenotype (Sica *et al*., 2010). Other studies have further suggested that B cells can promote lymph angiogenesis by enhancing metastasis in lymphoma and melanoma (Harrel *et al*., 2007; Ruddell *et al.,* 2011).

2.8.5 Role of induced regulatory T (iTreg) in breast cancer

Several subsets of regulatory T cells have been described in the literature. These include naturally occurring CD4+CD25+FoxP3+ cells that develop in the thymus (tTregs), peripherally-derived Tregs (pTregs) that are generated from FoxP3- conventional T cells at sites outside of the thymus, and induced regulatory T cells (iTregs) that are generated in vitro by stimulation of mouse conventional T cells with TGF-β. Cells in the pTreg group have been further classified as either central Tregs (cTregs), effector Tregs (eTregs), or tissue-resident Tregs. Additionally, CD4+FoxP3− type I regulatory T cells (Tr1), CD8+ Tregs, and follicular Treg cells (TFR) have been described (Woo *et al*., 2001).

Induced T regulatory (iTreg) cells are found in large number inside late stage tumors. Their function is thought to be inhibition of antitumor immune response by limiting production of IFN-γ from $CD8^+$ T cells and also preventing effector cell proliferation (Nishikawa *et al*., 2005). Treg in primary tumor have the ability to promote metastasis (Yang *et al*., 2012). Whiteside (2012) reported that accumulation of Tregs in tumors lead to poor prognosis due to suppression of anti-tumor immunity. Other studies have linked the frequency of iTreg among TIL to tumor grade and reduced patient survival (Whiteside, 2013; Lanca and Silva-Santos 2012).

During early stages of cancer development, tumors are infiltrated by tumor suppressive cells but this phenomenon seem to transform towards enhancement of tumor cell dissemination and metastasis (Smith and Kang, 2013). Breast cancer seen in Africa seem aggressive for it progresses very first, however no data on the link of infiltration of iTregs in tumor microenvironment. There was need to determine the correlation of the density and pattern of iTregs in breast cancer because it is a potential marker that could be targeted for immunotherapy.

2.9.0 Methods Used In Breast Cancer Subtyping

The prevalence of molecular subtyping in breast cancer has been increasing as the development of novel diagnostic tools to interrogate tumor biology has evolved. Traditional subtyping (such as IHC or FISH) assess a tumor by looking at cell surface characteristics, while molecular subtyping looks deeper at the functional level to see which genes are driving the tumor's behavior.

2.9.1 70-Gene signature (MammaPrint®)

The MammaPrint® test is performed and provided as a service by Agendia Laboratory. The test is a microarray based gene expression analysis of RNA extracted from breast tumor tissue. The test is a custom-designed array chip manufactured by Agilent Technologies using the Agilent oligonucleotide microarray platform which assesses the mRNA expression of the 70 genes in triplicate. The MammaPrint® microarray features eight 1900-feature sub arrays per glass slide which can each be individually hybridized. Per sub array 232 reporter genes are printed in triplicate, including the 70 genes which make up the MammaPrint® prognostic profile. Each sub array additionally includes 915 normalization genes and 289 spots for hybridization and printing quality control.

The analysis is based on several processes: isolation of RNA from frozen tumor tissue sections, DNAse treatment of isolated RNA, linear amplification and labeling of DNAse treated RNA, cRNA purification, hybridization of the cRNA to the MammaPrint® microaray, scanning the MammaPrint® microarray and data acquisition (feature extraction), calculation and determination of the risk of recurrence in breast cancer patients.

The MammaPrint® analysis is designed to determine the gene activity of specific genes in a tissue sample compared to a reference standard. The result is an expression profile, or fingerprint, of the sample. The correlation of the sample expression profile to a template (the mean expression profile of 44 tumors with a known good clinical outcome) is calculated and the molecular profile of the sample is determined (Low Risk, High Risk, Low Risk Borderline, High Risk Borderline).

Data analysis is performed according to a specific MammaPrint® algorithm (MammaPrint® Index). The algorithm calculates the similarity ("cosinor correlation") of the sample expression profile to a template, (the mean expression profile of 44 tumors with a known good clinical outcome) and determines the molecular profile of the sample (Low Risk, High Risk). This algorithm is designed and programmed by Agendia and compiled into a standalone software program, "X-Print Analysis Software". The "X-Print Analysis Software" loads a data file (CSV) which is created by the laboratory technician by extracting specific information from the laboratory database. The CSV data file contains: external sample ID, internal sample ID, Technician name, Bio-analyzer ratio, and RNA integrity number, location of straight and dye-swap data file (*www.agendia.com*. 2016*)*

2.9.2 21-Gene recurrence score (Oncotype DX®)

Oncotype DX (Genomic Health Inc., Redwood City, CA) is a clinically validated twenty-one-gene genomic assay that can quantify the risk of breast cancer recurrence. The gene panel includes five reference genes and sixteen cancer-related genes, including those associated with cell proliferation, invasion and hormone response. The test generates a recurrence score between 0 and 100 that correlates to the likelihood of disease recurrence within 10 years of diagnosis

The 21-gene assay is supported by strong evidence of clinical validity, i.e., that the Recurrence Score (RS) is associated with risk of distant recurrence in women with invasive breast cancer that is positive for hormone receptors, negative for human epidermal growth factor receptor 2 (HER2) amplification, and without lymph node involvement. Oncotype DX® adds additional risk information to conventional clinical classification of individual high-risk patients and identifies a subset of patients who would otherwise be recommended for chemotherapy but who are actually at lower risk of recurrence (average risk at 10 years, 7%-9%; upper bound of the 95% confidence intervals, 11% to 15%). The available evidence is therefore sufficient to determine that Oncotype DX® improves the net health outcome for women with hormone receptor–positive, HER2-negative, lymph node–negative invasive breast cancer. A woman who prefers to avoid the toxicity and inconvenience of chemotherapy and whose Oncotype DX® RS value shows that she is at low risk of recurrence might decide to decline chemotherapy (Sparano *et al.* 2008).

2.9.3 BluePrint® and TargetPrint®

The 80-gene expression assay BluePrint® discriminates three breast cancer molecular subtypes; Luminal-type, HER2-type and Basal-type, each with marked differences in long-term outcome and response to neoadjuvant chemotherapy. TargetPrint® is a method to measure estrogen receptor

(ER), progesterone receptor (PR), and HER2 as an alternative to IHC and FISH. Available evidence is insufficient to determine th\at BluePrint® and TargetPrint® improve the net health outcome in women with early-stage, invasive breast cancer. Clinical utility of BluePrint® is unknown, because it is unclear how this test will add to treatment decision making using currently available, accepted methods (e.g., clinical and pathologic parameters). The incremental benefit of using TargetPrint® as an alternative to current standard methods of measuring ER, PR, and HER2 has not been demonstrated, nor is it included in recommendations for testing issued by the American Society of Clinical Oncology and the College of American Pathologists (Nguyen *et al*. 2012)

2.9.4 Immunohistochemistry (IHC).

Immunohistochemistry or IHC staining of tissue sections (or immunocytochemistry, which is the staining of cells), is perhaps the most commonly applied immunostaining technique. While the first cases of IHC staining used fluorescent dyes, other non-fluorescent methods using enzymes such as peroxidase and alkaline phosphatase are now used. These enzymes are capable of catalysing reactions that give a colored product that is easily detectable by light microscopy. Alternatively, radioactive elements can be used as labels, and the immunoreaction can be visualized by autoradiography.

Tissue preparation or fixation is essential for the preservation of cell morphology and tissue architecture. Inappropriate or prolonged fixation may significantly diminish the antibody binding capability. Many antigens can be successfully demonstrated in formalin-fixed paraffin-embedded tissue sections. However, some antigens will not survive even moderate amounts of aldehyde fixation. Under these conditions, tissues should be rapidly fresh frozen in liquid nitrogen and cut

with a cryostat. The disadvantages of frozen sections include poor morphology, poor resolution at higher magnifications, difficulty in cutting over paraffin sections, and the need for frozen storage. Alternatively, vibratome sections do not require the tissue to be processed through organic solvents or high heat, which can destroy the antigenicity, or disrupted by freeze thawing. The disadvantage of vibratome sections is that the sectioning process is slow and difficult with soft and poorly fixed tissues, and that chatter marks or vibratome lines are often apparent in the sections.

The detection of many antigens can be dramatically improved by antigen retrieval methods that act by breaking some of the protein cross-links formed by fixation to uncover hidden antigenic sites. This can be accomplished by heating for varying lengths of times (heat induced epitope retrieval or HIER) or using enzyme digestion (proteolytic induced epitope retrieval or PIER).

One of the main difficulties with IHC staining is overcoming specific or non-specific background. Optimization of fixation methods and times, pre-treatment with blocking agents, incubating antibodies with high salt, and optimizing post-antibody wash buffers and wash times are all important for obtaining high quality immunostaining. In addition, the presence of positive and negative controls for staining are essential for determining specificity.

Applications of Immunostaining

The applications of immunostaining are numerous, but are most typically used in clinical diagnostics and laboratory research. Clinically, IHC is used in histopathology for the diagnosis of specific types of cancers based on molecular markers.

In laboratory science, immunostaining can be used for a variety of applications based on investigating the presence or absence of a protein, its tissue distribution, its sub-cellular

localization, and of changes in protein expression or degradation. In this study immunohistochemistry was used to determine expression of hormone receptors (ER, PR, HER2) Ki67 and immune infiltrates (CD4, CD8, CD20, CD25, CD68, CD163) in formalin-fixed paraffin-embedded breast tissues.

2.10 Conceptual Frame Work

The independent variables are risk factors that predisposes individuals to breast cancer or breast lumps that is not cancerous. The dependent variables are the breast cancer grades, histological types and the intrinsic subtypes determined by hormone receptors and the density of tumor infiltration lymphocytes together with intervening variables such as social and cultural practices (Fig 2.2).

Primary objective was to determine if breast cancer risk factors, hormone receptors and TILs influence breast cancer grade, histological type and intrinsic subtype. Secondary objectives was to determine if pathway A mediated by risk factors and intervening variables explain the tumor subtype; if Pathway B mediated by hormone receptors, TILs and intervening variables determines breast cancer subtype. Pathway C explains that risk factors predispose to breast lumps that are non-cancer and that breast lumps can predispose to breast cancer

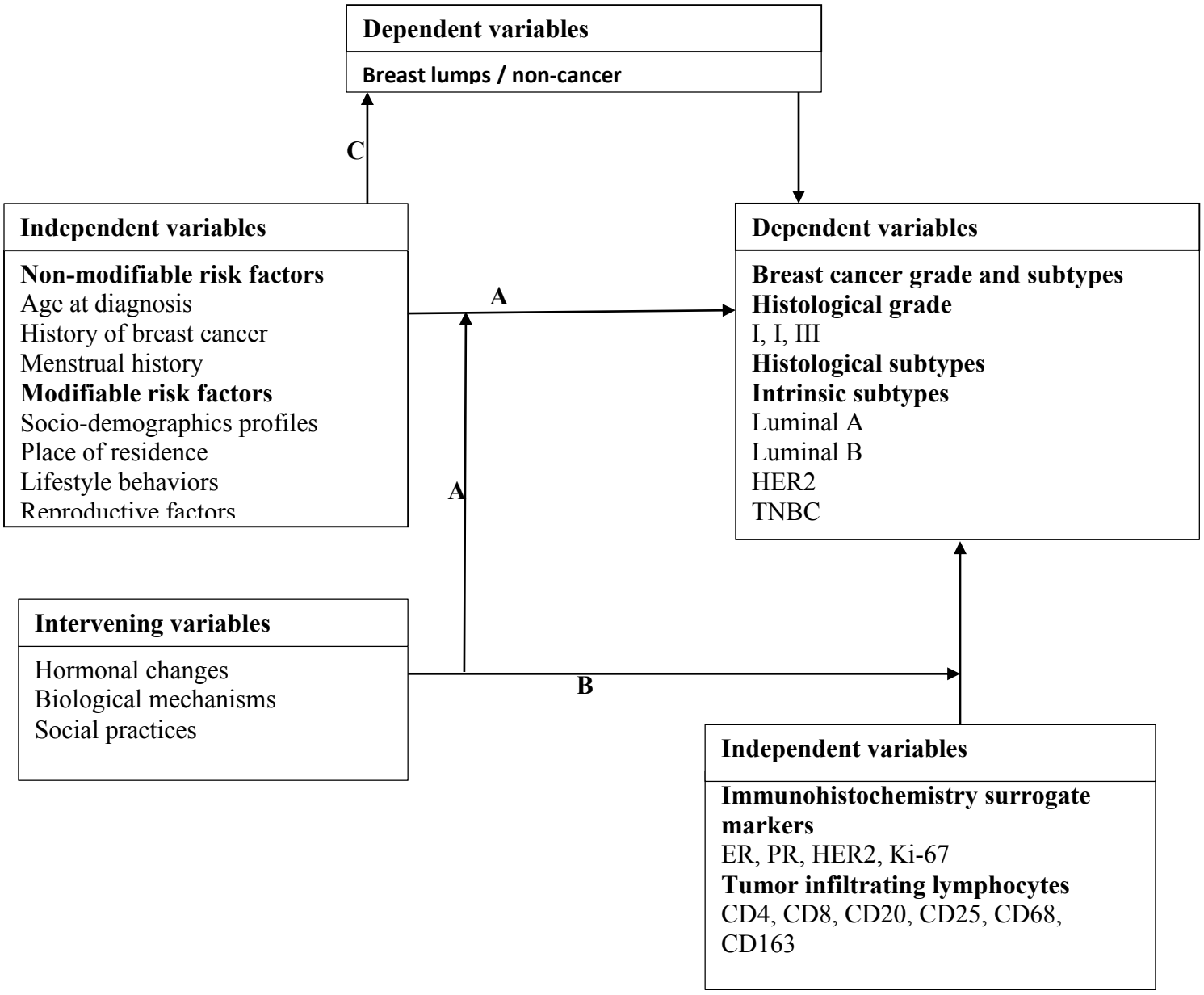

Fig. 2.2: Conceptual frame work

CHAPTER THREE: METHODOLOGY

3.1 Study Area/ Location

This study was carried out at Moi Teaching and Referral Hospital (MTRH) located in Eldoret town, Uasin Gishu County, North of Rift Valley province of western Kenya. It is about 320km North West of Nairobi. MTRH was started in 1917 as a 60 bed cottage Hospital. It was then declared a referral hospital in 1986 with a bed capacity of 324. In 1989 it become Kenya's second nation and teaching referral hospital with the establishment of the school of medicine. It serves as a referral hospital for the whole of western Kenya region including Rift Valley, Nyanza, and Western province.

The oncology center of excellence consists of cancer treatment and control activities, including cancer screening and prevention programs, as well as access to comprehensive palliative care services. The AMPATH oncology team was created in 2002, to address the growing burden of AIDS-defining malignancies in their HIV treatment program. This system has grown from a single nurse and a single physician to the current team comprised of 10 clinicians, 6 core nurses, and an oncology pharmacist. Clinical services are offered in 5 clinical sites - the central Moi Teaching and Referral Hospital Clinic in Eldoret, as well as at district health centers in Busia, Webuye, Chulaimbo, and Kitale.

3.2 Study Population

The study involved women who had breast lumps and MTRH- AMPATH oncology clinic from May 2011 to May 2013 were assessed. The target group was the patients who were to undergo surgery.

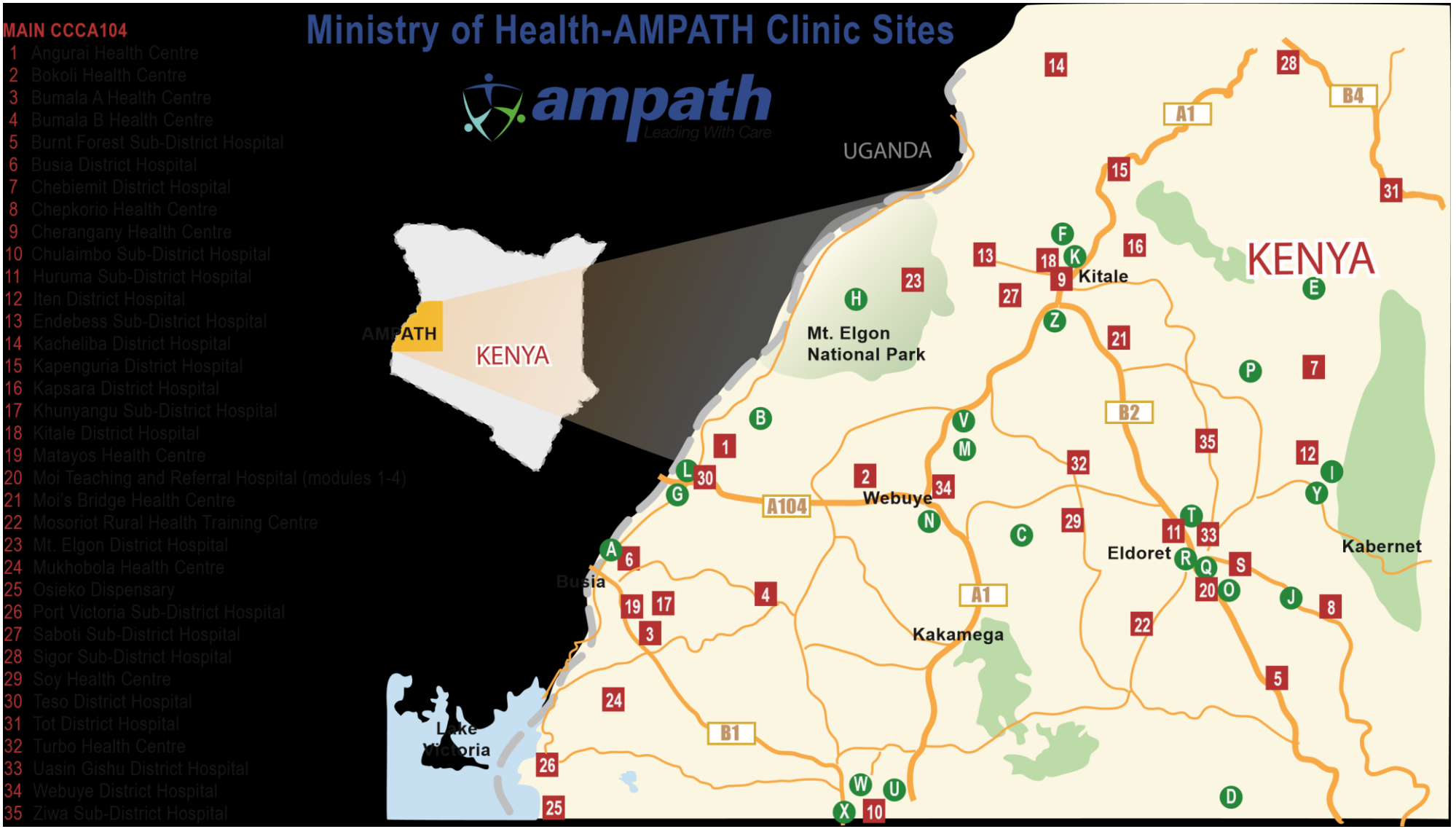

Fig 3.1: MTRH-AMPATH clinics in western Kenya

3.3 Study Design

A cross sectional comparative study design was used. Patients with breast lumps were referred to MTRH from all hospitals in western Kenya. After request for a biopsy heamotocylin and eosin (H&E) stain was done to make a diagnosis. Consent was obtained from histological proven breast cancer cases and normal controls with lumps that were cancerous. A questionnaire/interviewer was administered to the individuals who consented then their tissues blocks were used for immunohistochemical staining

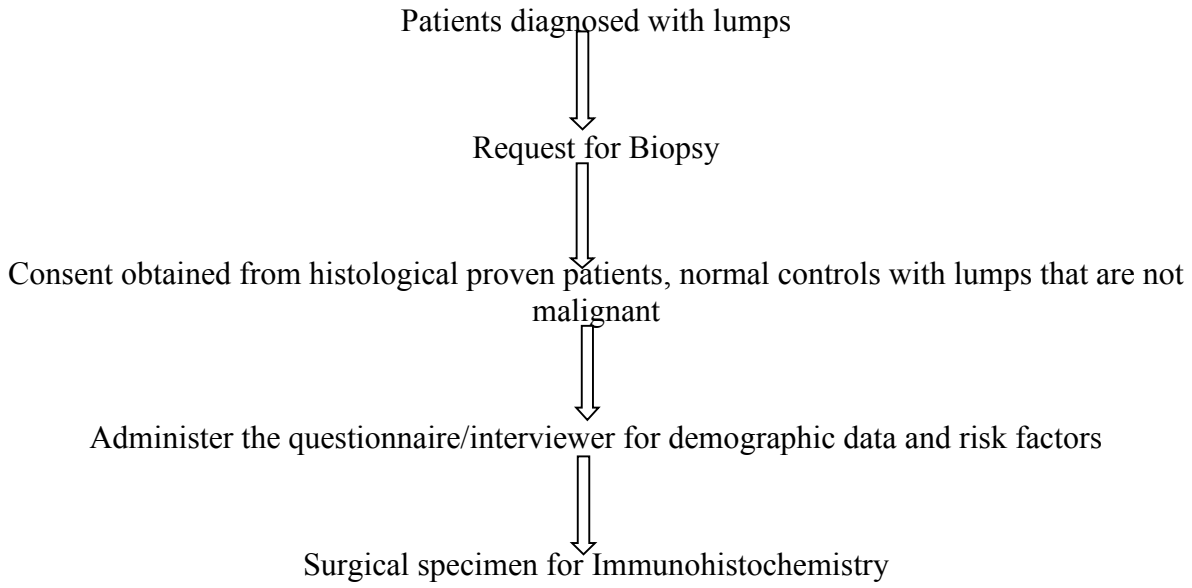

Fig 3.2 Chart indicating work flow

Patients with breast lumps were referred to MTRH from all hospitals in western Kenya. After request for a biopsy heamotoxylin and eosin (H&E) stain was done to make a diagnosis. Consent was obtained from histological proven breast cancer cases and normal controls with lumps that were cancerous. A questionnaire/interviewer was administered to the individuals who consented then their tissues blocks were used for imuunohistochemical staining

3.4 Sampling Procedure

Patients with histologically diagnosed breast cancer who met the inclusion criteria and the controls (individuals with non-malignant lumps) who visited the hospital for diagnosis and treatment were consecutively enrolled into the study after consenting (Appendix 1).

3.5 Eligibility Criteria

3.5.1 Inclusion criteria

a) Histologically confirmed breast cancer

b) No history of chemotherapy

3.5.2 Exclusion criteria

a) Patients who do not provide consent

b) Patients with history of other cancer

3.6 Sample Size Determination

The sample size of 164 was calculated using the following formula. Assuming a significance level of 95% and a two-sided test, there will be 80% power to detect an odds ratio of 2 or larger as significantly different from 1. This is equivalent to a risk factor occurring in 30% of the control group and 50% of the case group. For continuous variables, a differences of 0.4 standard deviations between the two groups using a two-sided test with significance level of 0.05 and 80% power was detected (Sullivan and Soe, 2007)

Therefore n (Each group)

$$= \frac{[P_1 (1-P_1) + P_2 (1-P_2)] [Z_{1-\alpha/2} + Z_{1-\beta}]^2}{[P_2-P_1]^2}$$

Where

P_1= Proportion of exposure among controls - 0.3

P_2= Proportion of exposure among Cases – P_1 (PR) = (0.3) (1.7)

PR = Prevalence ratio

$Z_{1-\alpha/2}$ = Value of the standard normal distribution corresponding to alpha e.g.

1.96 for 2 sided test at 0.05

$Z_{1-\beta}$ = Value of the standard normal distribution to desired power level e.g.

0.84 For 80% power.

$$= \frac{[(0.3)(0.7) + (0.51)(0.49)][1.96 + 0.84]^2}{(0.51-0.3)^2}$$

$$= \frac{(0.4599)(7.84)}{0.0441}$$

= 81.78

The minimum number of study participants required per group was 82 giving a sample size of 164.

3.7 Research Instrument/Data Collection Tool

Structured pre-tested interviewer-administered questionnaire (Appendix II) was administered to the breast cancer patients and the controls after consenting to participate in this study. This enabled collection of the following information; demographic characterization, name, age, gender, nationality, tribe, place of birth, village location, county, marital status, weight and height. Section two consisted of disease status, when diagnosis was made, if on treatment, tumor characteristics, left or right, axillary lymph nodes palpability. Section three consisted of family history and section four had risk factors that included age at first menarche, number of pregnancies, breast feeding, use of contraceptives , use of HRT, smoking, alcohol consumption and other environmental factors.

3.8 Validity and Reliability of Research Instrument

Pilot testing of the questionnaire was done at Mosoriot sub-county hospital to evaluate and improve the reliability of the questionnaire in generating appropriate information. The sample size for the pilot study was 16 (8 cases and 8 controls). That is 10% of the sample size. The split half technique was employed where the questionnaire items were divided into odd and even questions. Their scores were summed and correlated using the Pearson product moment correlation (PPMC). A correlation coefficient of r=0.732 was obtained and was considered reliable.

3.9.0 Data Collection on Risk Factors

3.9.1. Non-modifiable breast cancer risk factors

Non-modifiable risk factors are risk factors that cannot be changed and for this study included age at diagnosis, history of breast cancer, and menstrual history. The categories of non-modifiable risk factors for breast cancer development are shown in Table 2.1.

3.9.2 Modifiable breast cancer risk factors

Modifiable risk factors are the risk factors that can be changed because they represent lifestyle choices. Modifiable risk factors for this study included socio-demographic profiles, lifestyle behaviors, reproductive factors, and environmental factors. The categories of modifiable risk factors in terms of risk for breast cancer development are indicated in Table 2.1.

3.9.3 Tissue fixation

Specimens were fixed in 10% buffered formalin then processed in automatic tissue processor Leica TP 1020 (Leica microsystems Nussloch Gmbh Heidelbeger Str. 17-19 D-69226 Nussloch Germany). After formalin fixation the specimens were dehydrated through a graded series of

ethanol (one hour per step), then cleared in two changes of xylene (45 minutes each) and infiltrated through four changes of melted paraplast X-tra (Mc Cormick^{TM} Scientific) using a wax dispenser; thermal console and cryo console (Especialidals Medicans Mallorca 3 Espania).

The embedded tissue blocks were transferred from the MTRH hospital to the University of Notre Dame and submitted for further studies following IRB approval from both institutions. The tissue samples were subsequently melted down and re-embedded in Surgipath EM-400 paraffin (Leica Biosystems Inc.), using a Sakura Tissue TEK5 embedding station.

3.9.4 Haematoxylin and eosin staining

Thin section (3 μm) were cut using rotary microtone (Letz 1512) equipped with disposable knives. Histological sections were then stained with haematoxylin and eosin. These dyes show the parts of the cells that were then placed on a glass slide, then flattened on heated water bath then floated onto microscope slides and dried. The pathologist then read the slides under a microscope in a process of making histological diagnosis of both cases and controls then went ahead in grading the cases. Grading of the cases was done based on the Etston and Ellis (1991) grading (Table 2.2 and 2.3).

3.9.5 Construction of tissue microarrays (TMAs)

Tissue microarrays (TMAs) of the cancer and non-cancer breast tissue samples were constructed by punching cores from donor blocks with 1 mm diameter stylus and loaded to recipient blocks. Distance between tissue cores was also set at 1 mm. The TMA layout on the recipient TMA blocks was designed in advance to represent and distribute randomly across the TMAs the patient heterogeneity (i.e., cancer and non-cancer) identified by pathology. Tissue chosen to be included in the TMA was based on the pathology of the tissue determined from the H&E stained slides of

each tissue block; each area of interest was circled by a physician as guidance for punching. TMA blocks were constructed with Veridiam Advanced Tissue Arrayer VTA-110CC, which provided a well-controlled mechanism to locate precisely the desired area of tissue from a donor block, extract and deliver it quickly to the recipient block. Assembled TMA blocks were placed atop a glass slide and incubated at 37ºC overnight plus 42ºC for 2 hours to integrate the samples with the main block. Blocks were sectioned at 5µm thickness with Leica rotary microtome RM2125. Each of the two TMAs used for the staining had ~100 tissues per block with duplicates across the two TMAs. Ninety two tissue samples were represented, and all of the tissue samples analyzed were included on both the first and second TMA made and were then stained.

3.9.6 Immunohistochemistry (IHC) Staining

Before staining, dewaxing was done by placing the sections on IHC slides at 60°C for 30 minutes then passed through three exchanges of xylene and then alcohol. After deparaffinization and rehydration, the tissue were subjected to heat induced epitope retrieval (HIER). A working solution was prepared by diluting the envision^{TM} FLEX target retrieval solution (50x) concentration 1:50 in distilled water. Pretreatment (PT) link was then filled with sufficient quantity (1.5L) of working solution to cover the tissue sections, then was set to pre-heat the solution to 65°C. Tissue sections that were already deparaffinized and rehydrated were immersed to pre-heated Envision Flex target retrieval solution and incubated for 20 minutes at 97°C. Then the sections were left to cool in the PT link to 65°C

Each slide rack was removed from the PT link tank and immediately dipped slides into a jar/ tank with diluted room temperature FLEX TM wash buffer (20x) and left for 1-5 minutes. Slides were

then placed on a Dako autostainer Plus (Dako Colorado, Inc.) and proceeded with the staining as per the protocol in Table 3.1.

The primary antibodies used for analysis included antibodies that recognized estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Ki67, CD4, CD8, CD20, CD68, CD163, and CD25 (Table 1). The secondary antibodies used include EnVision ™ Flex+Mouse (Linker), (Dako) SM804; EnVision ™ Flex Peroxidase-Blocking Reagent (Dako) SM801; EnVision ™ Flex/HRP (Dako) SM802; EnVision ™ Flex Antibody Diluent (Dako) DM830; and Hercep Test ™ Peroxidase-Blocking Reagent (Dako) SK001 (Table 3.1).

Table 3.1 List of primary antibodies and their Incubation Time

Antibody	Vendor/Clone	Pretreatment	Dilution	Control Tissue	Detection/ Linker	Incubation time
ER	Dako IR084	TRS High pH	RTU	Breast Ca	Flex	20/20/10
PR	DakoIR068	TRS Low pH	RTU	Breast Ca	Flex + M	20/20/20/10
HER2	Dako SK001	Herceptest Antigen Retrieval		Herceptest	Herceptest	30/30/10
Ki67	DakoIR626	TRS High pH	RTU	Breast	Flex	20/20/10
CD4	DakoIR649	TRS High pH	RTU	Tonsil	Flex + M	15/10/10/10
CD8	DakoIR623	TRS Low pH	RTU	Tonsil	Flex + M	15/15/15/10
CD68KPI	DakoIR609	TRS High pH	RTU	Tonsil	Flex	20/10/10
CD20	DakoIR604	TRS High pH	RTU	Tonsil	Flex	10/10/10
CD25	NCL-CD25-305	TRS High pH	1:100	Tonsil	Flex+M	15/10/10/10
CD163	Vector Laboratories Inc.VP6017007	TRS High pH	1.100	Bone Marrow	Flex +M	15/10/10/10

3.9.6.1 Quality control of IHC

A known positive control specimen was used in each staining run to ascertain a proper performance of all the applied reagents. If a positive control specimen failed to demonstrate positive staining, labelling of test specimens were considered invalid hence were repeated.

A negative control reagent was used with each antibody to identify any nonspecific staining. If the nonspecific staining was not clearly differentiated from the specific staining of the test specimen that test was considered invalid.

3.9.6.2 Interpretation of IHC results

Diaminobenzidine (DAB) containing substrate working solution gave brown color at the site of the target antigen recognized by primary antibody. The brown color seen on the positive control at expected localization of target antigen was picked as positive. The status of the TILs was examined using Aperio Image Analysis Tools software. The intensity of the immune cells was scored according to the general intensity and density as: 0, none (0%), 1, low intensity (upto33%), 2, moderate intensity (34-66%) and 3, high intensity infiltrate (more than 66%). For Ki67, ≥ 14% (high intensity), ≤ 14% (low intensity).

3.10. Image scanning

Before loading slides with tissue sections on the Aperio ScanScope CS whole slide scanner, H&E and IHC stained slides were wiped clean with Kim wipe tissue wetted with alcohol. The slides were positioned onto the tray with the coverslip facing up and the slide label oriented to the left. Scanning objective by default was set at 20x. Using the scanner console software, the snapshot function was used to capture initial macro images of tissues and slide labels. Focus points, calibration spot, and the size of scanning area were adjusted with visual check. Images were scanned and saved onto a database controlled by eSlide manager software. Each included sample was represented at least twice (i.e., once on each TMA).

3.11 Image analysis

Immunohistochemistry (IHC) images were quantified with Aperio Image Analysis Tools software following the vendor's instructions. Customized analysis macros for each stain were first made with the provided nucleus, membrane, or cytoplasm algorithms, usually by adjusting some of the morphologic parameters and adjusting the thresholds for intensity setting. One representative area from each tissue core of the stained tissue sections was manually selected with ImageScope annotation tools and assigned with a position code on the TMA layout for easy identification. After marking out all the regions of interest on a TMA IHC slide, analysis with a proper macro was run and the output results, including cell numbers of each intensity levels, percentage of positive cells, H-score or membrane score depending on the corresponding algorithm, were exported from Image Scope's annotation as an Excel file for further data organization and processing.

3.12 Data Management and Analysis

Data entry and analyses was done using Statistical Package for Social Sciences (SPSS) version 16.0. Descriptive statistics (Frequencies, median, means and standard deviation) were mainly used in summarizing the data. Categorical variables were summarized using frequencies. Continuous variables were mainly summarized using mean for normally distributed variables and median for skewed distributions after normality checks using the Shapiro Wilks test. Clinic pathologic variables and selected categorical variables were compared by Chi-square test.

Mann Whitney U-test was used to compare median between two groups while Kruskal Wallis test was used to compare median between more than two groups. To test for association between tumor-infiltrating immune cells, Pearson correlation analysis was used. The study employed logistic regression model to identify factors associated with aggressive types of breast cancer.

Level of significance was set at $p < 0.05$, with a 95 % confidence interval. Graphics were generated using Graph pad prism 6.0. Results are presented in the form of tables, pie-charts and Bar- graphs

3.13. Ethical Considerations

The approval to conduct this research was obtained from the institutional Ethics and Research Committee (IREC) Moi Teaching and Referral hospital (MTRH) before commencement of the study (Approval number 000655). In addition Authorization and permission was also obtained from National Commission for Science, Technology and Innovation (NACOSTI) (Appendix III). The purpose of this study was explained to the participants in a language that they understood before seeking written informed consent (Appendix I). All the information that was obtained from the patients was kept confidential by not using any form of identification on the data collection tools. The participants were treated with respect and dignity and participation was on voluntary basis. They were also informed of their rights to withdraw at any stage of the study without jeopardizing their health care services. Completed data collection tools were kept under lock and key while data entered in the computer was protected using a password.

CHAPTER FOUR: RESULTS

4.1 Demographic characteristics of the study population

The demographics of the study population are summarized in Table 4.1. A total of 160 individuals in this study. Sixty nine patients had histologically confirmed breast cancer patients while 91 had breast non-malignant breast lumps (Table 4.1)

Table 4.1. Demographic and clinical characteristics of the study population

Cancer status		
Cancer (cases)	69	
Non Cancer (controls)	91	
Total	**160**	
	Cancer	
	N	**%**
Tumor Grade		
I	3	5.8
II	28	53.8
III	21	40.4
Tumor size (cm)		
≤ 2	7	20
2.1-5	7	20
>5	21	60
HER2 status		
Positive	7	14.3
Negative	42	85.7
Estrogen receptor status		
Positive	29	59.2
Negative	20	40.8
Progesterone receptor status		
Positive	19	39.6
Negative	29	60.4
Ki67		
<14%	7	14
≥14%	43	86
Mean age at diagnosis	48.4(SEM 16.8)	

A total of 160 participants were included. The mean age at diagnosis was 48.4(SEM 16.8). (SEM- standard Error of means).

4.2 Categories of Non-Modifiable Risk Factors.

Categories of non-modifiable risk factors are shown in Table 4.2. There was statistical significant variation between the various tribes (Fischer's exact test; p=0.001). Majority of the Layyah patients were cases 22(29.7%) followed by the Kalenjin 17 (23.0%) while the Kisii 7(10.3%), the other tribes had less than 6.0% incidence each.

Majority of the breast cancer patients 27(48.2%) were diagnosed at the age above 49 years placing them at medium category risk. Cases of breast cancer with high risk category (40-49 years) were 12 (66.7%) compared with 6(33.3%) of the non-cancer group. While the low risk group comprising of patients of less than 40 years were 20(33.3%). However age-group was not significantly (Fischer's exact test; p=0.802) related with cancer (Table 4.2).

Table 4.2: Non-Modifiable risk factors in cancer and non-cancer cases

Factor	Cases	Control	P-value
Tribe			
Kalenjin	17(23)	35(51.5)	
Luhya	22(29.7)	18(26.5)	<0.001
Kisii	4(5.4)	7(10.3)	
Luo	7(9.5)	4(5.9)	
Others	24(32.4)	4(5.9)	
History of B/C			
Yes	12(16.2)	20(29.4)	
No	56(75.6)	18(26.5)	<0.001
Other	6(8.1)	30(44.1)	
Menopause	53(51)	51(49)	0.707
No (<55)	21(55.3)	17(44.7)	
Yes(≥55)			
Age at diagnosis			
<40	20(33.3)	40(66.7)	
40-49 years	12(66.7)	6(33.3)	0.802
≥ 50 years	27(48.2)	29(51.8)	

Fischer's exact test: p<0.05. Various non-modifiable risk factors to cancer and non-cancer were compared. Only tribe and family history were significantly different in cancer and non-cancer groups (p<0.001).

4.3 Categories of modifiable risk factors

There was a significant relationship between marital status and development of breast cancer. (Chi square test; p=0.041). Being married places one at low risk of developing breast cancer (Table 4.3). The cancer patients who ever got married were 46(62.2%) compared to 53(77.9%) of the controls. The high risk group are those individual who never got married, 28(37.8%) of the cases and 15(22.1%) of the controls (Table 4.3).

Similarly 2(2.7%) of the cases were smokers compared to 6(8.8%) of the control (p=0.153). Almost all breast cancer patient and controls admitted that they used firewood (98.6% and 100%) respectively. Only 9(12.2%) of the cases used oral contraception (OC) compared to 11(16.2%) of the controls. This indicated a significant relationship between use of OC and development of cancer (p<0.001). Use of injection contraceptive (IC) was significantly related with cancer (p<0.001). However the duration of OC and IC did not show significant relationship. Parity, breast feeding, miscarriage, hysterectomy did not show a significant difference in the two groups (Table 4.3).

Table 4.3: Modifiable risk factors and level of risk in cancer and non-cancer

Characteristic	Case (%)	Control (%)	Risk Level	p-value
Marital status				
Yes	46(62.2)	53(77.9)	Low	0.041
No	28(37.8)	15(22.1)	High	
Life style behavior				
Alcohol use				
Yes	2(2.7)	13(19.1)	High	0.001
No	72(97.3)	55(80.9)	Low	
Smoking				
Yes	2(2.7)	6(8.8)	High	0.153*
No	72(97.3)	62(91.2)	Low	
Firewood				
Yes	73(98.6)	68(100)	High	1.000*
No	1(1.4)	0(0)	Low	
Reproductive factors				
Oral Contraceptive use				
Yes	9(12.2)	11(16.2)	High	
No	26(35.1)	55(80.9)	Low	<0.001
Duration of oral contraceptive use				
<1 yr.	0(0)	1(9.1)	Low	1.000 *
≥1 yr.	7(100)	10(90.9)	High	
Injection Contraceptive use				
Yes	9(12.2)	23(33.8)	High	<0.001
No	53(71.6)	45(66.2)	Low	
Duration Injection use				
<1	1(14.3)	6(30.0)	Low	0.080
≥1	6(85.7)	14(70.0)	High	
Parity				
0	4(16.7)	8(12.1)	High	
1-5	11(45.8)	27(40)	Medium	0.729
≥5	9(37.5)	31(47)	Low	
No. of children breastfed				
None	2(8.7)	8(12.3)	High	
≤4	12(52.2)	27(41.5)	Medium	0.664
≥5	9(39.1)	30(46.2)	Low	
Duration breastfeeding				
<6 months	2(8.7)	7(10.9)	High	
6-11months	1(4.3)	2(3.1)	Medium	0.924
≥12 months	20(87)	55(85.9)	Low	
Miscarriage	26(86.7)	56(86.2)	Low	1.000 *
0	4(13.3)	9(13.8)	High	
≥1				
Hysterectomy	71(95.9)	63(92.6)	High	0.48*
Yes	3(4.1)	5(7.4)	Low	
No				
HIV status	71(95.9)	64(94.1)	Low	0.710*
Negative	3(4.1)	4(5.9)	High	
Positive				
Combined Pill and injection	2(2.7)	3(4.4)	High	0.670*
Yes	72(97.3)	65(95.6)	Low	
No				
Live in house with mice	72(97.3)	68(100)	High	0.497*
Yes	2(2.7)	0(0)	Low	
No				

***** Fisher's exact test's: level of significance p≤0.05. Modifiable risk factors to breast cancer were compared in cancer and non-cancer cases. Only marital status and reproductive factors are risk factors to breast cancer.

4.4 Relating marital status, use of contraceptive and alcohol as risk factors for cancer

As indicated in Table 4.4, multiple binary logistic regression indicated that use of injection contraceptive put an individual to five times risk of breast cancer. Similarly Kalenjin tribe were three times more likely to develop breast cancer more than the other tribes. Having a history of breast cancer is put one to risk of developinf breast cancer (Table 4.4).

Table 4.4: Multiple Binary logistic regression

Factor	Regression coefficient(β)	AOR(95%CI)	P-value
Married	-0.604	0.546(0.115-2.595)	0.447
Use pill for contraception	-1.849	0.157(0.009-2.776)	0.207
Use injection for contraception	1.504	4.499(0.735-27.545)	0.104
Tribe (ref=Others)			
Kalenjin	1.161	3.192(0.661-15.404)	0.148
Luhya	-0.058	0.944(0.172-5.179)	0.947
History of B/C	0.110	1.116(0.267-4.673)	0.880
Alcohol use	-2.954	0.052(0.004-0.736)	0.029

AOR=adjusted Odds ratio

Those from the Kalenjin community were 3 times more likely to be cases compared to other tribes (OR; 95%CI: 3.192(0.661-15.404) though not statistically significant. Similarly, those using injection for contraceptive were almost 5 times more likely to be cases compared to those not using (OR; 95%CI: 4.499(0.735-27.545)).

4.5 Histological breast cancer subtype

Representation of the histological types of the cancer patients are summarized in Fig 4.1a. The distribution of cancer pathologies in the breast tissues analyzed after heamatoxylin and eosin (H&E) staining. Most (79% and 9%) of these patients were diagnosed with invasive ductal carcinoma (IDC) and mucinous IDC respectively.

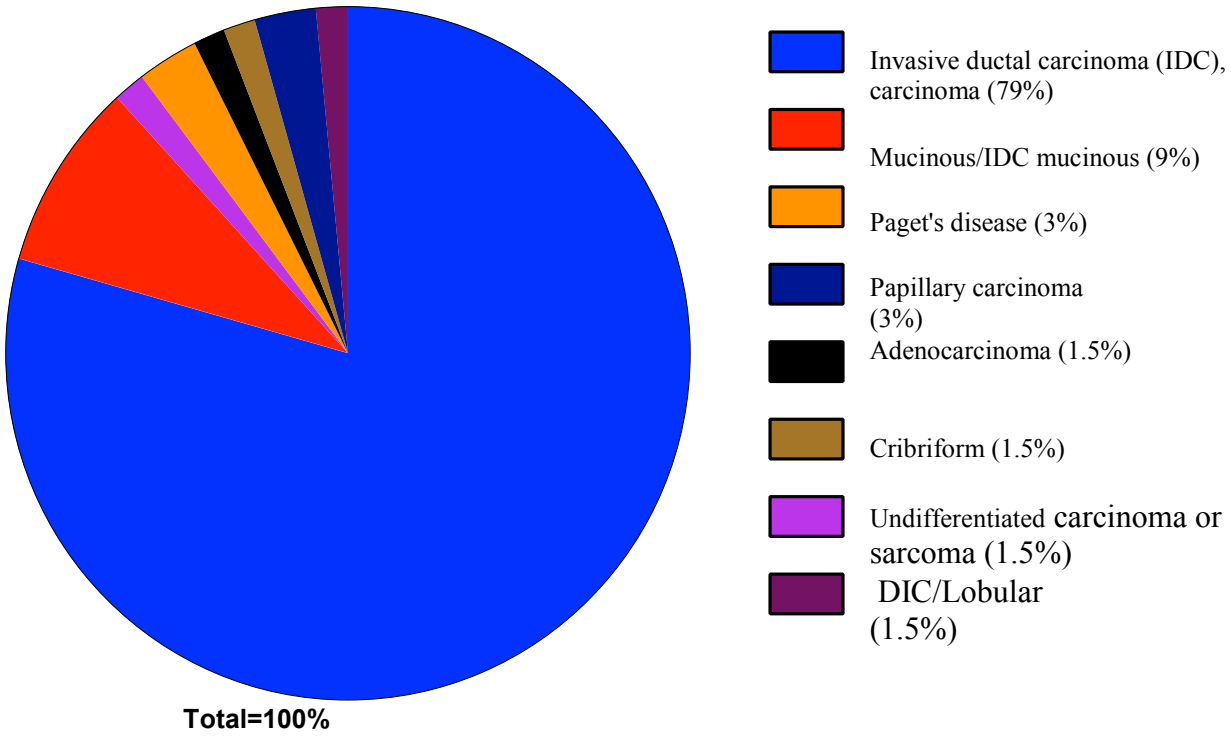

Fig.4.1a: Histological breast cancer types. Invasive ductal carcinoma (IDC) was the most (79%) likely to be diagnosed followed by mucinous and Papillary carcinoma (9% and 3% respectively). Adenocarcinoma, cribriform, undifferentiated carcinoma and lobular carcinoma were all at 1.5%.

Heamatoxylin and eosin staining of representative breast cancer samples analyzed for pathology. Both Patient 1 and Patient 2 have invasive ductal carcinoma (IDC). Patient 1 has some residual glandular organization and also has immune cell infiltration. Patient 2 is highly undifferentiated with immune cell infiltration and fibrotic stroma (Fig. 4.1b).

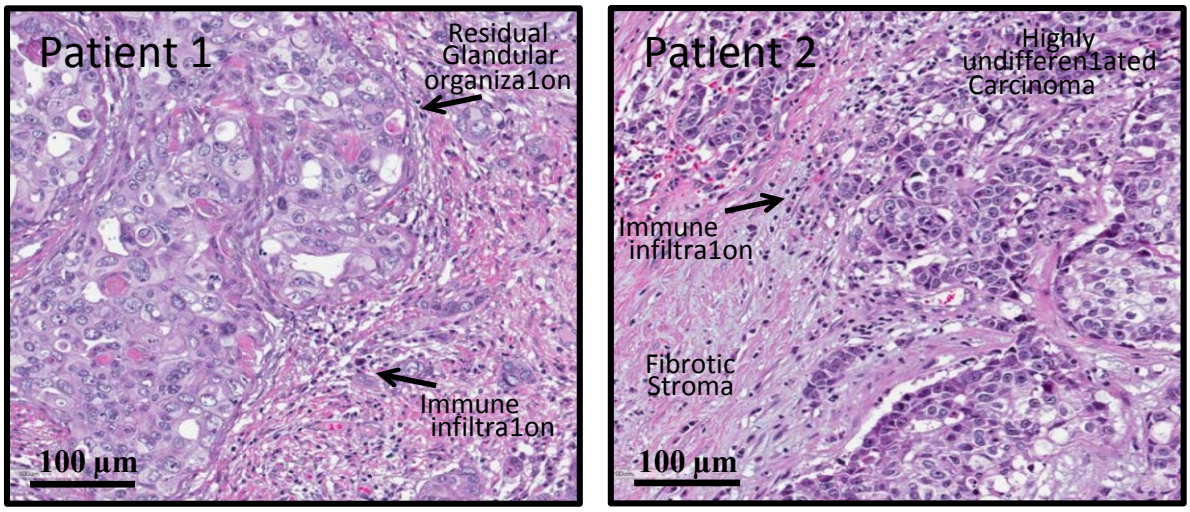

Fig. 4.1b Heamatoxylin and eosin (H&E) staining for IDC. Patient 1 has some residual glandular organization and also has immune cell infiltration. Patient 2 is highly undifferentiated with immune cell infiltration and fibrotic stroma

4.6 Intrinsic Breast Cancer Subtypes

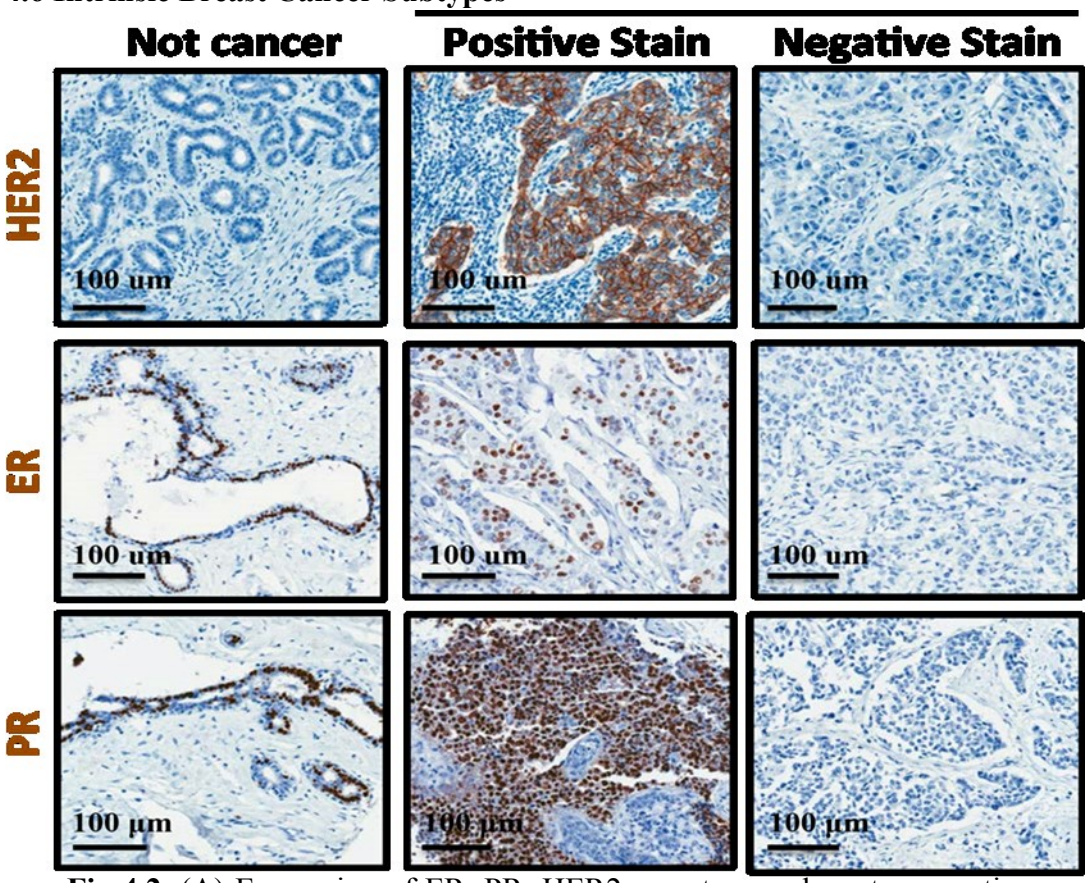

Fig 4.2. (A) Expression of ER, PR, HER2 receptors on breast cancer tissues. Representation of tissue samples from cancer and non-cancer tissues that were stained for HER2, ER, and PR receptor expression. Examples of tissue that stained positively and negatively for the receptors are included.

Immunohistochemistinry representative slides that is showing the staining for HER2, ER, and PR receptor expression (Fig. 4.2 (A). Fig 4.2(B) represent cancer and not cancer samples stained for the Ki67 proliferation marker. Because the graph is a log scale, any samples with unstained sections (i.e., zero) are not included. The bar represents the median of all samples in the indicated cohort and excludes any unstained samples. There was a significant increase ((P<0.0001; Mann-Whitney) of proliferative index (Ki67) in cancer tissue samples versus non cancer samples, indicating an increase in cellular proliferation as indicated in dot plot analysis. (Fig.4.2(C).

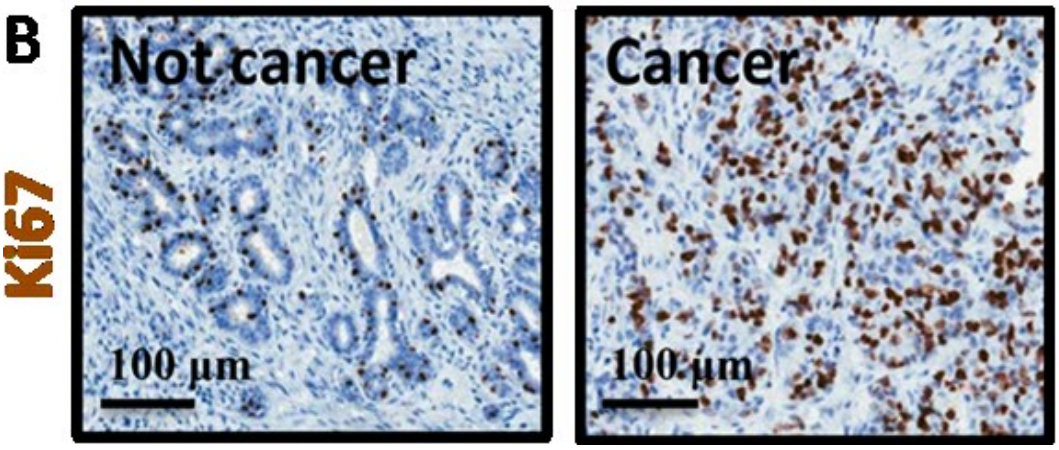

Fig 4.2; (B) Expression of Ki67 on cancer and non-cancer breast tissues. Representation of cancer and not cancer samples stained for the Ki67 proliferation marker

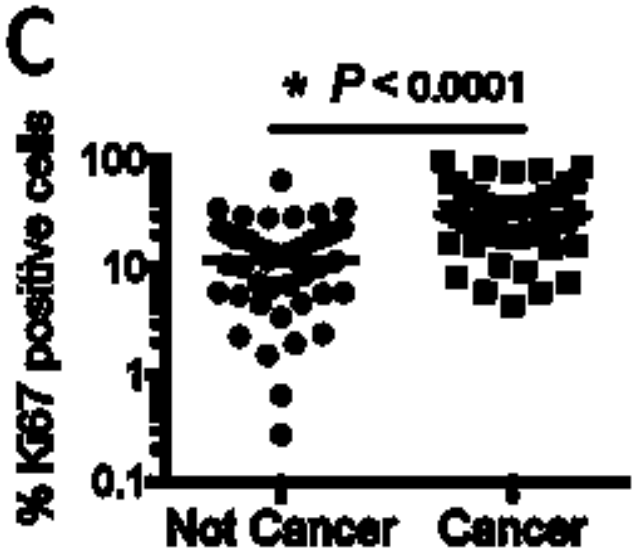

Fig 4.2; (C) Plot of expression of Ki67 in cancer and con-cancer breast tissues. Dot plot analysis of Ki67 positive cells in cancer vs. not cancer tissue samples. Ki67 staining is significantly increased (P<0.0001; Mann-Whitney. Because the graph is a log scale, any samples with unstained sections (i.e., zero) are not included in the graph. The bar represents the median of all samples in the indicated cohort and includes any unstained samples.

Classification of molecular breast cancer subtypes based on IHC4 score showed that, 26.4% were luminal A, 30.2% luminal B, 34.0 % were TNBC and 2.0% were HER2 overexpressed as summarized in Table 4.5

Table 4.5. Intrinsic breast cancer subtypes

Variable	Characteristics	N (%)
Luminal A	ER+ and/or PR+, HER2-, low ki67	14(26.4)
Luminal B	ER+ and/or PR+, HER2+ (or HER2- with high Ki67)	16(30.2)
Her2 Over expressed	ER- and /or PR-, Her2 +Any Ki67	2(2.0)
Triple-Negative	ER-, PR-, HER2- and any Ki67	18(34.0)
Unclassified	None	4(7.5)

ER+/-, estrogen receptor positive or negative; PR+/-, progesterone receptor positive or negative; HER2 +/-, human epidermal growth factor positive or negative.

4.7.0 Types of Tumor Infiltrating Leukocytes in Cancer and Non-Cancer Breast Tissues

4.7.1 Infitration of CD4+, CD8+, and CD20+ cells in breast tissue.

Examples of slides that were stained for T helper ($CD4^+$), T cytotoxic ($CD8^+$) cells and B ($CD20^+$) cells are shown in Fig. 4.3.

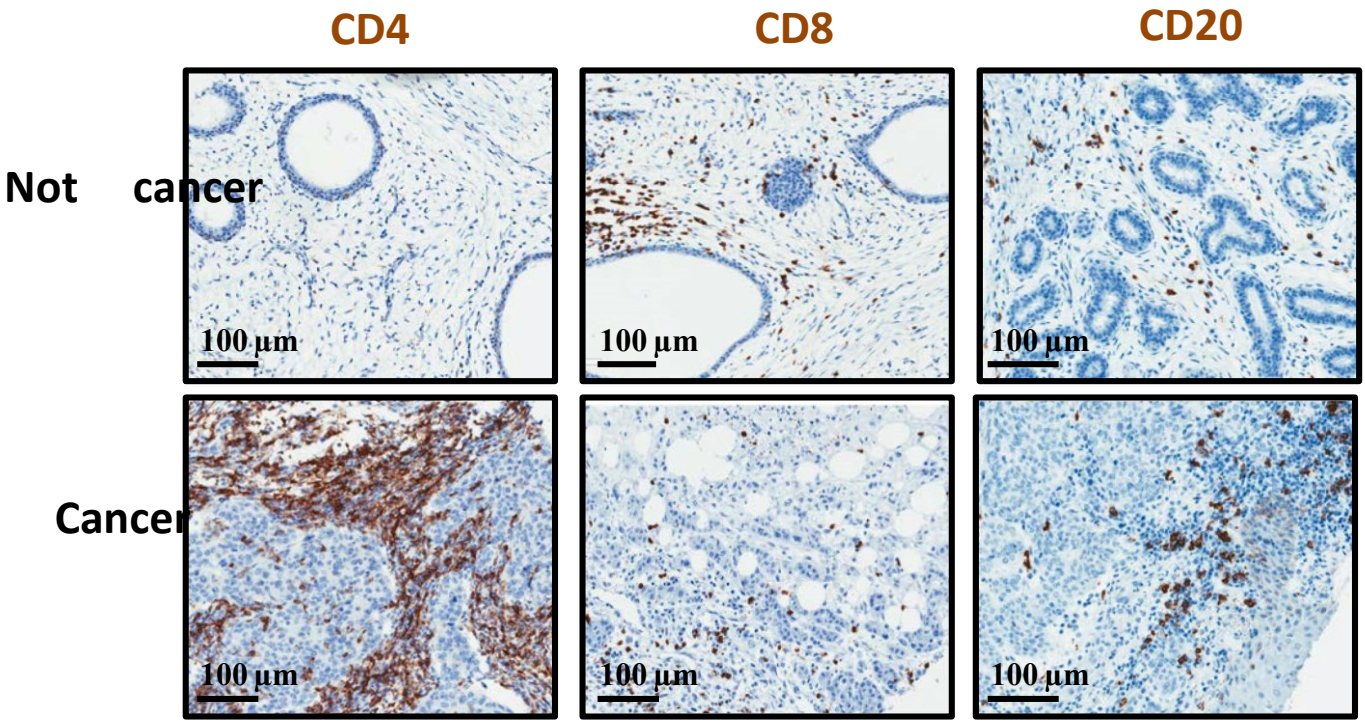

Fig. 4.3. Immunohistochemistry stains for $CD4^+$, $CD8^+$ and $CD20^+$ infitration in cancer and non-cancer breast tissues. Examples of immunohistochemistry stainings for $CD4^+$ T cells, $CD8^+$ T cells and $CD20^+$ B cells in cancer and non-cancer groups.

Data analysis comparing the non-cancer and cancer samples stained for T helper cell ($CD4^+$), cytotoxic ($CD8^+$) and B cells ($CD20^+$) infitration are shown in Fig 4.4. A significant increase was seen in T helper cell infiltration in the cancer samples shown by a higher percentage of $CD4^+$ stained cell area ($P = 0.03$; Mann-Whitney). Comparing non-cancer and cancer samples stained for CD8+ cytotoxic T cells showed no significant difference (n.s.; Mann-Whitney). Similarly there was no significant difference seen in $CD20^+$ cell infiltration in the cancerous samples, as shown

by percentage of $CD20^+$ stained cell area (n.s.; Mann-Whitney). Because the graph is a log scale, any samples with unstained sections (i.e., zero) are not included in the graph. The bar represents the median of all samples in the indicated cohort and includes any unstained samples (Fig 4.4).

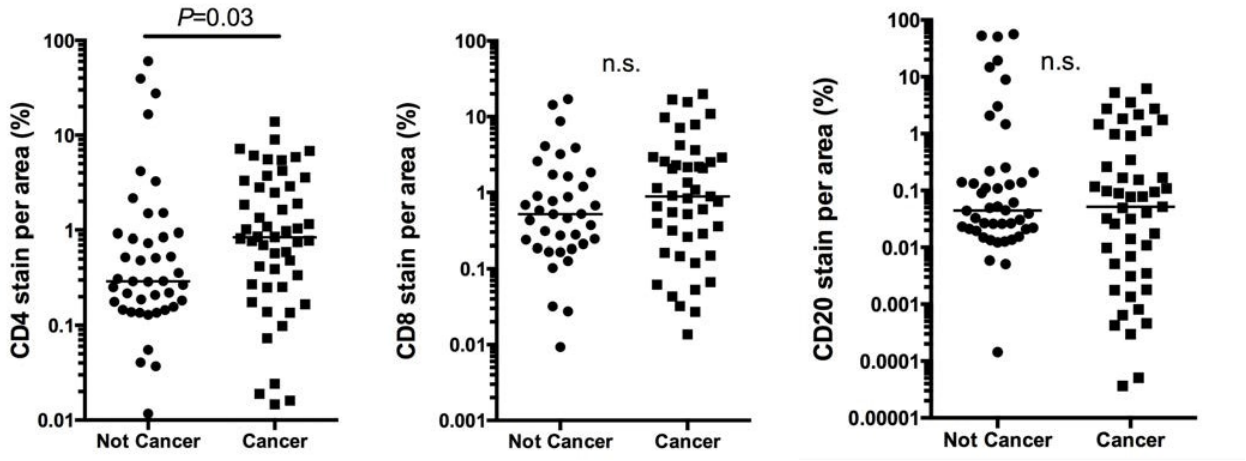

Fig. 4.4 Comparison of CD4+, CD8+ and CD20+ lymphocytes in cancer and non-cancer. A significant increase was seen in T helper cell infiltration in the cancer samples shown by a higher percentage of $CD4^+$ stained cell area. $CD8^+$ cytotoxic T cells and $CD20^+$ B cells showed no significant difference (n.s.; Mann-Whitney).

4.7.2 Infiltration of inducible regulatory T cells ($CD25^+$) in breast cancer tissues.

Immunohistochemistry stainings of noncancrous and cancerous samples stained for CD25+ inducible regulatory T cells (Fig.4.5). A significant increase was seen in regulatory T cell infiltration in the cancer samples, as shown by a higher percentage of positively stained cells (P = 0.03; Mann-Whitney), increased number of positively stained cells per area (P =0.01; Mann-Whitney), and a higher percentage of CD25 stain per area (P=0.0001; Mann-Whitney) (Fig. 4.6).

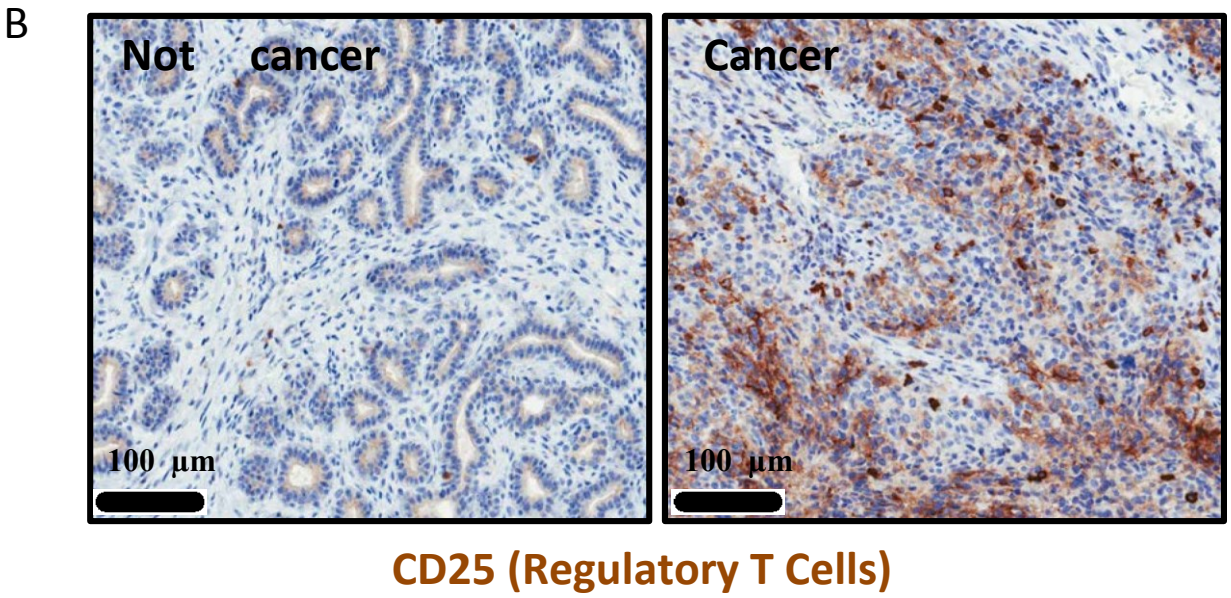

Fig 4.5: Immunohistochemistry stains for CD25 (iTreg) in cancer and non-cancer tissues. Examples of Immunohistochemistry stainings of noncancrous and cancerous samples stained for CD25+ regulatory T cells

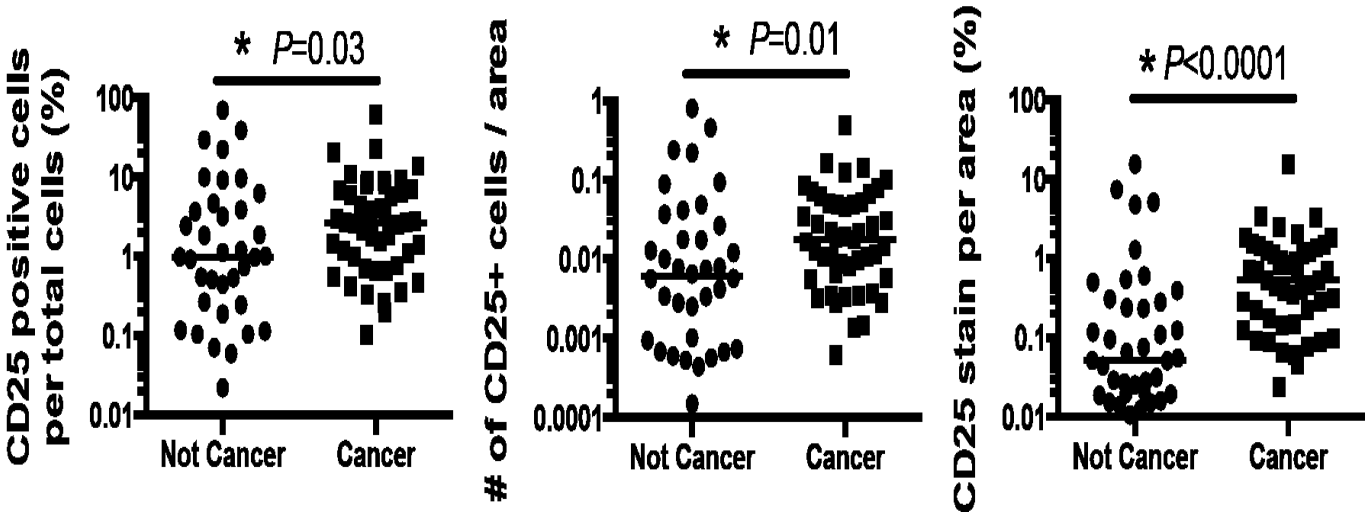

Fig 4.6. Comparison of CD25+ (iTreg) in cancer and non- cancer tissues. A significant increase was seen in regulatory T cell infiltration in the cancer samples, as shown by a higher percentage of positively stained cells

4.7.3 Macrophage infiltration of cancer and non-cancer breast tissues

Immunohistochemistry representative slides for the macrophage lineage CD68+ and CD163+ are shown in Fig. 4.7. Quantitative analysis of the staining indicates a significant increase in percent of $CD68^+$ stained area (P <0.0001; Mann-Whitney) (Fig. 4.8). Quantitative analysis of the IHC staining revealed a significant increase in percent of $CD163^+$ (M2) stained area ($P \leq 0.0001$; Mann-Whitney) in cancer breast tissues versus noncancer breast tissues (Fig. 4.8). Because the graphs are a log scale, any samples with unstained sections (i.e., zero) are not included in the graph.

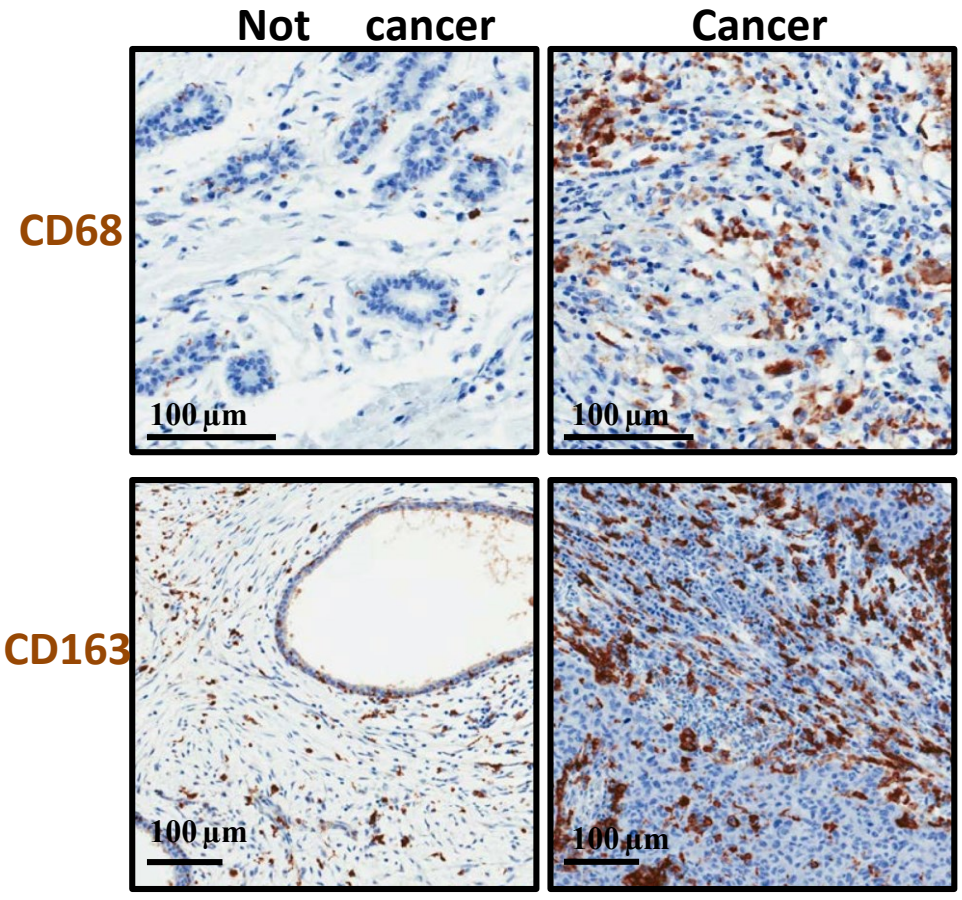

Fig. 4.7 Immunohistochemistry representative slides for the macrophage lineage $CD68^+$ and $CD163^+$ in cancer and non-cancer tissues.

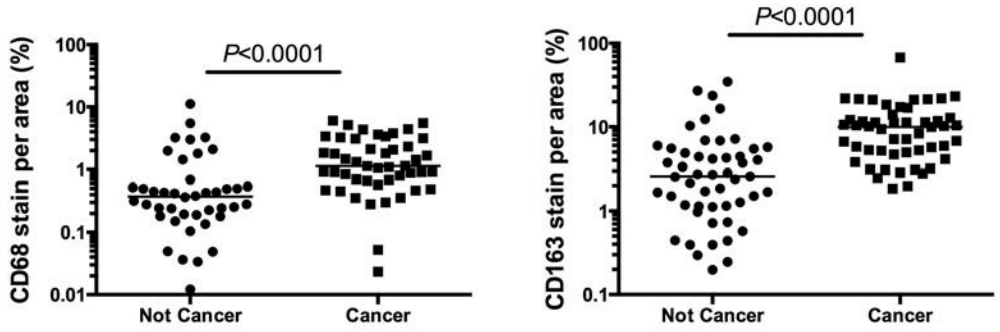

Fig. 4.8 Distribution of CD68+ and CD163+ macrophage lineage cancer and non-cancer breast tissues. Quantitative analysis of the staining indicates a significant increase in percent of CD68+ stained area (P <0.0001; Mann-Whitney). A significant increase in percent of CD163+ (M2) stained area (P ≤0.0001; Mann-Whitney) in cancer breast tissues versus non-cancer breast tissues.

4.8.0 Density of TILS in breast cancer intrinsic subtypes and grades.

4.8.1 Density of TILS in intrinsic subtypes (TN, luminal A, luminal B and HER2 overexpressed) subtypes.

Breast cancer tissue samples were stained for TILs, including CD68, CD163, CD4, CD8, CD20, and CD25. Each sample was scored for percentage of positively stained area for the indicated TIL (Fig 4.9). The positively stained area then was compared across all molecular subtypes Basal/TN, HER2, Luminal A, and Luminal B by Kruskal-Wallis followed by Dunn's multiple comparison tests; the stained area did not significantly vary by molecular subtype.

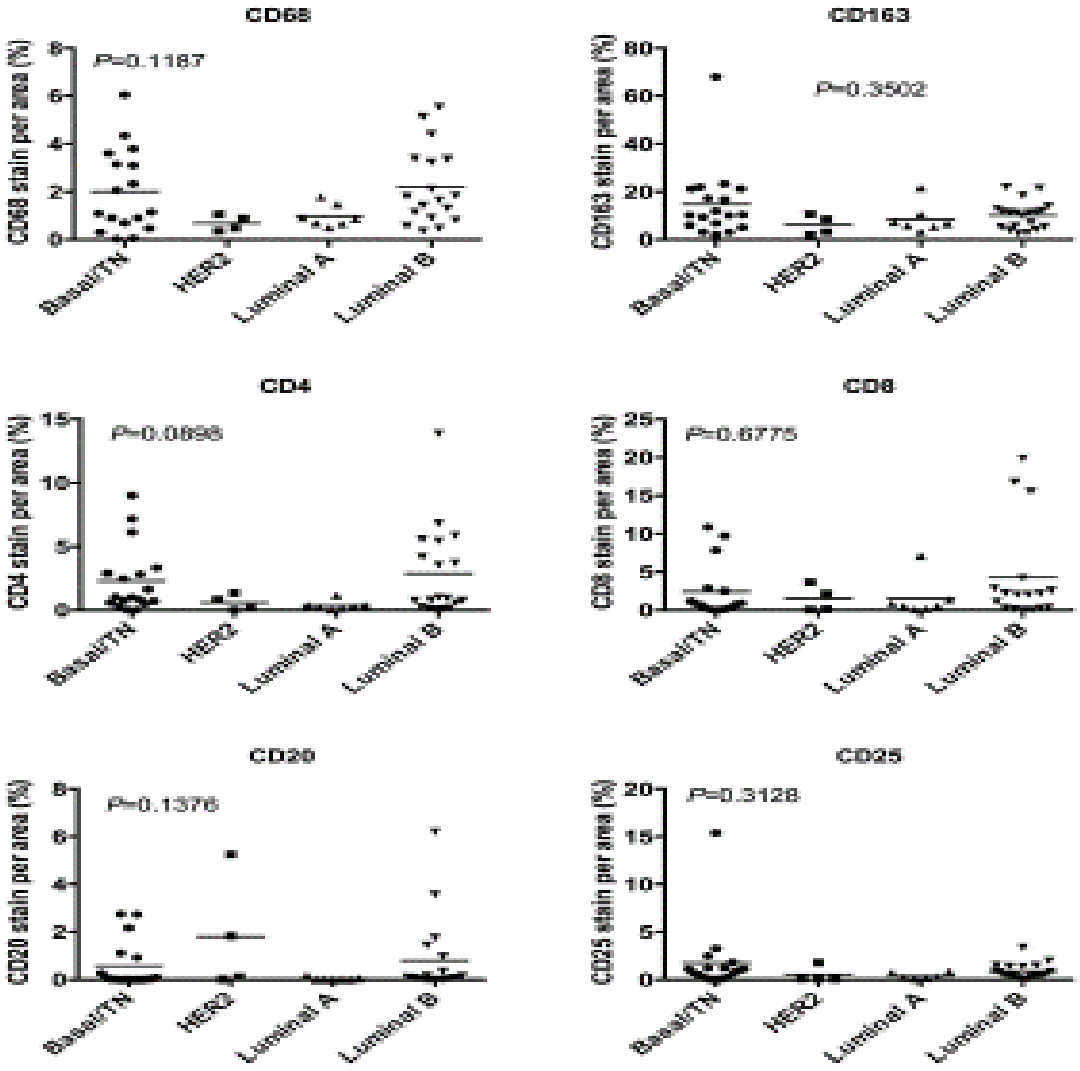

Figure 4.9. The density of TILs does not change across molecular subtype. The density of TILs was compared between intrinsic subtypes. The Percentage of TILs were similar for all molecular subtypes (Kruskal-Wallis followed by Dunn's multiple comparison tests).

4.8.2 Density of TILs in grade I, II and III of breast cancer

Breast cancer tissue samples were stained for TILs, including CD68, CD163, CD4, CD8, CD20, and CD25. The positively stained areas then were compared across tumor grades I, II, and III by Kruskal-Wallis followed by Dunn's multiple comparison tests; the stained area did not significantly vary by grade (Fig. 4.10).

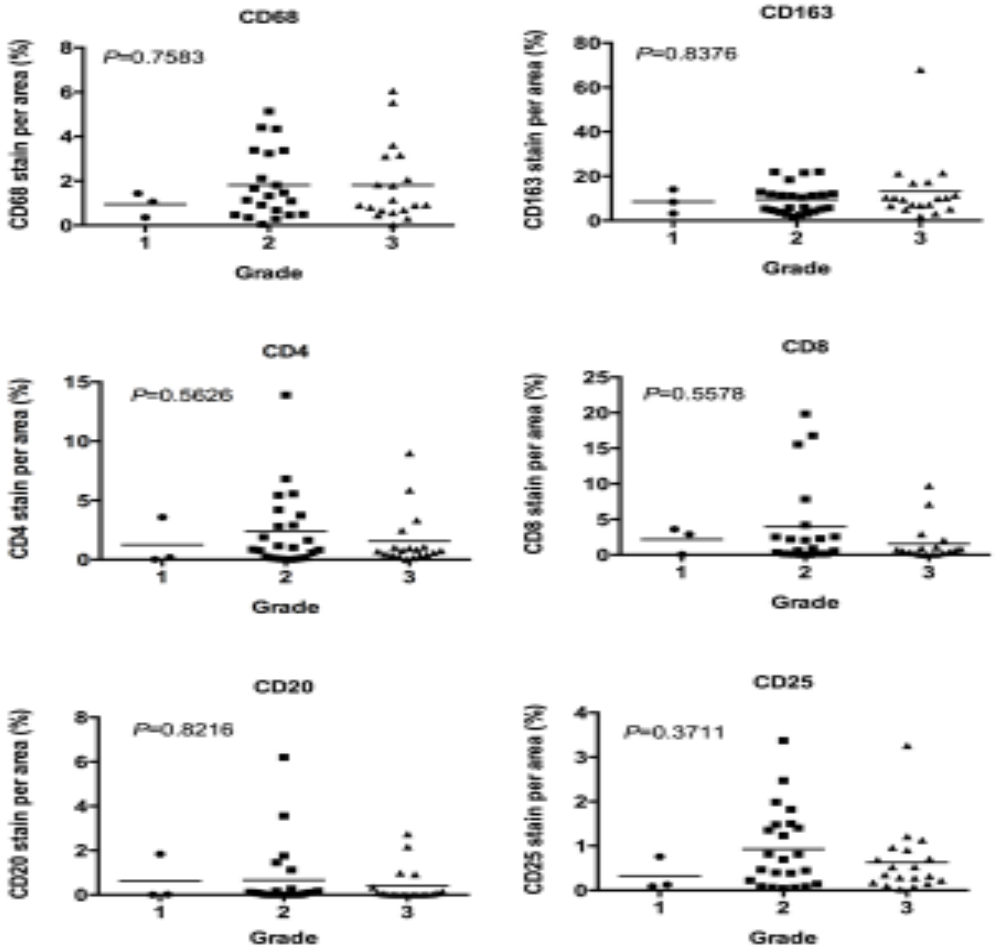

Fig. 4.10. The density of TILs does not change across tumor grades

Breast cancer tissue samples were stained for TILs, including CD68, CD163, CD4, CD8, CD20, and CD25. Each sample was scored for percentage of positively stained area for the indicated TIL. The positively stained areas then were compared across tumor grades I, II, and III (Kruskal-Wallis followed by Dunn's multiple comparison test).

4.9 Proliferation index of tumors in different intrinsic subtypes and grades

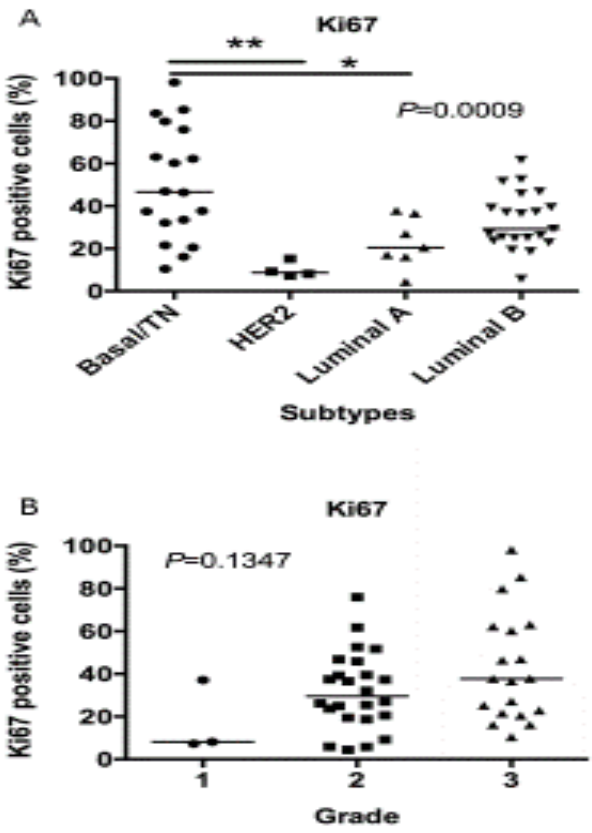

Fig 4.11: Ki67 status in Kenyan breast tumors by grade and molecular subtype.

Kruskal-Wallis (*P*=0.0009) followed by Dunn's multiple comparison tests (the *P* value is indicated by * and ** for *P*<0.05 and <0.01, respectively). There was no significant different of Ki67 status across tumor grades (*p*=0.1347).

Breast cancer tissue samples were stained for Ki67 and scored for percent Ki67 positive cells. Ki67 status then was compared across Intrinsic subtypes (A) and (B) by Kruskal-Wallis (*P*=0.0009) followed by Dunn's multiple comparison tests (the *P* value is indicated by * and ** for *P*<0.05 and <0.01, respectively) (Fig 4.11). There was no significant different of Ki67 status across tumor grades (*p*=0.1347).

4.9.0 Association of Risk Factors, TILs and Breast Cancer Subtypes

Table 4.6. Association of histological grades I, II and III with breast cancer risk factors

Factor	Grade		χ^2 value	p
	Low	High	value	
Age at diagnosis	53.1(SD17.1)	45.4(SD15.0)	1.599	0.094
Tribe				
Kalenjin	13(76.5)	4(23.5)		
Kikuyu	4(80.0)	1(20.0)		
Luo	2(25.0)	6(75.0)	7.935	0.094
Luyha	9(50.0)	9(50.0)		
Others	3(75.0)	1(25.0)		
Gender				
Female	31(63.3)	18(36.7)	4.700	0.060
Male	0(0.0)	3(100)		
History of BC				
No	19(61.3)	12(38.7)		
Yes	6(50.0)	6(50.0)	0.683	0.711
Don't Know	6(66.7)	3(33.3)		
Menopause				
<55 years	16(50.0)	16(50.0)	1.820	0.179
≥55 years	10(71.4)	4(28.6)		
Marital Status				
No	6(54.5)	5(45.5)	0.149	0.479
Yes	25(61.0)	16(39.0)		
Alcohol use				
No	24(55.8)	19(44.2)	1.722	0.423
Yes	25(66.7)	1(33.3)		
Use of firewood				
No	1(100.0)	0(0.0)	2.387	0.303
Yes	25(55.6)	20(44.4)		
Living a house with mice				
No	1(100)	0(0.0)	2.387	0.303
Yes	25(55.6)	20(44.4)		
Number of babies				
<5	7(77.8)	2(22.2)		0.500
>5	6(66.7)	3(33.3)		
Hysterectomy				
No	11(68.8)	5(31.2)	0.865	0.649
Yes	1(100.0)	0(0.0)		
Degree of BC history				
1st	1(25.0)	3(75.0)		0.075
2nd	4(100.0)	0(0.0)		

Association between risk factors and breast cancer grade. None of the tested modifiable and non-modifiable risk factors were associated with grade (Chi square test: Level of significance $p<0.05$)

Histological grade was classified as low (grade I and II) and high (grade III). When correlated grade and breast cancer risk factors it was found that patients who were diagnosed by low grade had a mean age of 53.1(SD 17.1) while those with high grade had a low mean age of 45.4(SD45.40). However it was no statistically significant p=0.094. Kikuyu tribe were likely to be diagnosed with low grade breast cancer (80.0%) and Luo were more likely to be diagnosed with high grade breast cancer (75.0%) (Table 4.6). Breast cancer risk factors were not significantly different in Luminal subtypes (Table 4.7).

Table 4.7: Association of Luminal A Subtype with Breast Cancer Risk Factors

Factor	Luminal A		χ2-value	p Value
	No	Yes		
Tribe				
Kalenjin	8(50.0)	8(50.0)		
Kikuyu	1(20.0)	4(80.0)	5.822	0.201[f]
Luo	6(75.0)	2(25.0)		
Luyha	11(73.3)	4(26.7)		
Others	3(75.0)	1(25.0)		
Histology of BC				
No	1.8(66.7)	9(33.3)	1.529	0.481
Yes	5(45.5)	6(54.5)		
Don't Know	6(60.0)	4(40.0)		
Menopause				
<55	14(51.9)	13(48.1)	2.766	0.096
≥55	11(78.6)	3(21.4)		
Gender				
F	27(60.0)	18(60.0)	0.052	1.000
M	2(66.7)	1(33.3)		
Married				
No	7(70.0)	3(30.0)	0.485	0.719
Yes	22(57.9)	16(42.1)		
Alcohol Use				
No	24(63.2)	14(36.8)	1.236	0.738
Yes	1(33.3)	2(66.7)		
Live a house with mice				
No	0(0.0)	1(100.0)	1.630	0.648
Yes	25(62.5)	15(37.5)		
Babies breastfed				
<5	3(42.9)	4(57.1)	1.165	0.401
≥5	22(64.7)	12(35.3)		

Association of breast cancer risk factors and luminal A subtype. None of the cancer risk factors was significantly associated with luminal A type (f – Fisher's exact test: Chi square test).

Similarly breast cancer risk factors showed no correlation to HER2 subtype (Table 4.8). Among the breast cancer risk factors, being Luo was found to be significantly associated with TBNC (p=0.016). None of the kikuyus' was TBNC and majority of those from Kalenjin community (87.5%) were not TBNC compared to 37.5, 46.7% for Luo, Luhya and others respectively (Table 4.9)

Table 4.8: Correlation of HER2 over-expressed subtype with breast cancer risk factors

Factor	Her2		Test	p Value
	No	Yes		
Tribe				
Kalenjin	16(94.1)	1(59.0)		
Kikuyu	5(100.0)	0(0.0)		
Luo	8(100.0)	0(0.0)	3.137	0.553
Luyha	14(93.3)	1(25.0)		
Others	3(75.0)	1(6.7)		
History BC				
No	26(92.9)	2(7.1)		
Yes	10(90.9)	1(9.1)	0.873	1.000
Don't know	10(100.0)	0(0.0)		
Menopause				
<55	26(96.3)	1(3.7)	1.348	0.287
≥55	13(86.7)	2(13.3)		
Age Group				
<40	12(93.3)	1(7.7)		
40-49	10(100.0)	0(0.0)	0.960	0.785
≥50	17(89.5)	2(10.0)		
Gender				
F	43(93.5)	3(6.5)	0.208	1.000
M	3(100.0)	0(0.0)		
Married				
No	8(80.0)	2(20.0)	4.210	0.102
Yes	38(97.4)	1(2.6)		
Babies Breast fed				
≤5	5(71.4)	2(28.0)	5.815	0.067
>5	34(97.4)	1(2.9)		

Association of breast cancer risk factors and HER2 subtype. None of the risk factors were significant to HER 2 subtype (Chi-square test: Level of significance p≤ 0.05).

Table 4.9: Association of TNBC subtype with breast cancer risk factors

Factor	TNBC		Test	P Value
	No	Yes		
Tribe				
Kalenjin	14(87.5)	2(12.5)		
Kikuyu	5(100.0)	0(0.0)		
Luo	3(37.5)	5(62.5)	10.931	0.016
Luyha	7(46.7)	8(53.3)		
Others	7(46.7)	8(53.3)		
History of BC				
No	18(66.7)	9(33.3)		
Yes	7(63.6)	4(36.4)	0.202	1.000
Don't know	7(70.0)	3(30.0)		
Menopause				
<55	18(66.7)	9(33.3)	0.360	0.548
≥55	8(57.1)	6(42.9)		
Gender				
F	31(68.9)	14(31.1)	1.600	0.223
M	1(33.3)	2(66.7)		
Married				
No	7(70.0)	3(30.0)	0.063	1.000
Yes	25(65.8)	13(34.2)		
Use of firewood				
No				
Yes	1(100.0)	0(0.0)	1.730	0.597
Alcohol use	25(62.5)	15(37.5)		
No				
Yes	23(60.5)	15(39.5)	2.593	0.220
Injection	3(100.0)	0(0.0)		
No				
Yes	20(66.7)	10(33.3)	3.214	0.395
Babies breast fed	2(40.0)	3(60.00		
≤5				
>5				
Age group	6(85.7)	1(14.3)	1.809	0.232
<40	20(58.80	14(41.2)		
40-49				
≥50	8(61.5)	5(38.5)	0.317	0.921
	7(70.0)	3(30.0)		
	11(61.1)	7(38.90		

Association of risk factors with TNBC. Only tribe showed a significant relationship with TNBC subtype. (Chi-square test level of significance $p \leq 0.05$).

CHAPTER FIVE: DISCUSSION

5.1.0 Risk Factors Associated With Breast Cancer

The known risk factors associated with breast cancer may be classified as non-modifiable and modifiable factors.

5.1.1.0 Non-modifiable risk factors

5.1.1.1 Age at diagnosis

Breast cancer is among the fastest rising non communicable disease in low and middle income countries such as Kenya. In the current study, the mean age at diagnosis was 48.4 years. This was comparable with mean age at diagnosis of 46 years found among Tanzanian women (Rambau *et al*., 2011) and 45 years of a Uganda study (Gakwaya *et al.,* 2008) but younger than 53.5 years recorded in Lesotho women (Lehlasoa 2011). The age range of the study population was 15 to 82 years which is slightly lower than the south African and also the Ugandan study that reported a range of 24 to 86 and 22 to 85 years respectively (Lehlasoa, 2011; Gakwaya *et al*. 2008).

When age at diagnosis was stratified, it was evident that 54.3% were below the age of 50 years compared to 75.7% of Tanzania, 46.1% of South Africa and 60% of Nigeria and similar to 54% recorded in Uganda (Rambau *et al*. 2011; Ogundiran *et al*., 2010 Gakwaya *et al*. 2008). When compared to the United States, Swanson and Lin (1994) reported that less than 23% of breast cancer diagnosis were made in women younger than 50 years. Breast cancer diagnosis before the age of 50 years is a risk factor that predisposes to recurrence (Chen *et al*. 1999). This study reports that more than half of the breast patients seen in Kenya are at risk of developing a second primary breast tumor because their first primary tumor developed at a younger age of less than 50 years.

Therefore proper management and follow up in these patients could help prevent recurrence of breast cancer.

The explanation to the young age at diagnosis is thought to be due to varied reasons. Akarolo-Anthony *et al*. (2010), reported that early onset breast cancer in African women is a demographic phenomenon that is justified by the fact that most African countries have a cone-shaped population pyramid with majority of their citizens being children and young adults with very little elderly population at the top. A lower median age of 20 recorded in the African population could explain why breast cancer among young women comprises of higher proportions of the cases presenting to the hospitals than among than the old (Akarolo-Anthony *et al*., 2010). In this study it is clear that demographic pattern alone may not explain why breast cancer was diagnosed at a younger age, there are other factors that could contribute to early onset of breast cancer in Africans; from the study's findings some Kenyan tribes seem to have higher incidence than other, also there is a general finding that some counties seem to have more cases than others. This study suggest that genetic and environmental risk factor could be associated to the early onset of breast cancer in African women implying that screening for breast cancer ought to be done early with the aim of early detection and treatment so as to increases chances of survival.

5.1.1.2 Age at menarche

Majority of the patients had their first menarche at an age older than 12 years placing them in a low risk category. Studies indicate that women who were younger at menarche (≤12 years) are at increased risk of developing breast cancer because early menarche exposes them to sex hormones for a longer period in their life time (Key *et al*., 2003; Sprague *et al*., 2008; Friedenreich, 2001; Sasco 2001).

From this study the median age at menarche was 14 years and is comparable to a study by Walker *et al.,* (1984), who recorded age at menarche as 14.7 years in rural black women in South Africa. Age at menarche in African women varies although generally these women experience menarche at older ages compared to non-Africans (Fregene & Newman 2005).

Reason for the difference in age at menarche depend on the interaction between genetic and environmental factors (Karapanou and Papadimitriou 2010). Change of lifestyle, nutrition and better health is expected to cause a decline in age at menarche (Karapanou and Papadimitriou 2010). There is need to conduct more studies that will inform on the factors association with early menarche as a risk factor of developing cancer in Kenyan population.

5.1.1.3 Age at menopause

Studies have indicated that after the age of 55 years the risk of breast cancer doubles in women generally (Sprague *et al.,* 2008) this is because long menstrual history increases life time exposure to sex hormones. In this study 28.4% of the cases were above 55 years while the majority (71.6%) were below the age of 55 years. Very few studies on menopausal history and risk of breast cancer in Africans have been documented. However this study report that breast cancer in Kenyan population is likely to develop early before menopause (>55 years) considering that the mean age at presentation was 54.3 years, this is higher than other studies that reported that the median age (46 years) at presentation is similar for black women in UK and in African (Opeyemi & Ganiyu 2012). This age is younger than the age at presentation in Caucasian women (67 years) (Elmore *et al.* 1998; Reis *et al*. 1999), the reason for this is not fully understood but it could be linked to breast cancer genes (BRCA 1 and 2) and their variants (Ghiasvand 2011).

Breast cancer in Kenya is no longer a disease of the old, but rather a disease of all ages therefore there is need to change the mind set by determining factors that are likely to affect the biology of our bodies thus predispose pre-menopausal women to developing this cancer.

5.1.1.4 Family history

A woman that has a close relative diagnosed with breast cancer puts her at a higher risk of developing the disease (Pakseresht *et al*. 2009). One is at double risk of developing breast cancer if one first- degree female relative (sister, mother, and daughter) is diagnosed with the disease. If two first- degree relatives have been diagnosed the risk is 5 times higher than average (Bevier *et al*., 2012). Majority of the patients (75.6%) in this study reported having no relative diagnosed with any type of cancer which correspond to low risk. This is comparable to a study by Lehlasoa (2011) which reported that 77% of their breast cancer patients had no family history. The findings from this study report that 16.2 % were at high risk of developing breast cancer for having a family history of breast cancer. This is higher than 5-6 % reported by several studies (Center *et al*., 2015; Pluchinotta *et al*., 2015; Balmana *et al*., 2009; Hoffman and Johnson, 1995) but comparable to 19.0% of Californian patients (Wrensch *et al*. 2003) and slightly lower at 13.3% reported for some African patients (Lehlasoa 2011). Since a greater number of people have no history of breast cancer suggest that breast cancer seen in Kenya could be as a result of somatic genetic mutation influenced by environmental but not hereditary changes? Therefore more genetic studies need to be conducted to ascertain this.

5.2.0 Modifiable Risk Factors

5.2.1.0 Socio-demographic factors

5.2.1.1 Marital status

The majority of the breast cancer patients had married at the time of interviews corresponding to a medium level of risk. Lone mothers have been recognized as vulnerable group to cancer and other chronic diseases, have fewer children, higher unemployment (Hemminki and Li, 2003). This study's findings showed higher risk of breast cancer among women who never ever married at the age between 31-40 years followed by those that were ≤ 30 years in the same category. Studies have reported that marital status remains a risk factor for breast cancer development and unmarried, delayed marriage, delayed first child birth are strong cofactors for development of breast cancer (Shaikh *et al*., 2014). This data suggest that the psychological and physiological changes associated with life events could be predisposing young African women to developing breast cancer (Price *et al*., 2001). It has been observed that some of these changes affect immunological function especially in individuals with stressful life events by enhancing development of breast cancer (Irwin *et al*., 1987; Schleifer *et al*., 1983). Furthermore it has been documented that unmarried patients were likely to present with metastatic cancer and have high chances of under treatment hence results to increased mortality rates (Aizer *et al*. 2013).

Stigma of being diagnosed with cancer, poverty and misinformation on treatment of cancer, are likely to trap lone breast cancer patients. This will in turn lead to late presentation at the hospital when chances of treatment have been lost leading to high rates of deaths. There is need of support of lone women diagnosed with breast cancer in terms of free and efficient counseling so as to improve the quality of life.

5.2.1.2 Tribe

Africans have low incidence of breast cancer and at the same time experience the highest mortality rate (Chlebowski *et al*., 2005). Kenya has a diverse ethnic group consisting of 42 tribes. These tribes are described with geographical and overlapping traditional living areas and habits. In this study Luyha and Kalenjin tribes were reported to have the highest incidence of breast cancer compared to all the other tribes who visited MTRH. Although Kalenjin is the fourth largest tribe overall and are living in the surroundings of the MTRH these alone may not explain why they were likely to be diagnosed with breast cancer. Parker *et al*. (2010) in their study found esophageal cancer was most common among the Kalenjin tribe than any other ethnicity. A study by Jabbour (2000) reported that Kalenjins have a slow heart rate even when running that is why they are good at long distance races; it is not clear if this affect health and disease. Therefore more studies needs to be done to find out which genetic and or environmental characteristics seem to predispose tribes like Kalenjins to developing cancers and tribe like kikuyu to resist cancers.

It is of great importance to look at environmental and cultural practices that could be contributing to these findings. Kalenjin are known to consume a lot of dairy products that are a source of saturated fats associated with chronic diseases (Stang, 2008). Collins *et al*., (2005) in their study showed that western dietary pattern composed of high loading of refined grains, high-fat dairy products, meat and processed meat, eggs, margarine, butter and mayonnaise, potato, French fries, sweets, soda and snacks that was significantly associated with breast cancer risk. No study has been done in Kenya that looks at the environmental and dietary factors link to cancers. The common occurrence of esophageal cancer in Kalenjins at an age of less 40 years despite the low prevalence rate of classical risk factors such as heavy drinking and tobacco smoking was associated with an old tradition of producing and consumption of fermented milk (Nieminen *et al*. 2012). In

their study Nieminen *et al*. (2012) reported that the fermented milk among the Kalenjins contains high levels of acetaldehyde (ACH) which is a mutagenic carcinogen in both animals and *in vitro* models at low concentration.

The association of prevalence of cancer and tribes in Kenya suggest that unique genetic, environmental and cultural practices for each tribe could be playing a big role in the prevalence of diseases such as breast cancer. Therefore, it is important to conduct studies on the role of genetic changes, environmental factors and cultural practices in the development of cancer in different tribes of Kenya.

5.2.2.0 Reproductive factors

5.2.2.1 Contraceptive use

Contraceptives were classified as carcinogenic agents by the IARC in 2005 (Cogliano *et al*., 2005). According to a report by collaborative group on hormonal factors in breast cancer (CGHFBC), (1996), use of oral contraceptive (OC) is associated with increased risk in breast cancer that disappears after 10 years following cessation. Current use of OC is thought to be the cause of a minority of breast cancers among premenopausal women (Gierisch *et al*., 2013). Oral contraceptives have hormones that may have a protective effect on cancers while on the other hand may stimulate mitotic activity in the breast tissue due to the mixture of estrogen and progesterone (Gierisch *et al*., 2013). Majority of the study participants 26 (70.3%, N=37) had not used OC at the time of interview placing them on low risk for breast cancer. This is comparable with a study by Puri *et al*., 2009. Of the 9 patients who used OC, the majority 7(77.8%, N=37) had used them for a period greater than one year (>1 year) as a result had high risk for breast cancer (Puri *et al*., 2009).

Majority (71.6%) of the study patients in the current study had not used hormonal injection for contraceptive as results had a low risk for breast cancer. On the other hand individuals who used hormonal injection for long were 71.6% placing them at a high risk for breast cancer. This agrees with a study by Beaber *et al*., (2014) that reported that, high dose estrogen formulation was linked to increased risk of breast tumors. However low dose estrogen pills and injection appears safest.

Bigger studies are required to conclusively ascertain the association of use of contraceptives and cancers. Such studies should be able to guide government in formulating policies that would only allow prescription of the safest hormonal contraceptive to its citizens.

5.2.2.2 Parity

There is evidence that giving birth in women confer a long-lasting protection on breast cancer. In this study majority of breast cancer women had given birth at least once in their lives hence may not link breast cancer development to parity. Out of these, 45.8% of the patients had given birth 1-5 times in life as a result had medium risk for breast cancer while 37.5% had more than five births placing them at low risk for breast cancer. In a previous study multiparous women had a 30% decrease in risk of breast cancer. This is supported by another study that recorded a 7% reduction in the relative risk of breast cancer for each birth independently from other pregnancy related factors (CGHFBC, 1996). In African population most women are multiparous and this could explain why breast cancer incidences are lower in this population compared to non- African population.

Only 16.7% of the study population were nulliparous thus placing them at high risk of breast cancer. Previously nulliparous have been recorded to have up to 25 % breast cancer risk as compared to a porous (Travis and Key, 2003). Although it has been shown that there is a long term

protective effect of parity, and specifically multiparity on breast cancer risk (Talamini *et al*., 1997; Yang *et al*., 2007), short term (5 to 10 years after each birth) is associated with a transient increase in breast cancer risk which levels off towards protection thereafter (Bruzzi *et al*., 1988). The relative risk is increased by 3 to 5 years following a full time birth. This is due to the fact that pregnancy induces differentiation of mammary glands thus making them less susceptible to carcinogenic stimuli yet on the other hand promote this effect by causing expansion of clones initiated cells that could promote cancer development (Vecchia and Pelucchi *et al*., 2012, Russo and Russo, 1994, Pike *et al*., 1983).

Age at first pregnancy has been associated with increased risk of developing breast cancer. MacMahon *et al*. (1970) in their study reported that the relative risk of a nulliparous women compared to those who gave birth at age 20 years was 0.5 but this rose to 1.3 for those with birth at age of 35 years. This means that having a baby early is protective from breast cancer and also having subsequent pregnancies that are close as 3 years could be protective (Bruzzi *et al*., 1970).

In Kenya today most women would want to have less children due to economic impact and other social challenges such as balancing between work, studies and raising up of children. This could explain why more young women in the study population tend to develop breast cancer more than in the past. More studies need to be done to determine the association between long intervals of births and stopping child bearing at a younger age with development of breast cancer.

5.2.2.3 Breastfeeding

Studies have shown that there is an inverse association between duration of breastfeeding and breast cancer risk in both low and high-income countries (Lord *et al*., 2008; Wrensch *et al*., 2003). The majority (87.5%) in this study had ever breastfed prior to the time of interview. Out of these

(52.2%) and (39.1%) had breastfed at most four and equal or more than five children respectively. Comparable to studies by Glade, (2008), Nagata, (2012) and AICR, (2008), majority of breast cancer patients in this study had breastfed for more than 12 months and as a result had low risk of cancer. The median duration of breast feeding was 18 months (min 12; max 24). Only 8.7% had not breastfed and or breastfed for short period (less than 6 months) placing them at high risk of breast cancer, this is comparable with a study by Ursin *et al*., (2005).

The relative risk of breast cancer decreases by 4.3% for every year of breastfeeding, in addition to 7% for each birth. Given that women in low income countries breastfeed for a longer period compared to high income countries, could explain why there are low incidences of breast cancer in the developing world. This is because breastfeeding for long delays return of mother's menstrual periods thus reducing a woman life time exposure to hormones such as estrogen (AICR, 2008). However the current increase in the incidence of breast and other cancer in the low income countries in part is associated with the fact that the developing world tend to copy the western lifestyle including shorter duration of breast feeding, longer spacing of births, and less number of children (Vecchia and Pelucchi, 2012).

This finding should encourage women, despite of challenges of work and career, to allow their children to breastfeed the longest for this is not only beneficial to the immunity of the baby and bonding between the baby and the mother, but also protects from developing breast cancer.

5.3.0 Lifestyle factors

5.3.1 Alcohol intake

There is convincing evidence that alcohol consumption increases the risk of cancer of the colorectal, breast, larynx, liver, esophagus, oral cavity and pharynx (Bagnardi *et al*. 2013).

Association between alcohol and breast cancer is linked to increased estrogen and androgen or increased levels of plasma insulin like growth factors that are produced by liver following alcohol consumption (Sarkar *et al*. 2001). In the current study use of alcohol was a significant risk factor of breast cancer (p=0.029; OR; 95%CI: 0.052). Majority of the drinkers who developed breast cancer were aged between 41-60 years. This indicates that alcohol consumption at this age bracket increase the risk of breast cancer.

Some epidemiological studies suggest that drinking alcohol during adolescence or early adulthood has a strong impact on BC risk. Romieu *et a*l. (2015) has shown that women who started drinking before their first full-term pregnancy (FFTP) had a higher risk than women who started afterwards. These effects were observed in hormone-receptor positive and –negative tumors pointing to non-hormonal pathways that need to be further investigated (Romieu *et al*., 2015).

The prevalence of alcohol use in Eastern Africa is 52% with young people forming the majority. Alcohol consumption could be one of the factors that has led to the increase of breast cancer prevalence in the young population. There is need to conduct bigger studies to determine carcinogenic effect of alcohol intake before FFTP and development the aggressive breast cancer subtypes seen in young patients in Africa and Kenya.

5.3.2 Smoking

Most epidemiological studies associated heavy smoking, smoking for long duration, smoking before a first full term pregnancy (FFTP) and passive smoking with increased risk of breast cancer in women with high levels of estrogen (Catsburg *et al*., 2015; Dossus *et al*., 2014b). In this study, majority (97.3%) had not smoked tobacco at the time of interview, 2.7% of the breast cancer

patients admitted that they have ever smoked tobacco, they were between an ages of 31-60 years. None of the patients smoked at a younger age of below 30 year nor at older than 60 years.

There were more cases who reported that they have been exposed to side stream smoking. The highest number of side stream smokers was seen in the category of 31-40 years. This category recorded the highest number of breast cancer cases suggesting that, there could be a link between passive smoking and breast cancer development. This supports other studies that reported an increased risk of developing breast cancer in premenopausal women who are passive smokers (Gram *et al*., 2005; Gray *et al*., 2009; Johnson *et al*., 2009).

There is a shift of heavy tobacco consumption from developed world to the more vulnerable low-resource countries such as Kenya (Sylla and Wild 2012). Tobacco consumption is responsible for 30% of cancers worldwide, therefore low-resource countries like Kenya should take this as a critical area that needs urgent attention preferably from the policy makers. Simple and efficient measures such as increasing taxes and enforcing strict pricing policies as well as restricting cigarrete smoking in public and providing educational information could combat this upcoming epidemic.

5.3.3 Wood smoke exposure

Exposure to wood smoke has previously been reported to increase the risk of developing esophageal cancer (Patel *et al*. 2013). In a similar study Kayamba *et al*. (2015), reported that HIV infection and domestic smoke exposure are risk factors for esophageal squamous cell carcinoma in Zambia. In the current study, almost all (98.6%) the patients were exposed to domestic wood smoke since birth up to the time of interview. This is known to increase levels of E-cadherin, a protein that is known to play a role in maintaining stable cellular environment (Gray *et al*., 2009).

Further studies have pointed out that women with breast cancer who lived in a region with more air pollution were more likely to have the alteration in the DNA in their tumor than those who live in a less-polluted regions (Gray *et al*., 2009; Michel *et al*., 2013).

Genetic analysis will tell if the breast cancer development and progression is as a result of DNA methylation or other effects of air pollution in Kenya.

Single or not married breast cancer patients needs free and efficient counselling and emotional support so as to improve their lives. Environmental and cultural practices for various tribes in Kenya could be playing a role in breast cancer development. Oral contraceptives that have high levels of estrogen could be associated with development of breast cancer in Kenya, prescription of safe contraceptive is imported. Social challenges and economic impact will make most women in Kenya have fewer children, long intervals of births, stop giving birth at a younger age hence risk developing breast cancer. Despite of challenges of work and career women should be encouraged to breast feed the longest for this prevent risk of breast cancer. Heavy alcohol consumption before FFTP could be associated with increased risk of breast cancer in the young population. Side steam smoking is a risk factors to breast cancer hence there is need to come up with policies that will curb smoking in public. Most Kenyans are exposed to wood smoke hence genetic analysis will tell if breast cancer is as a result of DNA methylation after wood smoke exposure or not.

5.4.0 Histological subtypes

Breast tumors have been classified into distinct histological subtypes, in the current study the majority (79.0%) of all breast carcinomas were invasive ductal carcinoma not otherwise specified (IDC-NOS), and this is consistent with a study by Bennis *et al*. (2012) that reported a prevalence rate of 87% in north-east Morocco. Another study in Tunisia reported that 83.7% of their new

cases were invasive ductal carcinoma (Missaoui, 2011). Another study within Africa has reported comparable results with IDC incidence rate of 70% in a Nigerian population (Opeyemi & Ganiyu, 2012).

It is clear that IDC remains the most common breast cancer histological type (Ebughe *et al*. 2013, but the question is what is the trend of incidence rate in African population today? It was reported that IDC increased at reducing rate in the US between 1980s and 1990s it increased by 4% followed by 3% between 1995 and 2004, this decline was associated with reduction in hormone therapy use. The current study reports that, IDC is the common (51.1%) type among grade II cases, this is higher than 24.6% that was recorded in Nigeria (Ebughe *et al*. 2013). Swart (2013) associated IDC with high grade, the reason for is not clear but it could be because of the ability of this type of tumor to metastasize to lymphatics (Swart 2013), making it have poor prognosis. This tumor is frequently associated with DCIS (Sinn, 2013), in that they have similar risk factors that include age, breast density, family history, and history of benign breast disease (Virnig *et al*., 2010; Kerlikowske, 2010).

Currently in Africa most women use hormonal therapy for family planning raising questions as to what extend this risk factor predisposes breast cancer patients to developing IDC. When one weighs between due loss of life due to cancer and reducing births that could be even protective against this disease, urgent intervention by providing alternatives methods of family planning other than the common use of hormonal contraceptive should be put in place so as to get rid of at least one risk factor.

The prevalence of papillary carcinoma was 3.8%, in the study population, this is higher than 1.2% reported by other studies (Swart 2013, WHO 2003, Natarajan *et al*. 2009). It is expected to be seen

more in women who are above 60 years (Swart, 2013). Paget's disease constituted 1.9% in the current study and is comparable to 1% recorded by Noel *et al*. (2010) and 1-4 % recorded by Swart (2013). However this disease has better prognosis compared to other types.

5.5.0 Intrinsic breast cancer subtypes

5.5.1 Luminal A breast cancer subtype

There is evidence that different intrinsic breast cancer subtypes presents in varied patterns among populations, age groups and varies with socio-economic categories and reproductive factors. Findings of different populations have shown that luminal A subtype is less presented 27-33% in African women (Olopade *et al*., 2008; Huo *et al*., 2009) but predominantly (>50%) in Asian, white and postmenopausal African American populations (Carey *et al*.,2006). Current results supports this finding for Luminal A subtype was at 26.4%, this is slightly lower than a prevalence rate of 38% reported in Uganda (Galukande *et al*., 2014) and 30% in Eritrea study (Tesfamariam and Roy, 2013). This variation could be as a result of number of markers used in subtyping. The current study adopted the IHC4 marker that classifies breast cancer into four subtypes; Luminal A, Luminal B, HER2 overexpressed and TNBC by using ER, PR, HER and Ki-67 markers while the other studies did not include Ki67.

Estrogen receptor positive tumors were 59.2%, this is comparable to a study from an African population that reported 60% (Tesfamariam and Roy, 2013). Estrogen receptor positivity breast cancer forms most of the Luminal subtype that is associated with post-menopausal breast cancer (Hainaut and Abedi-Ardekani, 2012). Majority (78.6%) of Luminal A subtype were premenopausal (≥55 years), this is high compared to 46% in Carolina Breast Cancer Study (Carey *et al*., 2006).

Metzger *et al*., (2013) reported that luminal A breast tumors have better prognosis hence long term survival if treated with tamoxifen, a target drug to estrogen receptor. Expression of ER, PR, and HER2 proteins and the Ki67 index appear to distinguish luminal A from luminal B breast cancer subtypes. In this study most of the luminal A subtype did not over express Ki67 (≥14%, p=0.001). This suggest that this subtype do not proliferate hence less aggressive, therefore may not need aggressive therapy. However, since molecular profiling is not done routinely in most Kenyan hospitals such cancer subtypes may end up been treated aggressively thereby lowering survival rate. The current study reports that hormonal receptor profiling may be useful in identifying the heterogeneity of clinical outcome in breast cancer which could help clinicians improve therapy for their patients.

5.5.2 Luminal B breast cancer subtype

Luminal B breast tumors were 30.2% in this study, this is a group that is characterized by relapse and therefore worse prognosis with respect to all the luminal subtypes (Sorlie *et al*., 2001). The prevalence of luminal B was higher in this study compared to a Uganda study and Eritrea studies that both reported 5% (Galukande *et al.,* 2014; Tesfamariam & Roy, 2013). This is a more aggressive form compared to luminal A and though they are hormone receptor positive and HER2 positive, they are known to be proliferating tumor since they display high Ki67 score (Ellis, 2014). In this study most Luminal B breast tumor overexpressed the proliferative index (Ki67 <14%; p>0.000). These tumors have been associated with P53 mutation, distant metastasis and are poorly responsive to both endocrine therapy and chemotherapy compared to luminal A tumors. The prognosis of this subtype is unknown in most African population. Like luminal A, luminal B is it commonly seen in premenopausal patients. This is not consistent with other studies that recorded

9% in African Americans and 18% in non-African American populations (Carey, 2006). The explanation to this could be associated to the number of markers used in determining the subtypes.

Overexpression of luminal B subtype in the study population is partly associated HER2 negativity. This is an aggressive type that needs aggressive treatment. Choice of treatment is best made if profiling of hormonal makers is done. Like for Luminal A, the findings from this study suggest that hormone receptor and Ki67 profiling could be useful in identifying the heterogeneity of clinical outcome in this type of breast cancer. However, there is need to look at the biology and response to treatment in Kenyan breast cancer.

5.5.3 HER2 overexpressed subtype

HER2 overexpressed tumor were the least represented. Among the cases 86% were HER2 negative. The level of HER2 positivity in the study group was slightly lower than 5% reported in the Eritrean study (Tesfamariam and Roy, 2013), this could be partly because HER2 equivocal cases were considered negative. Although 14% of the patients were HER2 positive, all this did not fall under this subtypes because some of these HER2 positive tumors will form other intrinsic subtypes particularly Luminal B (Ellis, 2014). This subtype has also been associated with large tumor size, regional and local metastasis and poor differentiation (Yang *et al*., 2007). Although there are target therapies to HER2 positive tumors this may not be of benefit to most patients because of availability and cost. In the absence of treatment, HER2 positivity is associated with high mortality rates compared to the other breast cancer subtypes.

These results should not be generalized since this study did not perform FISH to further sort the equivocal (2+) cases. There is need of studies to look at the prevalence of HER2 types in this population.

5.5.4 Triple Negative Breast Cancer (TNBC).

The present study did not sub classify TNBC into basal-like, but it described this subtype as ER-, PR-, HER2- and any Ki67 score. Similar to many studies this study reports elevated number (34%) of TNBC in the study population (Carey *et al*. 2006; Galukande *et al*. 2014; Huo *et al*. 2009). In this study it was evident that 72.0% of the premenopausal breast cancer were triple negative. This is consistent with other studies (Prat *et al*. 2013; Carey *et al*. 2006; Galukande *et al*. 2013) that reported high prevalence rate in young women of African origin.

The highest percentage of this subtype was seen among the Luo tribe, where 62.5% were of TNBC subtype. Similarly majority of this group presented with large tumors (>5cm) that tend to be aggressive. It is not clear why this particular group express an aggressive type of breast cancer. Gene expression analysis have indicated that triple-negative breast cancer are likely to have BRCA1 mutation (Sorlie *et al*. 2001; Lakhani *et al*. 2006). Occurrence of TNBC may have heterogenic oncogenesis that need to be uncovered by doing genetic studies in the varied ethnic groups in Kenya.

The current study found out that TNBC subtype was associated with high grade and high proliferative capacity which is consistent with other studies (Kreike *et al*. 2007; Otiriou 2009). The high proliferative capacity as measured by the expression of Ki67 in young African breast cancer patients is a clue that hereditary or sporadic mutation could be responsible in the development of this subtype. Since TNBC have no target treatment and such patients are put on chemotherapy that may not help much, there is a need to determine the prevalence of DNA mutation in the aggressive

breast cancer forms seen in Kenya, since this can be biomarkers that will help manage these tumors.

5.6.0 Tumor Infiltrating Leukocytes (TILs) In Cancer and Non-Cancer Breast Tissues

An increase in infiltration of macrophages, B cells, and T cells often increases with and correlates with pathological breast cancer progression, reduced survival, and response to therapy (DeNardo *et al*., 2009; Savage *et al*., 2008; DeNardo *et al*., 2011; Lin *et al*., 2001). These immune cells secrete factors that promote invasive and metastatic tumor growth. In this study, the breast cancer tissue samples tested had increased T cell and macrophage immune cell markers ($CD4^+$, $CD25^+$, $CD63^+$, and $CD163^+$) expression compared to benign tissue. Alternative (M2) macrophages or $CD163^+$ and regulatory T cells ($CD25^+$) in particular are associated with pro-tumor roles during breast cancer progression. In fact, M2 macrophages and Tregs have a complimentary and synergistic relationship to promote their plasticity (Biswas *et al*., 2010). Tregs are known to release cytokines that can differentiate monocytes/macrophages into $CD163^+$ M2 macrophages (Tiemessen *et al*., 2007). Classically activated (M2) macrophages also can secrete chemokines that promote the induction, differentiation, and recruitment of Tregs (Savage *et al*., 2008)

In normal tissues, Tregs prevent autoimmune responses; in tumors, Tregs can promote immunosuppression of $CD8^+$ T cells hence contributes to cancer cells evading their detection by the immune system (DeNardo *et al*., 2007). Moreover, infiltration of Tregs into breast tumors is prognostic of reduced survival in patients (Bates *et al*., 2006). Conversely, therapeutic strategies that eliminate to modulate Treg activity (such as anti-CD25 mAb and CTLA-4 antagonists) have had some success in treating melanoma patients (Shiao *et al*., 2011).

The Kenyan cancer patients with tumors that express high $CD25^+$ cells might benefit from a similar therapeutic strategy designed to overcome the inhibitory immune response and to improve the anti-tumor immune response for patients with high levels of Tregs within the tumor site. Future research will be required to determine if immune modulators that eliminate Tregs or increase $CD8^+$ T cell immunosuppressive activity will help in overcoming the aggressive breast cancer seen in Kenya.

5.7.0 Association of Tumor Infiltrating Leukocytes with Breast Cancer Grade and Intrinsic Subtypes

Tumors of most patients were of high grade (grade III). This study quantified significant increase in immune cell infiltration in cancer tissue compared to non-cancer tissue with the intention of determining if the density of individual TILs varied across intrinsic subtypes and grade. The results indicated that TIL densities were not significantly associated with the cancer subtype and tumor grade. While the immune cell infiltration significantly correlated with malignancy, this infiltration is independent of the molecular subtype or tumor grade. As a control in this study, proliferation within the tumor subtypes was examined, which previously has been shown to vary by subtype (Cheang *et al*., 2009; Voduc *et al*., 2010; Cuzick *et al*., 2011). Similar to other studies, the current study found out a significant difference in the proliferative potential of tumors from individual breast cancer subtypes as measured by the proliferative marker Ki67 (Trihia *et al*., 2003; Yang *et al*, 2012).

Even though the findings of this study reports no significant difference in immune cell infiltration in tumors across subtypes, individual TILs could hold clinical value as a biomarker or target that is applicable across all subtypes but specific to chemotherapy-resistant tumors. Future studies should determine the role of immune cell infiltrates in patient response to treatment. Since

chemotherapy is the primary line of therapy for treatment-resistant breast cancers, alternative immunomodulatory treatments may be second-line therapies for these patients.

The study population included very few tumors classified as HER2 positive (14%) by immunohistochemistry or as HER2 (2%) by molecular subtype (Tables 4.5). In Westernized treatment facilities, patients with HER2+ tumors generally are treated with trastuzumab. However, the Kenyan community has limited resources to provide expensive trastuzumab treatment. Therefore patients have few treatment options depending on markers that accurately will predict response to treatment. Interestingly, CD8 and CD25 could be such markers, since TILs are associated with improved distant metastases-free survival as well as increased rates of pathological complete response (pCR) after neoadjuvant trastuzumab and chemotherapy in patients with HER2 positive breast tumors (Denkert *et al*. 2010; Loi *et al.* 2014).

In contrast to the current study, which found no significant differences in immune cell density between tumor grades, other studies demonstrate differential lymphocyte infiltration in breast cancer tissues based on histological grade, with infiltration by CD4+ and CD8+ Th1 effector cells in lower grade tumors (Krell *et al*. 2012; Matkowski *et al*. 2009). This difference in results in the current study could be explained in part by the small number of patients per cohorts (N=3 for Grade I; N=21 for Grade II; N=21 for Grade III) and, therefore, are inconclusive. In addition, the study analysis scored for the number of TILs per cancer tissue and immediately adjacent stromal on a TMA but did not include TMA samples that were predominantly stromal tissue, rather than cancer cell containing tissue. This approach might self-select for a subset of the TIL population

and more appropriately should be analyzed by flow cytometry analysis from fresh tissue of single cell suspensions that are stained and analyzed for TIL markers.

The study reports that the immune response, as measured by the type and density of tumor infiltrating lymphocytes in different grades and subtypes of breast tumors, is not significantly different across breast cancer molecular subtypes. However, this finding does not rule out the possibility of TILs having a prognostic role in breast cancer progression or a predictive role in response to therapy, since this study does not include patient outcome or therapeutic response data. The immune response in breast cancer patients can have predictive therapeutic value, especially with response to chemotherapy (Garcia-Martinez *et al*. 2014). The presence of or the ratio comparing specific TILs within a tumor may represent immune homeostasis within the tumor and the tumor microenvironment (Loi *et al.* 2013). Although the role and mechanism of the individual TILs in the clinical and biological behavior of tumor are unclear, TILs seem to have predictive value for breast tumors in response to neoadjuvant chemotherapy (NCT) (Denkert *et al*. 2013; Ono *et al*. 2012).

Future studies will be required to collect survival data for these patient population and to run clinical trials to determine if TILs have prognostic and predictive value. Such studies will determine if the differences identified between TIL density in cancer and non-cancer tissue samples could contribute to response to therapy. Although additional validation is necessary, this study provides a rationale for future research in the development of immune cell panels that could be targeted in therapy and management of these deadly breast tumors**.**

CHAPTER SIX: SUMMARY OF THE FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of the findings

In summary, genetic and environmental factors influence the development of breast cancer at a younger age. The decline in age at menarche among the breast cancer patients could be associated with change in lifestyle, better nutrition and health services. Breast cancer in Kenya is a disease of all ages, hence there is need to further determine the risk factors associated with premenopausal breast cancer. Breast cancer in Kenya could be sporadic and not hereditary and only genetic studies will ascertain this.

Invasive ductal carcinoma is the most common histological subtype in the current study. Use of IHC4 score can characterized breast cancer into four subtypes namely liminal A, luminal B, triple negative breast cancer and HER2 overexpressed subtype. Tumor infiltrating lymphocytes may not be associated with breast cancer subtype but could have a predictive role in response to treatment.

6.2 Conclusions

1. Risk factors of breast cancer in Kenya include sporadic genetic changes that are triggered by environmental changes and cultural practices
2. Intrinsic breast cancer subtypes are triple negative breast cancer (34%), Luminal B (39.2%), Luminal A (26.4%), HER2 overexpressed (2.0%) while the most common histological subtype is invasive ductal carcinoma.
3. The tissue macrophages ($CD68^+$), alternative type macrophages ($CD163^+$), helper T cells ($CD4^+$) and inducible T regulatory cells ($CD25^+$) highly infiltrates breast tumors seen in western Kenya while proliferative index ($Ki67^+$) is highly expressed by triple negative breast cancer.
4. There were no significant difference in the type and density of TILs across molecular subtypes and grades.

6.3 Recommendations

6.3.1 Recommendations from the study

The following recommendations were made based on the findings of this study:

1. The findings from this study recommends that screening for the sporadic genetic changes should be done routinely, emotional support in form of free and efficient counselling should be given to breast cancer patients particularly those that are not married. There is need to ascertain the association between use of hormonal contraceptives and breast cancer and consequently efficient measures that restrict cigarette smoking in public could combat breast cancer in Western Kenya.
2. Routine profiling breast cancer by use of IHC4 score will enable the identification of patient subgroups with different treatment requirements.
3. Infiltration of CD25+ T cells (T reg) that is witnessed in high grade tumor plays a role in suppressing the adaptive immune response leading to poor prognosis thus modulation of immune response could manage aggressive breast cancer types like the ones seen in Western Kenya.

6.3.2 Recommendations for Further Research

1. More studies need to be done to determine the association between long intervals between childbirths ,cessation of childbirth at a younger age, living in a house with mice, exposure to wood smoke, with development of breast cancer
2. There is need for studies on the role of diet, social and cultural ethnic practices in the development of breast cancer.
3. Research on gene expression analysis aimed at finding out the level of BRCA mutation in breast cancer should be done.
4. Future studies to determine the role of immune cell infiltrates in breast cancer patients response to treatment.
5. Future studies on determine if type and density of TILs play a role in the prognosis in different molecular subtypes in African population.

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APPENDICES

APPENDIX 1: CONSENT TO PARTICIPATE IN THE STUDY

TITLE: CHARACTERIZATION OF BREAST CANCER MOLECULAR SUBTYPES IN RELATION TO LEUKOCYTES INFILTRATION AND TUMOR ASSOCIATED MACROPHAGE POLARIZATION IN WESTERN KENYA

Investigator: Rispah Torrorey

Department of Immunology,

School of medicine, College of health sciences,

Moi University, Kenya.

Contact number: +254 722 941 418.

We would like to invite you to participate in a research study that involves analysis of breast tissue. This study will also ask you questions that will help identify risk factors to the breast cancer. Please take some time to read the information presented here which will explain the details of this study. Please ask the study staff or doctor any questions about any part of this study that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part initially, without affecting future treatment in any way.

This research study was approved by the Institutional Review and Ethics Committee for Human Research at Teaching and Referral Hospital Moi University and it was be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, Guidelines on Ethics for Medical and Genetic Research of the Medical Research Council of Kenya.

What does this particular research study involve?

This study will search for existing molecular subtypes of breast cancer in our population, it will also look at Tumor infiltration leukocytes patterns in relation to hereditary factors that can influence the development of breast or related cancer types and also look for certain lifestyle factors (e.g. smoking factors in patients with post-menopausal breast cancer) that may differ from breast cancer in younger age groups. Individuals without breast cancer will also be included as controls to identify factors that could influence development of this cancer in generations to come.

Why have you been invited to participate?

As you are either a sufferer from breast cancer or someone without any form of cancer that could form part of the control group (to allow us to make comparisons), we would like to test certain hereditary and lifestyle factors to find out if patients have these risk factors more often than the control group (those without breast cancer).

What procedures will be involved in this research?

You will be asked to fill in a questionnaire with questions pertaining to age at menarche, use of hormone replacement therapy, etc. and a number of lifestyle factors (alcohol consumption, smoking etc.).

Are there any benefits to your taking part in this study and will you get told your results?

Your tissue will be stored and tested at a later time when batches of samples are available; this will be done to limit testing time and costs involved. This research will benefit people with the same condition in the future as they might be in a position to get treatment earlier or even preventive treatment.

How will your confidentiality be protected?

The specimens will be given a number only and only the principal investigator will have access to the original questionnaires with identifying information. The results of the study will be included in scientific articles and a PhD thesis, without revealing the identity of the study participants.

Will you or the researchers benefit financially from this research?

You will not be paid to take part in this study. However profits will be reinvested to supporting the cause of further research, which may bring benefits to your family or community in the future.

Declaration by participant

By signing below, I ……..……. agree to take part in the research study entitled: CHARACTERIZATION OF BREAST CANCER MOLECULAR SUBTYPES IN RELATION TO LEUKOCYTE INFILTRATION AND TUMOR ASSOCIATED MACROPHAGE POLARIZATION IN WESTERN KENYA

I declare that:

I have read or had read to me this information and consent form and I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this study is voluntary and I have not been pressurized to take part.

☐ I agree that my tissue sample can be stored, but I can choose to request at any time that my stored sample be destroyed. I have the right to receive confirmation that my request has been carried out.

OR

☐ Please destroy my samples as soon as the current research project has been completed. **(Tick the option you choose)**

Signed at (place)…….on (date) …….

…… ……
SIGNATURE OF PARTICIPANT *SIGNATURE OF WITNESS*

Declaration by investigator

I (name) …… declare that:

I explained the information in this document to ……..……...

I encouraged him/her to ask questions and took adequate time to answer them.

I am satisfied that he/she adequately understands all aspects of the research as discussed above.

I did/did not use an interpreter. (If an interpreter is used then the interpreter must sign the declaration below.

Signed at (place) .....…..…….. On (date) ……...……..

…… ……
SIGNATURE OF INVESTIGATOR *SIGNATURE OF WITNESS*

Declaration by Interpreter (if appropriate)

I (name) …… declare that:

I assisted the investigator (name) ……. to explain the information in this document to (name of participant) ……..

We encouraged him/her to ask questions and took adequate time to answer them.

I conveyed a factually correct version of what was related to me.

I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (place) .....…..…….. On (date) ……...…….. .

……..

Signature of interpreter Signature of witness

APPENDIX II: DATA COLLECTION FORM (QUESTIONAIRE)
SECTION I: Demographic characteristics

1. Name……AMRS ID…….……

2. Tel.No……Next of Kin…….……Tel. No…….

3. Age in years: ……

4. Gender

[ ] Male

[ ] Female

5. Nationality: ……

Tribe: ……..

6. Place of birth:

Village…….

Location……..

County …….

7. Marital Status: …….

SECTION II: Disease status

9.1 When were you diagnosed with breast cancer ……Can't remember □

9.2 How old were you when the diagnosis was made ……Can't remember □

9.3 Did you receive treatment for your breast cancer? Yes □ No□

10.0 Do suffer from any clinical condition (e.g. Diabetes, asthma etc.)? Yes □ No□

10.1 If yes name the condition…….

10.2 When were you diagnosed with the condition in 10.1? ……

10.3 Are you on treatment? Yes □ No□

SECTION III: Family History

	Breast cancer before age of 50	Ovarian cancer at any age	Other cancer e.g. endometrial and colorectal at any age	
Yourself				
Mother				
Father				
Sister(s)				
Brother(s)				
Daughter(S)				Please specify maternal of paternal
Son(s)				□ Maternal □ Paternal
Grandmother				□ Maternal □ Paternal
Grandfather				□ Maternal □ Paternal
Aunt(s)				□ Maternal □ Paternal
Uncle(s)				□ Maternal □ Paternal
Cousin(s)				□ Maternal □ Paternal
Others				□ Maternal □ Paternal

Check the box if you have in your family

□ Any female with both breast and ovarian cancer

□ Any female with bilateral breast cancer diagnosed under age 50
□ Is your family of Ashkenazi Jewish Descent?

12. Menarche, Pregnancy and breast feeding

12.1 How old were you when you started menstruating □□ Can't remember □

12.2 How many full term pregnancies have you had □□ Can't remember □

12.3 How many babies did you get that were born alive □□ Can't remember □

12.4 How many miscarriages did you have □□ can't remember □

12.5 If you have never been pregnant- do you know why you never fell pregnant (own words)……

…… Can't remember □

12.6 Did you breastfeed your children (if not go to question 13) Yes □ No□

12.6.1 How many children did you breastfeed □□ can't remember □

12.6.2 Can you remember about how long you breastfed your children (months)

…… Can't remember □

12.7 Are you beyond the "Change-of-Life" already (have your periods stopped?)

Yes □ No Do not know □

12.7.1 If yes, how many months □□ or years □□ Can't remember □

13. Contraceptive Use

13.1 Have you ever been on the "pill" for contraception Yes □ No□

(If not go to question 13.2)

13.1.1 If so, for how long (approximate months on different medications) □□

……

…….. Can't remember □

13.1.2 If you know the name of the product(s) please write the name(s)……..

…….. Can't remember □

13.1.3 How old were you when you started on "the pill"? ±……yrs. Can't remember □

13.2 Have you ever been on the "injection" for contraception? Yes □ (if not go to question 13.3)

13.2.1 If so, for how long (months) □□ can't remember □

13.2.2 If you can remember the name(s) - please write them down

……

…….. Can't remember □

13.2.3 How old were you when you started on the injection? ± ……..yrs. Can't remember □

13.3 Have you been on a combination of the "pill" and the "injection"? Yes □

(if not go to question 13.4)

13.3.1 If so, for how long (approximate months on different medications) on the pill □□ Can't remember □ If so, for how long (approximate months) on the injection □□ Can't remember □

13.3.2 If you can remember the name(s) - please write them down

…….. Can't remember □

13.4 Have you used any other form of contraception in your life?

Yes □ No □ Do not know □

13.4.1 If yes, please write down what you used ……

…….. Can't remember □

13.5 Why did you stop taking contraception? ……

…….. Can't remember □

14. Hormone Replacement Therapy

14.1 Are your menstruations (periods) still coming regularly (every month)?

Yes □ No □ Do not know due to hysterectomy□ Do not know □

14.1.1 If not, are they still coming but irregularly (perimenopausal)?

Yes □ No □ Do not know due to hysterectomy□ Do not know □

14.1.2 About how old were you when this happened? ……..yrs. Can't remember □ 14.1.3 If not, have they stopped altogether but for less than one year (perimenopausal)?

Yes □ No □ Do not know due to hysterectomy□ Do not know □

14.1.4 About how old were you when this happened? ……..yrs. Can't remember □

14.2 Have your periods stopped completely for more than a year (postmenopausal)?

Yes □ No □ Do not know due to hysterectomy□ Do not know □

14.2.1 About how old were you when this happened? …...…yrs. Can't remember □

14.3 Have you been on hormonal replacement therapy (HRT)?

Yes □ No □ …… can't remember □

14.3.1 If yes, about how old were you when this started? .. ……..yrs. Can't remember □

14.3.2 How long have you been on HRT? ……yrs. Can't remember □

14.3.3 Can you remember what kind of HRT? Can't remember □

estrogen alone □ Progesterone alone □ Combination □ Sequenced □

If you can remember the name(s), please write them down …….

……. Can't remember □

14.3.4 Why did you start on HRT?

Hot flushes □ Osteoporosis □ Urinary incontinence □ dry eyes or other

Post-menopausal symptoms □ other (specify) …….

…… Can't remember □

14.3.5 Have you ever been using anything else for post-menopausal symptoms?

Yes □ No □ can't remember □

14.3.5.1 What did you use? Please write down the name(s) …….

……..… Can't remember □

14.4 Did you undergo a hysterectomy?

Yes □ No □ can't remember □

14.4.1 How old were you when this happened? ……yrs. Can't remember □

14.4.2 Why did you have a hysterectomy (e.g. cancer, profuse bleeding) ……

…… Can't remember □

14.4.3 Were you ovaries left in?

Yes □ No □ can't remember □

15. Smoking

15.1 Do you smoke? Yes □ No □

15.1.1 If so, when did you start smoking? (Age) …….. Yrs. Can't remember □

15.1.2 How many cigarettes per day? ……..cigs. Or ……. Packets Can't remember □

15.1.3 What do you smoke? Cigarettes per packet □ rolled cigarettes □ can't remember □

15.2 If not, did you smoke before? Yes □ No □

15.2.1 If so, when did you stop …… yrs. Can't remember □

15.2.2 How many years did you smoke? ……..yrs. Can't remember □

15.2.3 How much per day? ……..cigs. or ……. Packets. Can't remember □

15.2.4 Why did you stop smoking? …….

…… Can't remember □

15.3 Side-stream smoke

15.3.1 Did anyone smoke in your house/room?

Yes □ No □ can't remember □

15.4.0 Do you use firewood while cooking? Yes □ No□

15.4.1 If yes which type ……

15.4.2 Does your kitchen have windows Yes □ No□ How many □□

15.4.3 Is there a ventilation vent or chimney? Yes □ No□

Any comments: …….

16. Environment

16.1 Have you ever or do you live in an environment with house mice?

Yes □ No □ can't remember □

16.2 Have you done any shift work in your life**?**

Yes □ No □ can't remember □

16.2.1 If so, for how long? ….……yrs. Can't remember □

16.2 .2Where did you work (e.g., factory) …… Can't remember □

16.3 Have you ever lived a house with mice? Yes □ No □ can't remember □

16.4 Do you use firewood? Yes □ No □ can't remember □

17. Alcohol Use

17.1 Do you take an alcoholic drink at times?

Yes □ No □ can't remember □

17.1.1 If yes, what kind of alcoholic beverage(s) do you drink? ……

…… Can't remember □

17.1.2 How much do you drink of each of these? ……

…… Can't remember □

17.1.3 How often do you drink (every day, weekends only) and how much per day? …… Can't remember □

17.1.4 When did you start drinking any alcohol? ……/ …….. Yrs. Can't remember □

17.2 If you do not drink at present, have you been drinking before?

Yes □ No □ can't remember □

17.2.1 If yes, what kind of alcoholic beverage(s) did you drink? ……

…… Can't remember □

17.2.2 How much did you drink of each of these? ……

…… Can't remember □

17.1.3 How often did you drink (every day, weekends only) and how much per day? …… Can't remember □

17.3 Do you have any other comments or statements you want to make e.g what do you think has contributed to this problem and why have you come to the hospital at this time?

……

……

……

……

……

Thank you!

APPENDIX III: IREC APPROVAL

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2011/03
Approval Number: 000655

4th July, 2013

Rispah Torrorey,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET, KENYA.

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
APPROVED
04 JUL 2013
P.O. Box 4606 ELDORET

Dear Mrs. Torrorey,

RE: CONTINUING APPROVAL

The Institutional Research and Ethics Committee has reviewed your request for continuing approval to your study titled:-

"Characterization of Breast Cancer Molecular Subtypes in Relation to Lymphocyte Infiltration and Tumor Associated Macrophage Polarisation in Western Kenya".

Your proposal has been granted a Continuing Approval with effect from 4th July, 2013. You are therefore permitted to continue with your study.

Note that this approval is for 1 year; it will thus expire on 3rd July, 2014. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Yours Sincerely,

06/06/2013

DR. W. ARUASA
VICE-CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc:

Director	-	MTRH
Principal	-	CHS
Dean	-	SOD
Dean	-	SOM
Dean	-	SPH
Dean	-	SON

APPENDIX IV: RESEARCH AUTHORIZATION

REPUBLIC OF KENYA

NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Telephone: 254-020-2213471, 2241349, 254-020-2673550
Mobile: 0713 788 787 , 0735 404 245
Fax: 254-020-2213215
When replying please quote
secretary@ncst.go.ke

P.O. Box 30623-00100
NAIROBI-KENYA
Website: www.ncst.go.ke

Our Ref: NCSTI/P/13/3029/30

Date: 2nd July, 2013

Rispah Torrorey Sawe
Moi University
P.O.Box 3900-30100
Eldoret.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on "***Characterization of breast cancer molecular subtypes in relation to lymphocyte infiltration and tumor associated macrophage polarization in Western Kenya,***" I am pleased to inform you that you have been authorized to undertake research in **Uasin Gishu County** for a period ending **3rd July, *2014.***

You are advised to report to **the County Commissioner, the County Director of Education and the County Coordinator of Health, Uasin Gishu County** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

DR. M. K. RUGUTT, PhD, HSC.
DEPUTY COUNCIL SECRETARY

Copy to:

The County Commissioner
The County Director of Education
The County Coordinator of Health
Uasin Gishu County.

APPENDIX V: PERMIT FROM MINISTRY OF HEALTH

PAGE 2

THIS IS TO CERTIFY THAT:
Prof./Dr./Mr./Mrs./Miss/Institution
Rispah Torrorey Sawe
of (Address) Moi University
P.O.Box 3900-30100, Eldoret.
has been permitted to conduct research in

Location
District
County

Uasin Gishu

on the topic: Characterization of breast cancer molecular subtypes in relation to lymphocytes infiltration and tumor associated macrophage polarization in Western Kenya.

for a period ending: 3rd July, 2014.

PAGE 3

Research Permit No. *NCSTI/P/13/3029/30*
Date of issue *2nd July, 2013*
Fee received *KSH. 2,000*

Applicant's Signature

for Secretary
National Council for
Science &Technology

APPENDIX VI: ANTIBODIES

Table S1. Antibodies used in this study							
Antibody name	**Antigen**	**Ab source**	**Positive control**	**Company**	**Catalog #**	**Dilution***	**Special Methods**
HER2	Her2 protein	rabbit	breast	sk001	90456	RTU	
ER alpha	Estrogen receptor	rabbit	breast	IRO84 DAKO	10075784	RTU	
PR	Progesterone receptor	mouse	breast	IR068 DAKO	10075666	RTU	
Ki67	Expressed during all active phases of cell cycle but absent in resting cells	mouse	tonsil	IR626 DAKO	93901	RTU	
CD68	Macrophages	mouse	tonsil	IR609 DAKO	85199	RTU	
CD163	M2 macrophages	mouse	bone marrow	VP-C374	6017007	1/100	
CD4	Thymocytes, T helper cells, and peripheral T cells	mouse	tonsil	IR649 DAKO	86211	RTU	no H202
CD8	Cytotoxic/suppressor T cells	mouse	tonsil	IR623 DAKO	87824	RTU	
CD20	B cells	mouse	tonsil	IR604 DAKO	92175	RTU	
CD25	Regulatory T cells. Type 1 transmembrane protein on activated B, T cells and some thymocytes.	mouse	tonsil	SK200 DAKO	10067590	RTU	
						*RTU=ready to use	

APPENDIX VII. CORRELATION OF TILS IN BREAST CANCER MOLECULAR SUBTYPES

Pearson's Correlation (Parametric)

	CD20 stain per area (%)	CD4 stain per area (%)	CD8 stain per area (%)	CD68 stain per area (%)	CD163 stain per area (%)	CD25 stain per area (%)	Ki67 positive (%)
CD20 stain per area (%)		2.698E-30	8.297E-08	0.004	0.002	4.635E-06	0.837
CD4 stain per area (%)	2.698E-30		1.041E-10	0.003	3.533E-05	7.926E-05	0.687
CD8 stain per area (%)	8.297E-08	1.041E-10		0.003	0.023	0.008	0.092
CD68 stain per area (%)	0.004	0.003	0.003		1.967E-11	0.006	0.022
CD163 stain per area (%)	0.002	3.533E-05	0.023	1.967E-11		0.011	5.353E-06
CD25 stain per area (%)	4.635E-06	7.926E-05	0.008	0.006	0.011		0.006
Ki67 positive (%)	0.837	0.687	0.092	0.022	5.353E-06	0.006	

Spearman Correlation (Nonparametric)

	CD20 stain per area (%)	CD4 stain per area (%)	CD8 stain per area (%)	CD68 stain per area (%)	CD163 stain per area (%)	CD25 stain per area (%)	Ki67% nuclei positive
CD20 stain per area (%)		1.097E-06	5.533E-07	0.001	0.0004	0.010	0.263
CD4 stain per area (%)	1.097E-06		8.81E-09	1.982E-12	5.443E-14	8.517E-12	0.002
CD8 stain per area (%)	5.533E-07	8.81E-09		0.0004	4.338E-08	0.001	0.034
CD68 stain per area (%)	0.001	1.982E-12	0.0004		2.762E-21	5.621E-16	7.375E-05
CD163 stain per area (%)	0.000	5.443E-14	4.338E-08	2.762E-21		1.276E-15	2.603E-08
CD25 stain per area (%)	0.010	8.517E-12	0.001	5.621E-16	1.276E-15		2.753E-05
Ki67 positive (%)	0.263	0.002	0.034	7.375E-05	2.603E-08	2.753E-05	

APPENDIX VIII: CORRELATION OF Ki67 WITH BREAST CANCER SUBTYPE AND GRADES

	P-value for Subtypes by Kruskal-Wallis	P-value for Grades by Kruskal-Wallis
Ki67*	0.0009	0.1347
CD68	0.1187	0.7583
CD8	0.6775	0.5578
CD4	0.0898	0.5626
CD163	0.3502	0.8376
CD20	0.1376	0.8216
CD25	0.3128	0.3711

APPENDIX IX: HORMONE RECEPTOR STATUS

Block #	ER FINAL	PR FINAL	HER2 FINAL
1	Negative	Negative	Negative
6	Negative	Negative	Negative
18	Negative	Negative	Negative
28	Negative	Negative	Negative
33	Negative	Negative	Negative
48	Negative	Negative	Negative
51	Negative	Negative	Negative
53	Negative	Negative	Negative
63	Negative	Negative	Negative
108	Negative	Negative	Negative
122	Negative	Negative	Negative
131	Negative	Negative	Negative
132	Negative	Negative	Negative
140	Negative	Negative	Negative
146	Negative	Negative	Negative
149	Negative	Negative	Negative
154	Negative	Negative	Negative
158	Negative	Negative	Negative
166	Negative	Negative	Negative
168	Negative	Negative	Negative
66	Negative	Negative	Positive
138	Negative	Negative	Positive
8	Positive	Negative	Negative
22	Positive	Negative	Negative
78	Positive	Negative	Negative
2	Positive	Negative	Negative
29	Positive	Negative	Negative
34	Positive	Negative	Negative
43	Positive	Negative	Negative
49	Positive	Negative	Negative
52	Positive	Negative	Negative
73	Positive	Negative	Negative
103	Positive	Negative	Negative
104	Positive	Negative	Negative
136	Positive	Negative	Positive
139	Positive	Positive	Negative
152	Positive	Positive	Negative
54	Positive	Positive	Negative
62	Positive	Positive	Negative
3	Positive	Positive	Negative
4	Positive	Positive	Negative
12	Positive	Positive	Negative
26	Positive	Positive	Negative
30	Positive	Positive	Negative
37	Positive	Positive	Negative
39	Positive	Positive	Negative
41	Positive	Positive	Negative
46	Positive	Positive	Negative
74	Positive	Positive	Negative
81	Positive	Positive	Negative
91	Positive	Positive	Negative
95	Positive	Positive	Negative
96	Positive	Positive	Negative
99	Positive	Positive	Negative
114	Positive	Positive	Negative
124	Positive	Positive	Negative
130	Positive	Positive	Positive
135	Positive	Positive	Positive
157	Positive	Positive	Positive

APPENDIX X: TMA 1, 2 AND 3 MAPPING

TMA 1	1	2	3	4	5	6	7	8	9	10	11	12	13
95	1	67	158	34	124	117	43	90	52	26	93	112	35
29	109	165	56	127	140	78	84	2	108	54	63	24	72
73	53	39	69	81	13	28	60	114	71	82	33	70	68
	64	87	89	36	11	145	41	146	19	131	20	132	136
	103	49	152	23	22	74	148	30	91	99	80	51	61
	138	76	37	110	135	154	169	62	96	12	104	59	119
	18?	167	8	48	46	149	168	166	50	79	157	31	122
	130	21	133	66	17	4	139	6	38	83	98	141	3

Cancer
Benign
Normal
Extra
Controls

TMA 2													
135	74	51	61	30	49	148	99	91	22	80	103	23	152
66	4	141	3	6	21	139	83	38	17	98	130	29	133
168	28	70	68	114	39	60	82	71	13	33	53	81	69
	117	112	35	90	67	43	26	52	124	93	1	34	158
	78	24	72	2	165	84	54	108	140	63	109	127	56
	149	31	122	166	167	95	79	50	46	157	18?	48	8
	154	59	119	62	76	169	12	96	73	104	138	110	37
	145	132	136	146	87	41	131	19	11	20	64	36	89

TMA 3	1	2	3	4	5	6	7	8	9	10	11
95	10	25	55	32	5		5	111	101	156	88
29	164	27	102	65	161		32	10	155	129	55
73	129	155	97	113	88		105	126	161	27	120
	156	106	118	123	101		125	40	113	102	118
	150	144	134	125	40		142	106	121	65	123
	58	15	92	121	120		164	25	144	159	97
	105	86	126	159	9		15	100	134	92	137
	143	111	142	137	100		9	150	143	86	58

Cancer
Benign
Normal
Extra
Controls

APPENDIX XI: PATHOLOGY REPORTS
Subtypes Vs Hormone (PR, ER &HER2)

Pathology	PR		ER		HER2	
	POS	NEG	POS	NEG	POS	NEG
Invasive Cribriform	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Invasive Ductal	18(42.9)	24(57.1)	1(25)	3(75)	5(11.4)	39(88.6)
Invasive Lobular	1(100)	0(0)	0(0)	0(0)	0(0)	1(100)
Mucinous	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Paget's disease	0(0)	1(100)	0(0)	1(100)	1(100)	0(0)
Papillary	1(100)	0(0)	0(0)	0(0)	1(100)	0(0)
P-Value	0.273		0.25		0.506	

There was no significant relationship between Pathology, PR, ER and HER2 (p=0.273, p=0.25 and p 0.506) respectively, since mean difference is significant at p=0.05.

Pathology Subtypes Vs hormone (PR, ER &HER2)

Pathology	PR		ER		HER2	
	POS	NEG	POS	NEG	POS	NEG
Invasive Cribriform	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Invasive Ductal	18(42.9)	24(57.1)	1(25)	3(75)	5(11.4)	39(88.6)
Invasive Lobular	1(100)	0(0)	0(0)	0(0)	0(0)	1(100)
Mucinous	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Paget's disease	0(0)	1(100)	0(0)	1(100)	1(100)	0(0)
Papillary	1(100)	0(0)	0(0)	0(0)	1(100)	0(0)
P-Value	0.273		0.25		0.506	

There was no significant relationship between Pathology, PR, ER and HER2 (p=0.273, p=0.25 and p 0.506) respectively, since mean difference is significant at p=0.05.

Pathology Report and tumor grade

Pathology	Tumor Grade		
	I	II	III
Invasive Ductal	2(4.4)	23 (51.1)	20 (44.4)
Invasive lobular	1(100)	0(0)	0(0)
Paget's disease	0(0)	1(100)	0(0)
Papillary	0(0)	0(0)	2(100)

There was no significant relationship between Pathology and Tumor grade in which the P-value is 0.612. Which shows that it is not assuming the null hypothesis using the asymptotic standard error assuming the null hypothesis of 0.05?

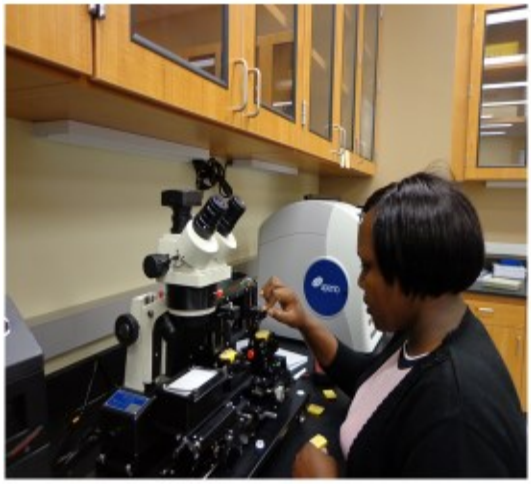
Pathology report and tumor Size

Pathology	Tumor size		
	<=2	2.1-5	>5
Invasive Ductal	13(39.4)	16 (48.5)	4 (12.1)
Invasive lobular	1(100)	0(0)	0(0)
Paget's disease	0(0)	1(100)	0(0)
Papillary	0(0)	0(0)	2(100)
Mucinous	1(50)	1(50)	0(0)

There was no significant relationship between Pathology and Tumor Size in which the P-value is 0.334. Which shows that it is not assuming the null hypothesis using the asymptotic standard error assuming the null hypothesis of 0.05?

APPENDIX XII: PHOTOS OF TMA MACHINES, APERIO SCANNER AND MARKED SLIDE

TMA Construction.



Veridiam semi-automated tissue microarrayer

H&E stained slides from donor blocks were circled out the areas of interest

Tissue cores - 1mm diameter
- recipient blocks.

sectioned at 5µm thickness

Image Scanning

Aperio ScanScope

20x. Using scanner console software

saved onto a database controlled by eSlide manager software

Quantification of IHC images was performed with Aperio Image Analysis Tools software as instructed by vendor's manuals

CD 68 Marked for Quantification