EVALUATION OF SERUM LEVELSOF RANKL, NF-κBAND OXIDATIVE STRESS BIOMARKERS AMONG POSTMENOPAUSAL PATIENTS WITH BREAST CANCER ATTENDING AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NIGERIA

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JULY, 2023

DECLARATION

I declare that the work in this dissertation titled "**Evaluation of Serum Levels of RANKL, NF-κB and Oxidative Stress Biomarkers Among Postmenopausal Patients with Breast Cancer Attending Ahmadu Bello University Teaching Hospital Zaria, Nigeria**." is a record of my own work and has not been submitted for the award of a degree, diploma or any other qualification at this or any other institution. All information and excerpt from the work of others have been duly acknowledgement and a list of references provided in the text.

Abubakar Maru ABDULLAHI _____ _____ _____

Signature Date

CERTIFICATION

This dissertation titled "EVALUATION OF SERUM LEVELS OF RANKL, NF- κB AND OXIDATIVE STRESS BIOMARKERS AMONG POSTMENOPAUSAL PATIENTS WITH BREAST CANCER ATTENDING AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NIGERIA". Submitted by, Abubakar Maru ABDULLAHI meet the regulations governing the award of Master of Science (M.Sc.) in Biochemistry of the Ahmadu Bello University, and is therefore approved for its' contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to Almighty Allah, who granted me the wisdom, knowledge and understanding to succeed in this program.

Also to my lovely parents, friends and family for their support and words of encouragement.

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With heart full of thanks and honour I submit myself to the Almighty God for his favour, grace and mercy upon my life.

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ABSTRACT

Breast cancer is a major cause of morbidity and mortality among postmenopausal women in developing countries, including Nigeria. Receptor activator of NF-κB ligand (RANKL) and nuclear factor kappa-B (NF-κB) have been implicated in breast carcinogenesis. Considering racial disparities in breast cancer pathogenesis and response to therapy, this study examined serum levels of RANKL, NF-κBand oxidative stress biomarkers among postmenopausal breast cancer patients attending Ahmadu Bello University Teaching Hospital (ABUTH). Socio-demographic characteristics were assessed using semi-structured questionnaires and clinical parameters were assessed frommedical records. RANKL and NF-κB levels were analysed using ELISA while MDA and GSH levels were determined using Ellman and Niehaus &Samuelssen methods respectively. The mean age of the respondents was 54.7 ± 6.7 years, while the modal age was 45-50 years. Triple negative breast cancer subtype accounted for 40% of the patients, while 83.3% had invasive carcinoma histological type. Serum RANKL was significantly ($P<0.05$) higher (40%) in breast cancer patients when compared to apparently healthy controls, but lowest in triple negative patients when compared to other subtypes. NF-κB concentration was significantly ($P<0.05$) higher (83.7%) in breast cancer patients but highest among triple negative and HER2-enriched patients when compared to apparently healthy control. Oxidative stress markers (GSH and MDA) were significantly ($P<0.05$) higher in breast cancer patients when compared to apparently healthy control. A significant ($P<0.05$) association between RANKL, NF-κBlevels and disease severity (stage I to stage IV) was also observed. Collectively, the results showed that RANKL, NF-κBand oxidative stress biomarkers serum levels were associated with disease severity among the postmenopausal breast cancer patients, necessitating further longitudinalstudy with larger sample size on their possible prognostic potential.

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LIST OF ABBREVIATION

ABUTH - Ahmadu Bello University Teaching Hospital

ADH - Atypical ductal hyperplasia

AH = apparently healthy

ALH - Atypical lobular hyperplasia

ANOVA – Analysis of variance

BC- Breast cancer

BCSCS - Breast cancer stem cells

BRCA - Breast cancer associated genes

c-Myc - Cellular myelocytomatosis

DCIS - Ductal carcinoma *in situ*

DMBA - 7, 12-dimethylbenzanthracene

EMT - Epithelial-mesenchymal transition

ER- Oestrogen

FEA - Flat epithelial atypia

GRRs - Glycine rich regions

GSH = Reduced glutathione

HATs - histone acetyltransferases

HER2 = human epidermal growth factor receptor 2

HER2- Human epidermal growth factor receptor 2

HRT - hormone replacement therapy

IARC - International Agency for Research on Cancer

IDC - Invasive Ductal Carcinoma

ILC - Invasive lobular Carcinoma

LA = Luminal A

LB = Luminal B

LCIS - Lobular carcinoma *in situ*

MDA = Malondialdehyde

MPA - Medroxyprogesterone acetate

NEMO - NF-κB essential modulator

NF-κB - Nuclear factor kappa-B

ODF - Osteoclastic differentiation factor

OPG - Osteoprotegerin

OPGL - Osteoprotegerin ligand

OR - Odd Ration

PR- Progesterone

PTHrP - Parathyroid hormone related protein

RANKL- Receptor activator nuclear factor kappa-B ligand

RHD - Rel homology domain

RR – Relative Risk

TMB - H_2O_2-tetramethylbenzidin

TN = triple negative

TNF- Tumour necrosis factor

TNRSF11 – Tumour necrosis factor receptor superfamily

TRANCE - TNF-related activation-induced cytokine

UDH - Usual epithelial ductal hyperplasia

CHAPTERONE

INTRODUCTION

1.1 Background

Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body (National cancer institute, 2021) Breast cancer is a multifactorial disease categorized among the major causes of morbidity and mortality among pre and post-menopausal women in many countries of the World (Muhammad *et al*., 2018). It constitutes major public health issue among women globally, with 2.3 million newly diagnosed cases in 2020 (WHO, 2020). The prevalence of breast cancer is higher among women as it mainly affects females with a lifetime risk amounting to a staggering total of 10% while approximately 15-20% of all cases are associated with genetic predisposition (Joko-Fru*et al.*, 2020).

Reports show that there is an increasing rate of mortality among breast cancer patients in Sub-Saharan Africa, and Nigeria contributes greatly to the recorded mortality cases in the region (Azubuike *et al*., 2018). In Nigeria, breast cancer is the leading cause of mortality and morbidity among women with 14,274 death and 28,380 new in 2020 (GLOBOCAN, 2020).The management of breast cancer depends on various factors, including the stage of the cancer, the subtypes of the cancer and the person's age (Lavdaniti *et al*., 2019). Treatments are more vigorous when the prognosis is worse or there is a higher risk of recurrence of the cancer following treatment. Breast cancer is usually treated with surgery, which may be accompanied by chemotherapy or radiation therapy, or both. A multidisciplinary approach was found to be preferable (Saini *et al*., 2011). Hormone receptor-positive cancers are often treated with hormone receptor-blocking therapy over several years

(Holmes *et al*., 2010). Monoclonal antibodies, or other immune-modulating treatments, may be administered in certain cases of metastatic and other advanced stages of breast cancer (Holmes *et al*., 2010).

Breast cancer is a heterogeneous condition consisting of multiple subtypes with distinct morphologies (Prat *et al*., 2015; Aliyu *et al*., 2018). Breast cancer is subdivided into different groups based on their origin. Luminal A and B originate from the mammary duct luminal epithelium with consistent hormone receptor expression (Prat *et al*., 2015). The luminal A tumours have higher expression of oestrogen receptor related genes and lower expression of proliferative genes (ER+, PR+, HER2-) when compared to luminal B cancers (ER+, PR+, HER2-) (Reis-Filho& Pusztai, 2011). Tumours classified as HER2 overexpressing are a group of aggressive breast cancers that are associated with poor prognosis, expressed only HER2 and termed as HER2-enriched (Schramm *et al*., 2015). Basal like tumours are tumours that originate from mammary basal myo-epithelium and are classified as group of aggressive breast cancers that are characterized by negative expression of hormone receptors (ER-, PR-, and HER2-) thus termed Triple negative breast cancer (Shao *et al*., 2017). Triple negative breast cancer follows an aggressive clinical course with difficulty of standard targeted systemic therapy (Shao *et al*., 2017). It is characterized by rapid growth and is commonly seen among black women and women of African descent (Grubb *et al*., 2017). The tumours are reported to be larger than other subtypes and metastasis among patients is seen to have a tendency towards extending to visceral organs (Shao *et al*., 2017).

Receptor activator nuclear factor kappa-B ligand (RANKL) is a type II transmembrane protein and a member of tumour necrosis factor (TNF) superfamily, produced chiefly by osteoblastic lineage cells and stromal cells (Dougall, 2012). RANKL exists in membrane and in a soluble form (sRANKL) which is cleaved from the cellular form by metalloproteases and TNF-α converting enzyme (Nakashima *et al.,* 2011) or released as primary secreted isoform

(Woo *et al.*, 2000). Various factors can induced soluble or membrane bound RANKL such as oxidative stress, oestrogen, vitamin D_3, parathyroid hormone related protein (PTHrP), or cytokines (such as TNF-α and interleukins 1, 11 and 17) (Palmqvist *et al*., 2002; Kido *et al*., 2003).

As a signalling intermediary biomolecule, RANKL has also been implicated in mammary cell proliferation, breast cancer initiation and metastasis to bone (Infante *et al*., 2019). Receptor Activator of Nuclear factor Kappa beta Ligand (RANKL) is an important molecule that plays crucial role not only in bone metabolism and osteoporosis (Mada *et al*., 2017), but also in the development of the mammary gland during pregnancy (Rao *et al*., 2018). Downstream of RANKL, Nuclear Factor kappa Beta (NF-kB) is a transcription factor that controls physiological functions that are observably altered during breast cancer (Tsubaki *et al*., 2013). NF-kB contributes to breast cancer via mechanisms involving mammary cell proliferation, inflammation, resistance to apoptosis and metastasis (Park, 2017). Altered NF-kB expression has been associated with larger tumour size and aggressive disease progression in a number of breast cancer patients (Prajoko & Aryandono, 2014; Sarkar *et al*., 2013).

Oxidative stress, thatis an imbalance between oxygen free-radical generation and antioxidant scavenging, plays an important role in the initiation, progression, and invasion of breast cancer. Excessive formation of oxygen free radicals can cause oxidative damage to biomolecules and consequently result in mutagenesis, lipid peroxidation, and carcinogenesis (Hecht *et al*., 2016; Yue and Wang, 2015). The prime targets of reactive oxygen species (ROS) are the polyunsaturated fatty acids (PUFA) and some of the proteins found in cell membrane (Stanicka *et al*., 2015). Additionally, impaired lipid hydro-peroxides yields a wide range of end-products including malondialdehyde (MDA). On the other hand, lipid peroxidation induced by oxygen-free-radical can led to malignant transformation (Aldini,

2010; Halliwell, 2007; Nechuta *et al*., 2014). Several studies have shown that oxidative stress increases in breast cancer (Balliet *et al*., 2011; Fazilaty *et al*., 2013).

1.2 Statement of Research Problem

Breast cancer is the most commonly diagnosed cancer amongst women across the world with about 2.3 million newly diagnosed cases in 2020 (WHO, 2020). In Nigeria, according to GLOBOCAN breast cancer is the leading cause of cancer mortality and morbidity among women with 14,274 deaths and 28,380 new cases annually (GLOBOCAN, 2020). Significant improvements have been made in the treatment of breast cancer during the last decades, mainly due to the advances made in breast cancer research. However, sadly, due to the increased incidenceof breast cancer cases across the world, breast cancer has become more and more prevalent in recent years (Yedjou *et al.,* 2019). This development is attributed to lifestyle changes regarding reproductive behavior, weight gain, and hormone replacement therapy (Vogel, 2017). Thus, breast cancer is becoming an even more global pandemic and equally one of the main challenges facing modern scientific research.

Higher level of RANKL and NF-kB among postmenopausal women with breast cancer cases has been widely reported across races and geographical region (Yedjou *et al.,* 2019). Indeed, increased in their expression have been implicated in osteoporosis among postmenopausal women with breast cancer, leading to an increase in percentage mortality (Infant *et al*., 2018). However the serum levels of RANKL, NF-kB and oxidative stress biomarkers among breast cancer patients across the Northwestern region of this country have not been established.

1.3 Justification

RANKL and NF-κB are both implicated in breast cancer development and its metastasis to different part of the body especially in postmenopausal women with tendency to have osteoporosis. RANKL is also reported to protect breast cancer cells from apoptosis in response to DNA damage, as well as control the self-renewal and anchorage-independent growth of tumour-initiating cells (Infante *et al*., 2019). RANKL through the activation of NF-κB pathway induced epithelial-mesenchymal transition (EMT) which occurs during cancer progression and metastasis. RANKL and NF-κB are both reported to serve as useful circulating biomarkers in bone related disease (Mada *et al*., 2017). Whether or not there is a higher level of RANKL and NF-kB in relation to disease progression among breast cancer patients of different subtypes attending ABUTH Zaria, remains to be explored. Therefore, identifying possible biomarkers that can serve as indicators for breast cancer is one of the most important yet unresolved needs in this part of the country. This research work was aim to evaluate RANKL, NF-κB and oxidative stress biomarkers among postmenopausal breast cancer patients attending ABUTH. Zaria, Nigeria.

1.4 Aim of the Study

This work was aim at evaluating serum levels of RANKL, NF-κB and oxidative stress biomarkers among postmenopausal patients with breast cancer attending Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, Nigeria.

1.5 Specific Objectives

The specific objective are to:

i. To determine the socio-demographic factors of the postmenopausal breast cancer patients.

ii. To determine the frequency ratio of the different subtypes of breast cancer (Luminal A, Luminal B, $HER2^{+/-}$ and Triple negative) using immunohistochemistry history.

iii. To determine the serum level of RANKL and NF-κB among the postmenopausal breast cancer patients and apparently healthy controls

iv. To determine the level of some oxidative stress biomarkers among the postmenopausal breast cancer patients and apparently healthy controls

1.6 Null Hypothesis

There are no changes in the serum levels of RANKL, NF-kB and oxidative stress biomarkers among postmenopausal patients with breast cancer attending Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, Nigeria

CHAPTER TWO

LITERATURE REVIEW

2.1Epidemiology and Global Burden of Breast Cancer

Breast cancer (BC) is a heterogeneous disease that arises from accumulated genetic and epigenetic changes in cells as a result of both inherited and environmental risk factors (Ginsburg, *et al*., 2011). Globally, breast cancer is the most prevalent cancer (7.8million up to 5 years alive after diagnosis) and the leading cause of cancer related morbidity and mortality among women (IARC, 2020). It accounts for approximately 2.3 million newly diagnosed cases estimated in 2020, contributing about 11.7% of the total cancer incidence burden and about 6.9% (685 000 deaths) worldwide compared to 2.1 million new cases and 627,000 deaths in 2018, because the prognosis is relatively favourable, at least in more developed countries. (Ferlay *et al*., 2021; Bray *et al*., 2018; IARC, 2020). Breast cancer has an incidence rate of 47.8% and a mortality rate of 13.6% per 100,000 across the world, but the incidence rates far exceed those of other cancers in both developed and developing countries (GLOBOCAN, 2020). The highest incidence rates in women are seen in Australia, New Zealand,Western Europe and North America, (IARC: Cancer Epidemiology Database, GLOBOCAN. 2020).

The International Agency for Cancer Research (IARC) estimated that the incidence of breast cancer ranged from 33 per 100 000 women in central Africa to 50 per 100 000 women in southern Africa in 2020(Ferlay *et al*., 2021). Although the incidence of breast cancer appears to be relatively low in Sub-Saharan Africa (SSA), survival from the disease is also generally poor in the region, with high mortality recorded in many settings (Ferlay *et al*., 2021; Jedy Agba *et al*., 2016). The poor survival of breast cancer patients in SSA has been associated

with late presentation, poor health care infrastructure and lack of adequate funding, amidst other competing public health challenges (Pace and Shulman, 2016).

Moreover, in some isolated countries with relatively good breast cancer care services in SSA, inequity and unaffordability of the services provided have been widely reported (Pace and Shulman, 2016; Al-Haddad *et al*., 2015). Consequently, late presentations and advanced stages at diagnosis of several breast cancer cases have been prevalent, likely explaining the higher mortality rates reported (Jedy Agba *et al*., 2020). With ageing, population growth, and adoption of unhealthy lifestyles, the burden of breast cancer is projected to double in Africa by 2030 (Ferlay *et al*., 2019), especially in the absence of effective public health policies and interventions (Ferlay *et al*., 2019). The perturbing part however was the fact that while incidence in the African region (although rising) was lower than in other continents except Asia, its age-standardized mortality rate ranked highest globally with Nigeria, the most populous African nation, having the highest mortality rate (Joko-Fru*et al*., 2020).

The high rate occurrence of breast cancer mortality may be credited to the fact that breast cancer is generally more aggressive and associated with higher mortality among non-Caucasian race, a partiality that becomes more apparent after menopause (Torre*et al*., 2017) This racial trend was more clearly highlighted by Joko-Fru*et al*. who showed an 18% increase in breast cancer mortality among non- Caucasian with a 3% decrease among Caucasians over the same period in the USA (Joko-Fru*et al*., 2020) A possible contributing factor may be racial genetic differences in metabolism of commonly used drugs (Metcalfe*et al*., 2010). A genome- wide association studies in women of African ancestry (Feng *et al*., 2014) suggest a significant number of common shared variant loci predisposing these women to breast cancer.

According to GLOBOCAN database breast cancer is the leading cause of cancer related morbidity and mortality among women in Nigeria (Figure 2.1), with 28,310 (38.8%) of all cancer new cases and 14,274 death in 2020 (IARC, 2020; Ferlay *et al*., 2021). The recent ASR of 49.0% as reported by the GLOBOCAN database shows that There was a significant increase in the incidence of breast cancer compared to historical records (13.7 per 100,000 women per year for 1960–1969, 24.7 per 100,000 women per year between 1998 and 1999 (GLOBOCAN, 2020: Joko-Fru *et al*., 2020). These data therefore suggested an approximate increase by 70.3% over the four decades and an increase of approximately 20.1% per decade. The recent ASR of 49.0% reported equates to approximately a 120% increase between the years 2010 and 2020. The figure was higher than the GLOBOCAN estimate for 2018 (38.7 per 100,000)(Joko-Fru *et al*., 2020). The most worrisome aspect of this development was the fact that even though Nigeria was ranked second in terms of breast cancer incidence, its age-standardized mortality ratio was the highest in Africa (25.9 per 100,000, compared to 18.8 per 100,000 for Mauritius).

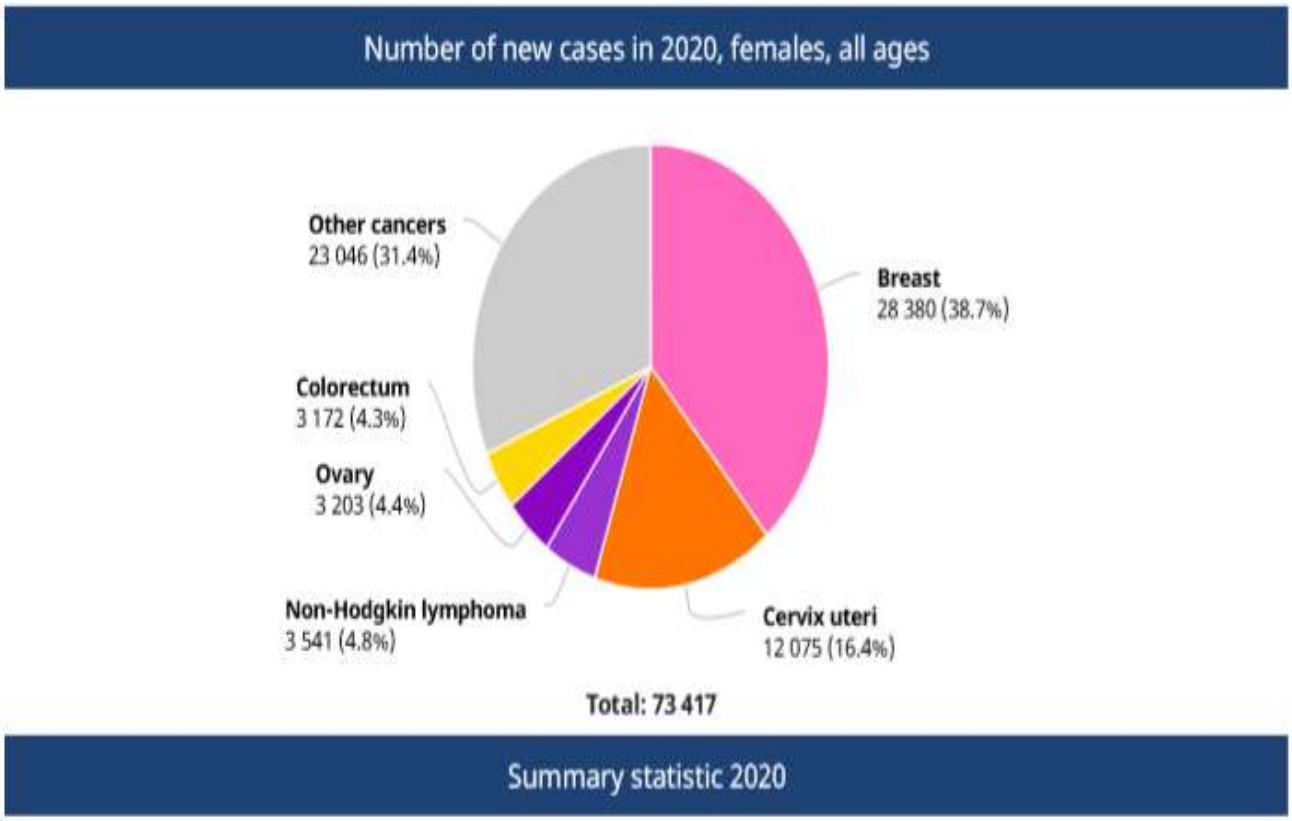

Figure 2. 1: Chart showing number of new cancer cases in Nigeria in 2020 (X1000), Adult population (GLOBOCAN, 2020)

2.2 Pathogenesis of breast cancer

Breast tumours usually start from the ductal hyper-proliferation, and then develop into benign tumours or even metastatic carcinomas after constant stimulation by various carcinogenic factors (Shah *et al*., 2014). Tumour microenvironments such as the stromal influences or macrophages play vital roles in breast cancer initiation and progression. The mammary gland of rats could be induced to neoplasms when only the stroma was exposed to carcinogens, not the extracellular matrix or the epithelium (Maffini *et al*., 2004; Sonnenschein and Soto, 2016). Macrophages can generate a mutagenic inflammatory microenvironment, which can promote angiogenesis and enable cancer cells to escape immune rejection (Qian and Pollard, 2010; Dumars *et al*., 2016). Different DNA methylation patterns have been observed between the normal and tumour-associated microenvironments, indicating that epigenetic modifications in the tumourmicroenvironment can promote carcinogenesis. For example CpG hyper-methylation of *RASSF1A*, *PEMT*, *SFRP*, and *RKIP* in breast tumours (Zhao *et al*., 2016; Basse and Arock, 2015). Recently, a new subclass of malignant cells within tumours called the cancer stem cells (CSCs) are observed and associated with tumour initiation, escape and recurrence. This small population of cells, which may develop from stem cells or progenitor cells in normal tissues, have self-renewal abilities and are resistant to conventional therapies such as chemotherapy and radiotherapy (Zhang *et al*., 2017).

Breast cancer stem cells (BCSCS) were first identified by Al-Hajj and even as few as 100 BCSCS could form new tumours in the immune-compromised mice (Al-Hajj *et al*., 2003). BCSCS are more likely to originate from luminal epithelial progenitors rather than from basal stem cells (Molyneux *et al*., 2010). Signalling pathways including Wnt, Notch, Hedgehog, p53, PI3K and HIF are involved in the self-renewal, proliferation and invasion of BCSCS (Valenti *et al*., 2017). However, more studies are needed to understand BCSCS and to

develop novel strategies to directly eliminate the BCSCS. There are two hypothetical theories (Figure 2.2) for breast cancer initiation and progression: the cancer stem cell theory and the stochastic theory (Polyak, 2007; Sgroi, 2010). The cancer stem cell theory suggests that all tumour subtypes are derived from the same stem cells or transit-amplifying cells (progenitor cells). Acquired genetic and epigenetic mutations in stem cells or progenitor cells will lead to different tumour phenotypes (Figure 2.2A). The stochastic theory is that each tumour subtype is initiated from a single cell type (stem cell, progenitor cell, or differentiated cell) (Figure 2.2B). Random mutations can gradually accumulate in any breast cells, leading to their transformation into tumour cells when adequate mutations have accumulated. Although both theories are supported by plenty of data, neither can fully explain the origin of human breast cancer (Yi-Sheng *et al*., 2017).

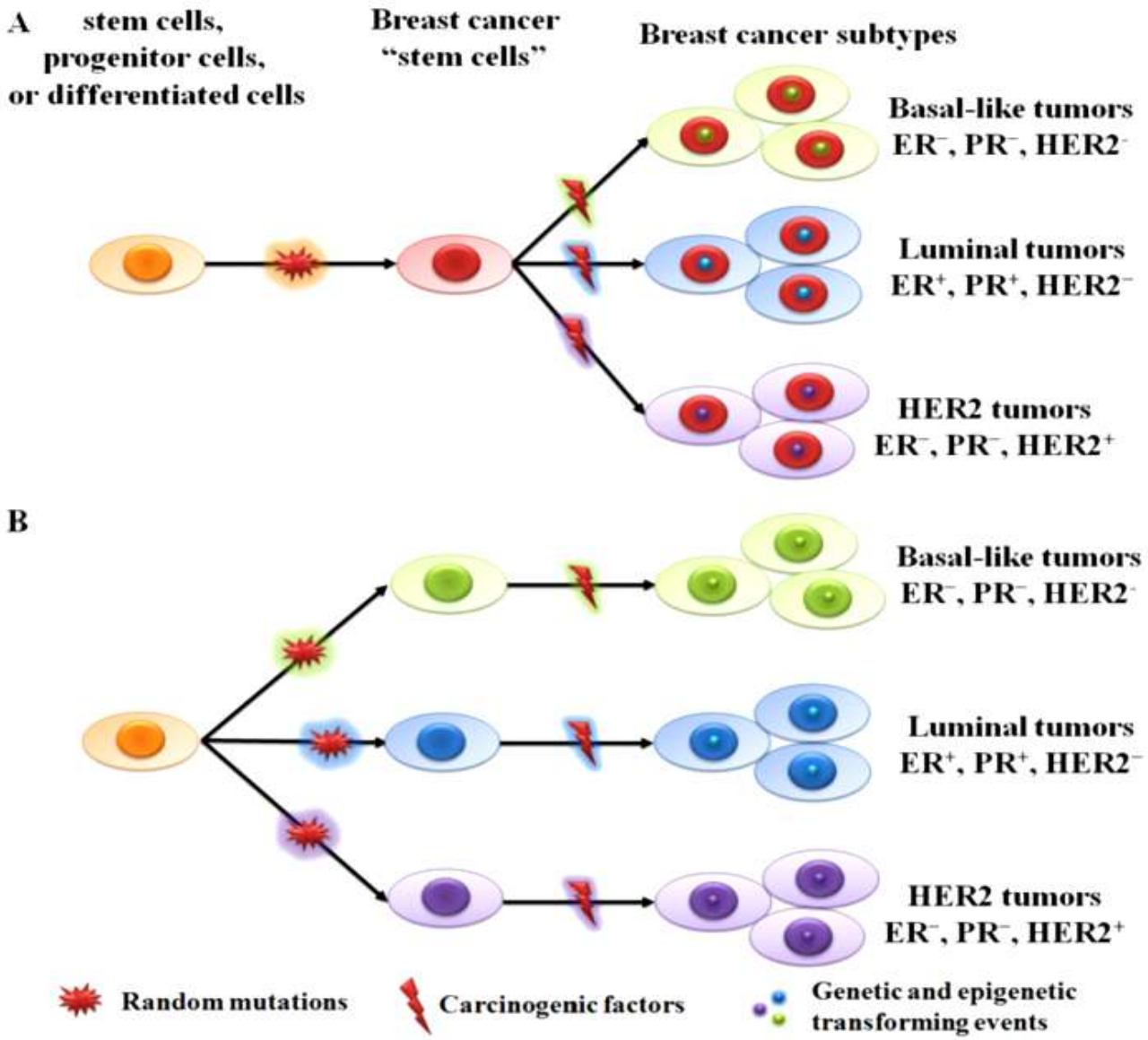

Figure 2. 2: Two hypothetical theories of breast cancer initiation and progression

(A) All subtypes of tumour are derived from the same stem cells or progenitor cells. Different tumour phenotypes are then determined by subtype-specific transforming events. (B) Each tumour subtype is initiated from a single cell type (stem cell, progenitor cell, or differentiated cell). Random mutations can gradually accumulate in any breast cells, leading to their transformation into tumour cells when an adequate number of mutations have accumulated (Yi-Sheng *et al*., 2017).

2.3 Genes related to breast cancer

Many genes have been recognized in relation to breast cancer. Irregular amplification and Mutations of both oncogenes and anti-oncogenes play key roles in the development of tumour initiation and progression. Some of these genes are discussed below.

2.3.1 Breast cancer associated genes (BRCA1/2)

Breast cancer associated gene 1 and 2 (*BRCA1* and *BRCA2*) are two famous anti-oncogenes for breastcancer risk. *BRCA1* and *BRCA2* are located onchromosome 17q21 and 13q12, respectively. Theyboth encode tumour suppressor proteins. BRCA1deficiency leads to the dysregulation of cell cyclecheckpoint, abnormal centrosome duplication, geneticinstability and eventually apoptosis (Deng, 2006; Dine and Deng, 2013). BRCA1expression is repressed by "pocket proteins" such asp130, p107 and the retinoblastoma protein in an E2F-dependent manner. The *BRCA1* gene has been shown to form a loop between the promoter, introns, and terminator regions, which regulates the expression of this gene via interactions with its own promoter (Tan-Wong *et al*., 2008; Hegan *et al*., 2010).

BRCA2 protein regulates recombination repair in DNA double-strand breaks by interacting with RAD51 and DMC1 (Sanchez *et al*., 2017; Martinez *et al*., 2016). BRCA2-associated breast cancers are more likely to be high-grade invasive ductal carcinomas, but with a luminal phenotype (Bane *et al*., 2007). The risk of breast cancer could be increased greatly if an individual inherits deleterious mutations in either *BRCA1* or *BRCA2* genes. *BRCA1/2* mutations are inherited in an autosomal dominant manner even though the second allele is normal. Totally, about 20-25% of hereditary breast cancers and 5-10% of all breast cancers are caused by *BRCA1/2* mutations (Balmana *et al*., 2011; Paluch-Shimon *et al*., 2016). A meta-analysis by Chen showed that breast cancer risk ratio in women older than 70 years

carrying *BRCA1* or *BRCA2* mutations was 57% and 49%, respectively (Chen and Parmigiani, 2007).

2.3.2 Human epidermal growth factor receptor-2 (HER2)

Human epidermal growth factor receptor 2, also known as *c-erbB-2*, is an important oncogene in breast cancer and located on the long arm of human chromosome 17 (17q12). The homologene in mice is *Neu*, which was first identified in 3-methylcholanthrene induced rat neuroblastoma cells (Davis *et al*., 2014). The expression of *HER2* gene is activated mainly through the gene amplification and re-arrangement. HER2 protein is an epidermal growth factor receptor (EGFR) of tyrosine kinase family and form heterodimers with other ligand-bound EGFR family members such as Her3 and Her4, thus to activate downstream signalling pathways (Harbeck and Gnant, 2017). Knockout of *HER2* in mouse models disrupts normal mammary duct formation. Overexpression of HER2,which is detected in about 20% of primary breast cancers, increases the number of cancer stem cells by PTEN/Akt/mTORC1 signalling, and indicates poor clinical outcomes (Davis *et al*., 2014; Elizalde *et al*., 2016).

2.3.3Cellular myelocytomatosis (c-Myc)

This gene is located on the long arm of chromosome 8 (8q24) and encodes for the Myc protein, a transcription factor containing the bHLH/LZ (basic Helix-Loop-Helix Leucine Zipper) domain. Genome-wide screening shows that 15% of all genes are regulated by the Myc protein mainly through binding on the E-box consensus (CACGTG) and recruiting histone acetyl-transferases (HATs) or DNA methyl-transferases (Green *et al*., 2016; Poole and van Riggelen, 2017). Some of the Myc-regulated genes such as *MTA1*, *hTERT* and *PEG10* play a vital role in breast cancer initiation and progression. The overexpression of c-Myc is predominantly observed in the high-grade, invasive stage of breast carcinomas, while

no c-Myc amplification is detected in the benign tissues (Chen and Olopade, 2008; Jung *et al*., 2017).

2.4 Risk Factors for Breast Cancer

2.4.1 Genetic predispositions

Basically, a risk factor is something that affects an individual's chance of getting a disease, in this case breast cancer. Some of the major risk factors for breast cancer are beyond individual's control (Howell *et al*., 2014; Anderson *et al*., 2014; Barnard *et al*., 2015; Kaminska *et al*., 2015; sun *et al*., 2017; Ozsoy *et al*., 2017). For example, simply being a woman is the main risk factor for breast cancer as this disease is about 100 times more likely to occur in women than in men. Aging inevitably increases one's risk of breast cancer as evinced by the fact that most breast cancers are diagnosed in women age 55 and older. Beyond the inherent risks of gender and aging as they relate to breast cancer, it has been well documented that a woman's risk of developing breast cancer nearly doubles if she has a first-degree relative (mother, sister, or daughter) diagnosed with breast cancer. Close to 15% of US women who suffer from breast cancer also have a family member who has been diagnosed (Veronesi *et al*., 2005).

Overall, about 5-10% of breast cancers are linked to gene mutations inherited from a parent. The most common cause of hereditary breast cancer is an inherited mutation in the BRCA1 or BRCA2 gene (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012). Statistically, women with a BRCA1 mutation have a 55-65% lifetime risk of developing breast cancer. For women with a BRCA2 mutation, the lifetime risk is 45%. On average, a woman with a BRCA1 or BRCA2 gene mutation has about a 70% chance of getting breast cancer by age 80. The effect of the mutation is related to how many other family members have breast cancer, as breast

cancer risk goes up as more family members are affected. In the US, BRCA mutations are more common in Jewish people of Ashkenazi (Eastern European) origin than in other racial and ethnic groups although anyone can have these mutations. Women with one of these two mutations are also more likely to be diagnosed with breast cancer at a younger age, as well as to have cancer in both breasts. The impact of the BRCA1 and BRCA 2 mutation expands beyond just breast cancer as having mutations in either of these genes is associated with an increased ovarian cancer risk as well. Conversely, BRCA1 mutations are found less frequently in breast cancers occurring in men while BRCA2 mutations are associated with a lifetime breast cancer risk of only about 6.8% (Veronesi *et al*., 2005;Polyak, 2007; Allison, 2012).

Although less common and less drastic in their increase of breast cancer risk than the BRCA mutations, inherited mutations in many other genes can also lead to breast cancer development (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012). Some of the mutated genes include ATM (inheriting 2 abnormal copies of this gene causes the disease ataxia-telangiectasia), TP53 (inherited mutations of this gene cause Li- Fraumeni syndrome with an increased risk of breast cancer, as well as some other cancers such as leukemia, brain tumours, and sarcomas), CHEK2 (a CHEK2 mutation can increase breast cancer risk about 2-fold), PTEN (inherited mutations in this gene can cause Cowden syndrome which is accompanied by a higher risk for both non-cancerous and cancerous tumours in the breasts, as well as growths in the digestive tract, thyroid, uterus, and ovaries), CDH1 (inherited mutations cause hereditary diffuse gastric cancer with an increased risk of invasive lobular breast cancer), STK11 (mutations in this gene can lead to Peutz-Jeghers syndrome with a higher risk of many types of cancer, including breast cancer), and PALB2 (PALB2 gene makes a protein that interacts with the protein made by the BRCA2 gene, resulting in

mutations in this gene causing a higher risk of breast cancer) (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012).

2.4.2 Non genetic risk factors for breast cancer

The aetiology of breast cancer is multifactorial and from descriptive epidemiological data it has evidently appeared that breast cancer is a disease of rich societies which have picked up the Western lifestyle, characterized by a high-caloric diet rich in animal fat and proteins, joint with a lack of physical exercise. Various risk factors have been identified which include age, hereditary, dietary (diet and obesity), gynaecological (oral contraceptives, hormone replacing therapies, endogenous hormone levels, age of menarche and menopause, parity and mammographic density), life style (physical activity, smoking and alcohol), oxygen reactive species, radiation and environmental pollutants (Yi-Sheng Sun *et al*., 2017). A schematic diagram of risk factors is depicted in a pyramid-style structure (Figure 2.3).

2.4.2.1 Aging

Besides sex, aging is one of the most important risk factors of breast cancer, because the incidence of breast cancer is highly related to the increasing age. In 2016, approximately 99.3% and 71.2% of all breast cancer-associated deaths in America were reported in women over the age of 40 and 60, respectively (Siegel *et al*., 2017). Therefore, it is necessary to have a mammography screening ahead of time in women aged 40 or older.

2.4.2.2 Reproductive factors

Reproductive factors such as early menarche, late menopause, late age at first pregnancy and low parity can increase the breast cancer risk. Each 1-year delay in menopause increases the risk of breast cancer by 3% (Ritte *et al*., 2012). Each 1-year delay in menarche or each

additional birth decreases the risk of breast cancer by 5% or 10%, respectively (Washbrook, 2006; Dall and Britt, 2017).A recent Norwegian cohort study showed that a hazard ratio (HR) is 1.54 between late (≥35 years) and early (<20 years) age at first birth (Horn *et al*., 2013). Reproductive factors are strongly associated with the ER status, with differences in the odds ratios (OR) between ER+ and ER– breast cancer for parity (OR: 0.7 vs. 0.9 for ≥3 births vs. nulliparous) and age at the first birth (OR: 1.6 vs. 1.2 for age ≥30 vs. <25 years) (Rosato *et al*., 2014).

2.4.2.3 Oestrogen

Both endogenous and exogenous oestrogens are associated with the risk of breast cancer. The endogenous oestrogen is usually produced by the ovary in premenopausal women and ovariectomy can reduce the risk of breast cancer (Endogenous *et al*., 2013). The main sources of exogenous oestrogen are the oral contraceptives and the hormone replacement therapy (HRT). The oral contraceptives have been widely used since the 1960s and the formulations have been upgraded to reduce side-effects. However, the odd ration(OR) is still higher than 1.5 for African American women and Iranian populations (Soroush *et al*., 2016; Bethea *et al*., 2015). Nevertheless, oral contraceptives do not increase the risk of breast cancer in women who stop to use them for more than 10 years (Washbrook, 2006). HRT involves the administration of exogenous oestrogen or other hormones for the menopausal or postmenopausal women. A number of studies have shown that the use of HRT can increase breast cancer risk.

The Million Women Study in the UK reported a relative risk (RR) of 1.66 between current users of HRT and those who never used it (Beral, 2003). A cohort study of 22,929 women in Asia demonstrated higher risks (HRs) of 1.48 and 1.95 after HRT use for 4 and 8 years, respectively (Liu *et al*., 2016). However, the risk of breast cancer has been shown to

significantly decrease after two years of stopping HRT (Narod, 2011). The recurrence rate is also high among breast cancer survivors who take HRT, and the higher risk for a new breast tumour is 3.6 (Fahlen *et al*., 2013).

Since the adverse effects of HRT were published in 2003 based on the Women's Health Initiative randomized controlled trial, the incidence rate of breast cancer in America has decreased by approximately 7% due to the reduction in the use of HRT (Ravdin *et al*., 2007).

2.4.2.4 Lifestyle

Modern lifestyles such as excessive alcohol consumption and too much dietary fat intake can increase the risk of breast cancer (Chen *et al*., 2011). Alcohol consumption can elevate the level of oestrogen related hormones in the blood and trigger the oestrogen receptor pathways. A meta-analysis based on 53 epidemiological studies indicated that an intake of 35-44 grams of alcohol per day can increase the risk of breast cancer by 32%, with a 7.1% increase in the RR for each additional 10 grams of alcohol per day (Hamajima *et al*., 2002; Jung *et al*., 2016). Modern western diets contain too much fat and excess intake of fat, especially saturated fat, is associated with mortality (RR=1.3) and poor prognosis in breast cancer patients (Makarem *et al*., 2013).

Although the relationship between smoking and breast cancer risk remains controversial, mutagens from cigarette smoke have been detected in the breast fluid from non-lactating women. The risk of breast cancer is also elevated in women who both smoke and drink (RR=1.54) (Knight *et al*., 2017). Up to now, accumulating evidences demonstrate that smoking, especially at an early age, has a higher risk on breast cancer occurrence, this is because Cigarette smoke is very rich in carcinogens and reactive oxygen species and may be considered as one with high risk in breast cancer (Mitruen and Hirvonen, 2003; Catsburg *et al*., 2015; Kispert and McHowat, 2017).

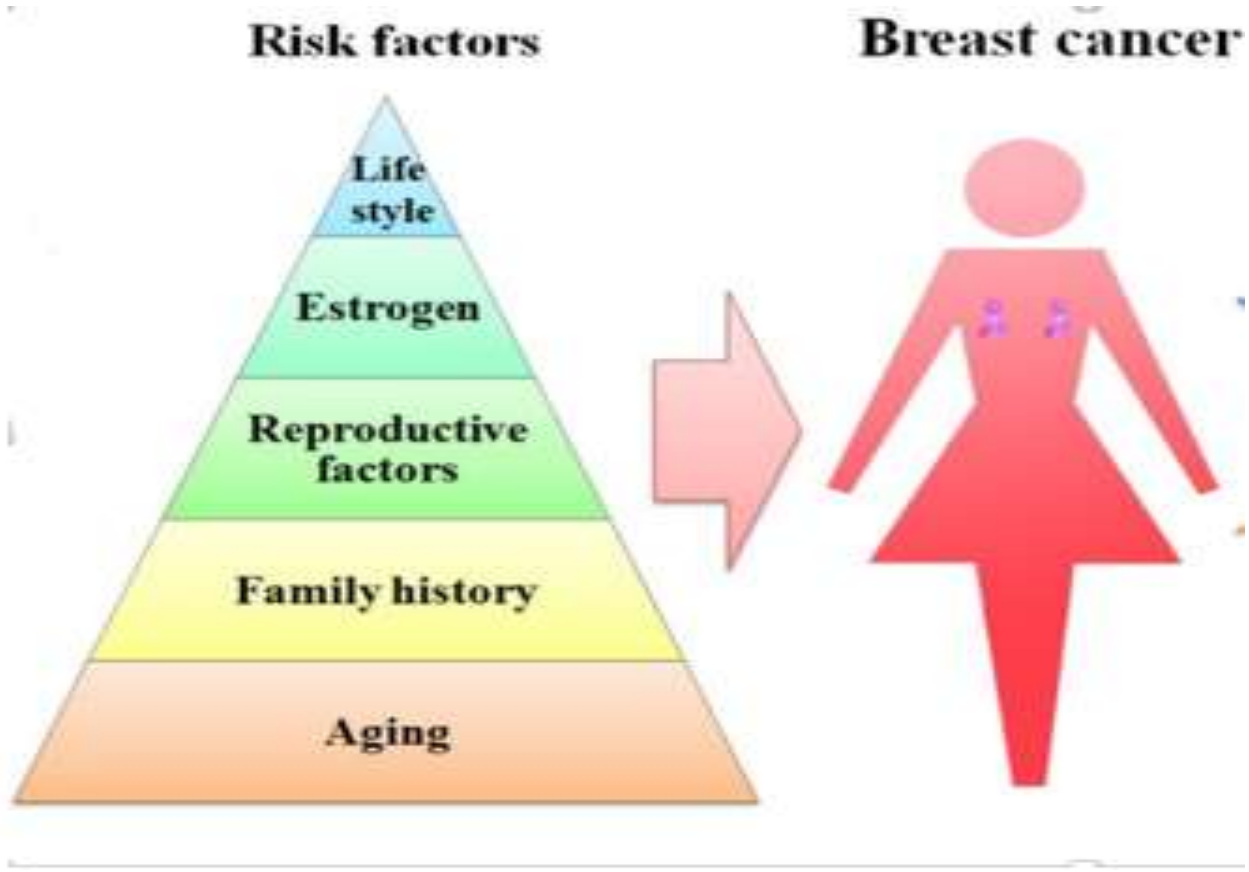

Figure 2. 3: Aetiological factors involved in the development of breast cancer (Ji-sheng *et al*., 2017)

2.5 General characteristics of Breast Cancer

2.5.1 The normal Breast

There are about 15 to 20 sections called lobes in a female's breast and each lobe is made of many smaller sections known as lobules, which in turn have groups of tiny glands or milk-producing glands that can make milk. It is also made up of ducts, tiny tubes that carry the milk from the lobules to the nipple, and stroma fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels (American Cancer Society booklet).

2.5.2 The lymph (lymphatic) system of the breast

The lymph system is very essential in breast cancer research in the sense that it is one of the ways breast cancers can spread to several parts (Figure 2.4). Lymph nodes are small, bean-shaped collections of immune system cells that are connected by lymphatic vessels. These vessels are like small veins, except that they carry a clear fluid called lymph in place of blood away from the breast. They also contain Lymph tissue fluid and waste products, in addition to immune system cells. Breast cancer cells can enter lymphatic vessels and begin to grow in lymph nodes(Bombonati and Sgroi, 2011). Most lymphatic vessels in the breast connect to lymph nodes under the arm (axillary nodes), some lymphatic vessels that connect to lymph nodes inside the chest are called internal lymph nodes, and those either above or below the collarbone are called supraclavicular or infra-clavicular nodes (American Cancer Society booklet).

There are several types of breast cancer, but some of them are quite rare. Currently, the majority of all breast cancers worldwide are the ductal and lobular subtype .However, the ductal subtype accounts for the majority of the diagnosed cases, constituting for about 40–

75% (Rakha *et al*., 2006). In addition, several linear models of breast cancer initiation, transformation and progression have been reported, there are two models for the ductal subtype The first ‘ductal’ model, reported by Lerwill (Lerwill, 2008) recognizes flat epithelial atypia (FEA), to atypical ductal hyperplasia (ADH) and then ductal carcinoma *in situ* (DCIS), which is the non-obligate precursors of the advanced invasive and metastatic ductal carcinoma. In the second model, usual epithelial ductal hyperplasia (UDH) was proposed as an intermediate stage of progression between FEA and DCIS (Pati*et al*., 2020). In the case of lobular subtype, atypical lobular hyperplasia (ALH) and lobular carcinoma *in situ* (LCIS) was also proposed as the non-obligate precursor lesions to invasive lobular carcinoma. (Gonzalez*et al*., 2022).

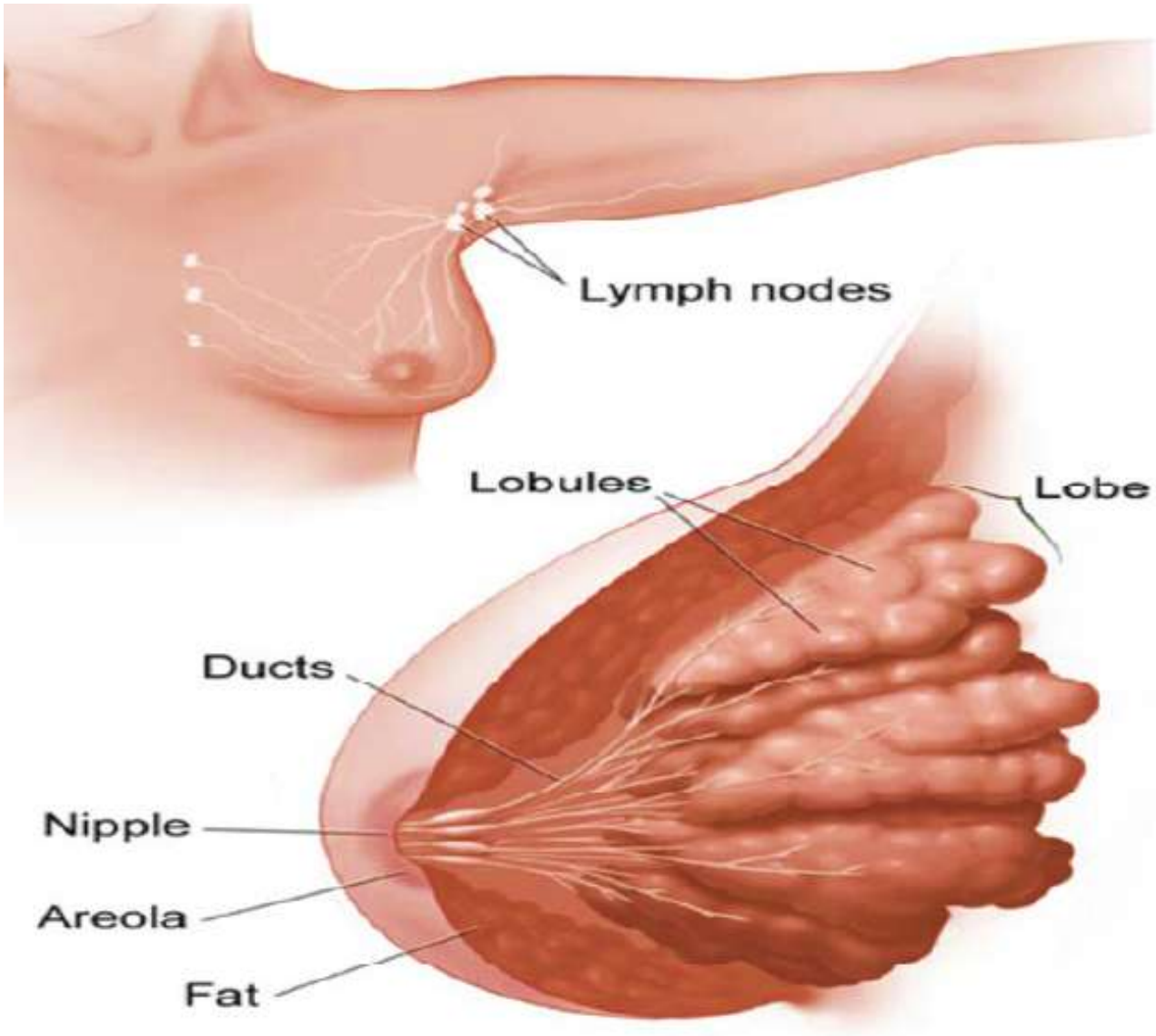

Figure 2. 4: Picture showing lobes and ducts inside the breast and lymph nodes near the breast (National Cancer Institute Booklet, 2012)

2.6 Classifications of Breast Cancer

2.6.1 Types of breast cancer based on pathology, invasiveness and prevalence

There are many types of breast cancers as it can present in distinct areas of the breast, such as the ducts, the lobules, or the tissue in between. The type of breast cancer is determined by the specific cells that are affected. Based on which cell origin is involved, breast cancers can be divided into two broad classifications, carcinomas and sarcomas (WHO, 2020). Carcinomas are breast cancers arising from the epithelial component of the breast, which consists of the cells that line the lobules and terminal ducts responsible for making milk. Sarcomas are a much rarer form of breast cancer (<1% of primary breast cancer) arising from the stromal components of the breast, which include myo-fibroblasts and blood vessel cells. These groups are not always sufficient categories as, in some cases, a single breast tumour can be a combination of different cell types (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012).

Most breast cancers are carcinomas. Within the large group of carcinomas, there are many different types of breast cancer identified based on their invasiveness relative to the primary tumour sites. Accurately being able to distinguish between the various subtypes is vital as they each have different prognoses and treatment implications. Based on criteria of pathological features and invasiveness, common breast cancers can be divided into three major groups: non-invasive (or *in situ*), invasive, and metastatic breast cancers (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012).

2.6.1.1 Non-invasive (or in situ) breast cancer

Ductal carcinoma in situ (DCIS; also called intra-ductal carcinoma): As one of the most common types of breast cancer, DCIS is a non-invasive or pre-invasive breast cancer, which develops inside of pre-existing normal ducts. (Veronesi *et al*., 2005; Polyak, 2007; Allison,

2012). While DCIS is itself not invasive, in situ carcinomas have high potential to become invasive cancers, so early and adequate treatment is important in preventing the patient from developing an invasive cancer.

2.6.1.2 Invasive or infiltrating breast cancer

Invasive breast cancers have cancer cells that invade and spread outside of the normal breast lobules and ducts, growing into the surrounding breast stromal tissue. (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012). About two-thirds of women with an invasive form of breast cancer are 55 years or older when they are diagnosed. Invasive carcinomas have the potential to spread to other sites of the body, such as the lymph nodes or other organs and to form metastases thus entering the classification of metastatic breast cancers. (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012). Based on the tissue and cell types involved, invasive breast cancers are further divided into following two types:

*2.6.1.2.1: Invasive Ductal Carcinoma (IDC):*IDC is the most common type of breast cancer with about 80% of all breast cancers being constituted by invasive ductal carcinomas. (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012). The IDC classification includes several subtypes: tubular carcinoma of the breast, medullary carcinoma of the breast, mucinous carcinoma of the breast, papillary carcinoma of the breast, and cribriform carcinoma of the breast. (Veronesi *et al*., 2005;Polyak, 2007; Allison, 2012).

2.6.1.2.2: Invasive Lobular Carcinoma (ILC): ILC is the second most common type of breast cancers and accounts for approximately 10-15% of all breast cancers. (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012). Although ILC can affect women at any age, it is more common in older women. ILC tends to occur later in life than IDC, e.g. in the early 60s as opposed to the mid-to late 50s for IDC. (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012). Together,

90-95% of all breast cancer cases fall into invasive subcategories. IDC and ILC cancers each exhibit distinct pathologic features. Lobular carcinomas grow as single cells arranged individually, in single file, or in sheets, and they have distinct molecular and genetic aberrations that distinguish them from ductal carcinomas. Ductal and lobular carcinomas may have different prognoses and treatment options and are thus important to clearly differentiate from one another.

2.6.1.3 Metastatic breast cancer

Metastatic breast cancers, also known as stage IV or advanced breast cancers, are late stage breast cancers, which have spread to other organs in the body. (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012). Metastases from breast cancers can be found in lymph nodes in the armpit, and/or in distant sites such as the lung, liver, bone and brain. Even after the primary tumour is removed, microscopic tumour cells or micro-metastases may remain in the body, which allows the cancer to return and disseminate. Clinically, patients may initially be diagnosed with metastatic disease (or de novo metastatic breast cancers), or they may develop metastases months or years after receiving initial treatment. The risk of breast cancer returning and metastasizing is not clearly understood or predictable as it varies from person to person, largely depending on the unique molecular biology of the tumour and the stage at the time of the original diagnosis. Unfortunately, approximately 30% of the women diagnosed with early-stage breast cancer will develop a metastatic form of the disease. (Veronesi *et al*., 2005; Polyak, 2007; Allison, 2012).

2.6.1.4 Molecular subtypes of breast cancer

Molecular Breast cancer is a highly heterogeneous disease for its histology, epidemiology, and molecular properties. Six molecular subtypes of breast cancer have been identified

according to their gene expression profiles and their identification and classification was of fundamental importance for our understanding of tumour genesis and progression: normal breast-like, luminal A and B, basal-like, claudin-low, and HER2/ERB2 overexpressing. The origin of luminal A and B tumours seems to be the mammary duct luminal epithelium, with consistent hormone receptor expression. Basal-like cancers form a heterogeneous group of breast cancers, which probably arise from progenitor cells different from those involved in other breast cancers. HER2/ERB2 overexpressing breast cancers represent a group of aggressive breast cancers that are associated with poor prognosis. Finally, claudin-low are a peculiar group of aggressive breast cancers that are characterized by negative expression of ER, PR, and HER2 (triple-negative), and by the acquisition of mesenchymal/sarcomatoid and/or squamous metaplasia of malignant breast epithelium. This classification also reflects a different metastatic potential of these various breast cancer types. Bone is the most frequent metastatic site for all breast cancer subtypes, with the exception of basal-like. Luminal A tumours are those with the lowest tendency to metastasize; luminal/HER2 and HER2-positive breast cancers were more metastatic than luminal A cancers, particularly at the level of brain, liver, and lung metastases; the basal-like tumours displayed a higher tendency to metastasize at the level of brain and lung, but a lower tendency at the level of liver and bone; finally, triple-negative tumours metastasize at the level of all sites (Kennecke *et al*., 2010; Ugo *et al*., 2020).

Other investigators have grouped and classified breast cancers according to the expression of the important functional markers oestrogen receptor (ER), progesterone receptor (PR), and HER2, allowing the identification of tumour subtypes with different outcomes (Wirapati *et al*., 2008). These markers may be also used to additionally characterize the molecular subtypes: luminal A subtype is defined as ER+ and/or PR+, HER2-; luminal B subtype is defined as ER+ and/or PR+, HER2+; basal-like subtype is defined as ER-, PR-, HER2-; and,

HER2 subtype is defined as ER-, PR-, HER2+. Thus, luminal A breast cancers are highly ER+ and PR+, HER2- have usually low proliferative rates and a low Ki67 index, have a NST (no special type), tubular cribiform or classic lobular histology and have a good prognosis. Luminal B breast cancers can be subdivided into HER2- and HER2+: the HER2- tumours are usually ER+ (lower expression than in luminal A tumours), have high proliferation rates, a high Ki67 index, a micro papillary and lobular pleomorphic histology, and exhibit an intermediate prognosis; luminal B, HER2+ breast subtypes of breast cancer cancers are usually ER+, PR+, have a high Ki67 index and an intermediate prognosis. HER2-enriched non-luminal breast cancers have NST histology, a high Ki67 index, an aggressive tumour phenotype, and an intermediate prognosis. Triple-negative breast cancers (TNBCs) largely correspond to basal-like and claudin-low subtypes, have a NST histology or a special histology (metaplastic, adenoid cystic, medullary-like), a high Ki67 index, and a poor prognosis (Ugo *et al*., 2020).

These three clinically adopted markers for the classification of primary breast cancers are used to help decisions regarding therapy in the metastatic setting. The ER, PR, and HER status often changes during disease progression; in fact, a recent study that was carried out on a large cohort of patients estimated that at relapse 32%, 41%, and 15% of patients change their ER, PR, and HER2 status, respectively (Lindström *et al*., 2012). Importantly, women with ER-positive tumours that changed to ER-negative tumours had a significantly 48% increased risk of death when compared with women with stable ER-positive tumours (Lindström *et al*., 2012).

Table 2. 1: Molecular/intrinsic subtypes of breast cancer

Subtypes	Molecular signatures	characteristics	Treatment options
Luminal A	ER+, PR+, HER2-, low Ki67	70% most common prognosis	Hormonal therapy Targeted therapy
Luminal B	ER+, PR+, HER2±, high Ki67	10% - 20% Lower survival than luminal A	Hormonal therapy Targeted therapy
HER2 enriched	ER-, PR-, HER2+,	5% - 15%	Targeted therapy
Triple Negative	ER-, PR-, HER2-,	15% - 20% More common in black women diagnosed at young age worst prognosis	Limited targeted therapy
Normal-like	ER+, PR±, HER2-, low Ki67	Very rare Low proliferation gene cluster expression	Hormonal therapy Targeted therapy

2.7 Clinical staging and survival rates of breast cancer

Once breast cancer is diagnosed, tests are performed to determine the stage of the disease, which will impact the treatment patients receive. The clinical staging of breast cancer is identical across breast cancer subtypes according to the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) Tumour, Node, and Metastasis (TNM) breast cancer staging system: Stage 0, Stage I, Stage II, Stage III and Stage IV.

Table 2. 2: Anatomic stage groups of breast cancer

Stages		Definition
Stage 0		Ductal Carcinoma In Situ
Stage I	IA	Primary invasive tumour with a size of ≤20 mm No nodal involvement
	IB	Nodal micro-metastases (>0.2 mm, <2.0 mm) with or without ≤20 mm primary tumour
Stage II	IIA	Movable ipsilateral Level I, II lymph node metastases with ≤20 mm primary tumour; Or > 20 mm, ≤50 mm tumour with no nodal involvement
	IIB	Movable ipsilateral Level I, II lymph node metastases with >20 mm, ≤50 mm tumour; Or > 50 mm tumour with no nodal involvement
Stage III	IIIA	Movable ipsilateral Level I, II lymph node metastases with >50 mm tumour; Or any size primary tumour with fixed ipsilateral Level I, II or internal lymph node metastases
	IIIB	Primary tumour with chest wall and/or skin invasion
	IIIC	Any size primary tumour with supraclavicular or ipsilateral Level III lymph node metastases; Or with ipsilateral Level I, II and internal lymph node metastases
Stage IV		Any case with distant organ metastasis

Note: Lobular carcinoma in situ is now considered benign thus removed from the breast cancer staging system

Source*:AJCC Cancer Staging Manual, 2010. Eighth Edition, the American College of Surgeons (ACS), Chicago, IL, USA. With reprint permission of ACS*

2.8 Receptor Activator of Nuclear Factor Kappa-β Ligand (RANKL)

Receptor activator of nuclear factor kappa-B ligand (RANKL) was discovered in the year 1997 as a member of tumour necrosis factor receptor superfamily (TNRSF11), it is also known as TNF-related activation-induced cytokine (TRANCE), Osteoprotegerin ligand (OPGL) (Nelson *et al*., 2012) and osteoclastic differentiation factor (ODF)(Ono *et al*., 2020). It was discovered by four different independent groups. While two groups described it (RANKL) as an key factor in T cell and dendritic cell biology (Bissell and Hines, 2011; Wada*et al*., 2006), the other two groups believed it to be an important factor involved in inducing osteoclast differentiation and activation in vitro (Molon *et al*., 2016;Plaks *et al*., 2015;Slavic *et al*., 2018).

The initial confusion was solved by studies on knockout mice which provided essential proof that RANKL plays a vital role in bone physiology in vivo (Slavic *et al*., 2018). RANK, which is also known as TRANCE-R or TNFRSF11A was discovered in 1997 as receptor expressed on T cells regulating the interaction between T cells and dendritic cells via its ligand RANKL (Nelson *et al*., 2012). It was until two years later that RANK was recognized as the receptor that facilitates RANKL-induced osteoclast activation and differentiation (Boyce *et al*., 2015). The third molecule OPG, which was discovered earlier in 1996, acts as a molecular decoy receptor for RANKL (Slavic *et al*., 2018). The name osteoprotegerin (OPG) means ‘‘protector of the bone’’ describing a key in vivo function, which is the inhibition of osteoclast development and activation and thereby inhibition of bone loss (Okamoto, 2021)).

RANKL is a homo-trimeric type II membrane protein with no signal peptide and exist in three isoforms due to alternative splicing of the same gene (Ikeda *et al*., 2001). Among these isoforms, RANKL1 is the full length RANKL, RANKL2 is a shorter form of RANKL1 in which a part of the intra-cytoplasmic domain is missing and RANKL 3 is a soluble form of

RANKL, with the N-terminal part of the amino acids removed (Ikeda *et al*., 2001). A soluble RANKL is as a result of cleavage from membrane-RANKL induced by various enzymes such as the metalloproteinase, dis-integrin, and TNF-α converting enzyme (TACE) (Lum *et al*., 1999) or ADAM-10, MMP-7, MMP-14 (Hikita *et al*., 2006; Georges *et al*., 2009). RANKL is expressed by a wide variety of tissues such as the brain, skin, intestine, skeletal muscle, kidney, liver, lung and mammary tissue, but is more highly expressed in bone tissue (Infante*et al*., 2019), lymphoid organs and the vascular system (Collin-Osdoby *et al*., 2001). The predominant function of RANKL is the control of bone remodelling. Certainly, RANKL efficiently regulates the bone resorption process by stimulating osteoclast differentiation and osteoclast survival (Mada *et al*., 2017).

2.8.1 RANKL couple sex hormones to mammary stem cells

The mammary gland is organized into two main cell types, namely the luminal and myo-epithelial lineage. Luminal cells can be subdivided into ductal and alveolar cells and are mainly responsible for the mammary secretion of fluids and nutrients. Myo-epithelial lineage cells are also referred to as basal cells because they are located adjacent to the basement membrane and can exert contractile functions, thereby guiding the milk through the epithelial tree (Fu *et al*., 2014). While it was thought for a long time that mammary progenitors reside in a quiescent stem cell niche, it has become clear that mammary progenitor cells undergo proliferation and differentiation during each oestrus cycle (Joshi *et al*., 2015; Asselin-Labat *et al*., 2010). Moreover, mammary stem cell numbers change during the course of each oestrus cycle, during pregnancy as well as during ageing, thereby allowing the mammary gland to adapt to altered physiological states (Joshi *et al*., 2015; Asselin-Labat *et al*., 2010).

Mammary stem cells are highly enriched in a basal epithelial population, self-renew and are able to generate all mature cell types of the mammary gland; a single mammary stem cell is

able to reconstitute a fully functional mammary gland, which can even undergo further development and milk production during pregnancy (Shackleton *et al*., 2006; Fu *et al*., 2014). Although the mammary stem cell enriched subsets in mouse and human lack expression of oestrogen and progesterone receptors, these cells are highly responsive to steroid sex hormones. During the oestrous phase of each cycle, progesterone induces the expansion of mammary stem cells through paracrine mechanisms (Yu-Jia *et al*., 2015). Similarly, administration of exogenous oestrogen and progesterone also increases the numbers and repopulation capacities of mammary stem cells. By contrast, ovariectomy or treatment with aromatase inhibitors significantly reduces mammary stem cell activity also during pregnancy a dramatic increase in mammary stem cell numbers as well as enhanced repopulation capacity can be observed (Joshi *et al*., 2015; Recouvreux*et al*., 2020).

2.8.2 RANKL and the mammary gland

One of the most surprising phenotypes that was discovered in the RANKL knockout mice, is the key role in mammary gland biology. Whereas mammary glands after birth and during puberty are normal in these mutant mice, deletion of *Rank* or *Rankl* results in a complete block of mammary gland development during pregnancy (Rao, *et al*., 2018). As a consequence, mice in which Rank or Rankl is genetically inactivated display a severe lactation defect resulting in the death of a new born. Mammary gland development follows a series of well-defined processes (Chen*et al*., 2019). At birth small mammary gland anlagen are present which expand throughout the fat pad during puberty forming new branches and end buds. Thereby mammary gland development strongly depends on the sex hormone oestrogen. At mid-pregnancy the formation of a lactating mammary gland is initiated. The establishment of a lactating mammary gland is regulated by a hormonal regulatory mechanism and depends mainly on progesterone, prolactin and parathyroid hormone related

peptide (PTHrP)(Rao, *et al*., 2018).. Most importantly, progesterone drives proliferation, further expansion and differentiation of mammary epithelial cells into lobulo-alveolar milk-secreting end bunds (Chen *et al*., 2019). Rankl mutant females undergo normal mammary gland development during adolescence, however they display a complete block in the development of a lactating mammary gland (Rao, *et al*., 2018).

Transplantation of Rankl deficient mammary epithelium into SCID mice did show that the defect was caused by a cell-autonomous effect since the development of a lactating mammary gland was still arrested in the Rankl sufficient recipients. However local injection of recombinant Rankl could restore lactation in Rankl deficient females (Chen *et al*., 2019). Rank mRNA is constitutively expressed in low levels in the mammary gland of non-pregnant and pregnant females (Rao, *et al*., 2018).

However, during pregnancy Rank protein not mRNA levels are massively increased, starting around pregnancy day 15.5 (P15.5). After day 1 of lactation Rank protein is decreased to normal levels again. By contrast, Rankl expression underlies strong fluctuations during the oestrous cycle in non-pregnant mice (Rajaram*et al*., 2015). In general high Rankl expression can be detected during the oestrous phase whereas RANKL expression cannot be detected during pre-oestrous. Importantly RANKL expression during the oestrous cycle correlates with progesterone receptor expression (Rajaram*et al*., 2015). During pregnancy Rankl mRNA expression is strongly up-regulated starting at about pregnancy day 12.5 (P12.5) and decreases again around pregnancy day 19.5 (Chen *et al*., 2019). Functionally, Rankl induces expansion of mammary stem cells during the oestrous cycle (Asselin-Labat *et al*., 2010; Joshi *et al*., 2015).

Rankl expression in the mammary gland is not only very tightly controlled temporally but also spatially(Rao, *et al*., 2018). Whereas RANKL protein is mainly localized in luminal

mammary epithelial cells RANK protein is mainly detected in basal mammary epithelial cells (Asselin-Labat *et al*., 2010; Joshi *et al*., 2015).

This phenotype results from increased proliferation of mammary epithelial cells mediated by RANK/RANKL leading to impaired differentiation of lobulo-alveolar structures(Rao, *et al*., 2018). Moreover, due to constitutive overexpression of RANK in the mammary gland these mice develop hyperplasia at advanced age. Forced expression of RANKL also induces ductal side-branching and the formation of lobulo-alveolar structures in nulliparous female mice, independent of any pregnancy hormones (Biswas*et al*., 2022).

These studies clearly emphasize the importance of a very tight spatial and temporal control of Rank and Rankl expression in mammary gland biology. Most importantly, Rankl mutant mice exhibit a complete defect in formation of a lactating mammary gland in pregnancy (Biswas*et al*., 2022; Schramek *et al*., 2010). The lactation defect observed in Rankl and Rank null mice most closely resembles the lactation defect observed in progesterone receptor B (PRB) knockout mice (Biswas*et al*., 2022). PRB knockout mice not only fail to develop lobulo-alveolar structures during pregnancy but also exhibit decreased RANKL expression. Most importantly, ectopic RANKL expression can rescue the defect in ductal side branching and lobulo-alveolar differentiation of mammary epithelial cells during pregnancy (Biswas*et al*., 2022).

Thus RANKL is a critical regulator of PR action in mammary epithelial cells and controls morphogenetic changes induced by progesterone. Recent work has shown that PR induces ELF5 via RANKL in order to mediate the expansion of hormone receptor negative mammary epithelial cells (Lee *et al*., 2013). The detailed mechanism is still unknown and requires further research. However, it has become clear that RANKL is a crucial downstream molecule in progesterone receptor signalling. Besides progesterone, prolactin is essential for

the development of a functional lactating mammary gland during pregnancy. Similar to PRB-knockout mice prolactin (PRL) and prolactin receptor (PRLR) knockout mice fail to develop milk secreting lobulo-alveolar structures during pregnancy and exhibit decreased RANKL expression in mammary epithelial cells (Onji, M. and Penninger, 2023). Mechanically, PRL mediates proliferation of mammary epithelial cells via the induction of STAT5 and RANKL (Sigl*et al*., 2016).

Accordingly, STAT5 knockout mice also display a block in the formation of lobulo-alveolar structures during pregnancy (Sigl*et al*., 2016). Besides PR-B, PRL and PRLR knockout also mice lacking, NF-kB kinase subunit alpha (IKKa, CHUCK), Inhibitor of DNA binding protein 2 (Id2) or cyclin D1 (CCND1) also show a mammary gland phenotype similar to RANKL knockout mice (Onji, M. and Penninger, 2023). In all of these mutant mice, mammary glands develop normally during puberty, but fail to develop a functional lactating mammary gland during pregnancy. Detailed analysis of the response of mammary epithelial cells to a hormonal stimulus has shown that progesterone induced proliferation occurs in two different phases (Beleut *et al*., 2010). The immediate phase of proliferation takes place within the first 24 h after a progesterone stimulus and depends on the cell cycle progression protein cyclin D1. The second phase of proliferation continues for 8 days and is independent of cyclin D1 but depends on RANKL-induced proliferation (Beleut *et al*., 2010).

The lactation defect of IKKa knock-in mice in which the kinase function of IKKa has been deactivated can be rescued with forced expression of cyclin D1 (Cao *et al*., 2001). RANKL therefore induce differentiation and proliferation of mammary epithelial cells via the NF-κB-cyclin D1 axis. It is also worth mentioning that IKKa kinase dead mice display a delayed onset of NeuT-dependent and progestin-induced mammary tumours (Cao *et al*., 2007).

2.8.3 RANKL and its function in mammary stem cells

RANKL also play an important role in mammary stem cell biology. For a long time mammary stem cells have been thought to be quiescent and only active during development and pregnancy. However recently it was recognized that mammary stem cells are actively cycling during the whole reproductive period of a woman and their numbers change during aging, pregnancy and the menstrual cycle (Asselin-Labat *et al*., 2010; Joshi *et al*., 2015). High progesterone levels during the luteal phase of the reproductive cycle serve to prepare the female body for a potential pregnancy. As a consequence the mammary gland undergoes pregnancy-like development during each oestrous cycle in response to oestrogen (Silberstein *et al*., 2006).

Mammary stem cells reside in the basal cell compartment and are defined by their ability to repopulate a whole functional epithelial mammary Tree (Shackleton*et al*., 2006; Sting *et al*., 2006; Van Keymeulen *et al*., 2011). During mouse diestrous, which is comparable to the human luteal phase, CD24+CD49fhi basal mammary stem cells are markedly expanded and the mammary gland undergoes alveologenesis (Asselin-Labat *et al*., 2010; Joshi *et al*., 2015). Similarly, there are 11 times more numbers of mammary stem cells during pregnancy of a mouse. In contrast, mammary stem cells are strongly reduced in ovariectomized mice (Asselin-Labat *et al*., 2010; Joshi *et al*., 2015). In all these situations, RANKL play important roles, relaying the signal from the sex hormone progesterone to the mammary epithelium. Pregnancy as well as progesterone treatment of non-pregnant female mice leads to up-regulation of RANK protein expression mainly in basal mammary epithelial cells and RANKL expression in luminal mammary epithelial cells (Asselin-Labat *et al*., 2010; Joshi *et al*., 2015).

Although the exact mechanism is not fully understood yet, all data so far suggest that RANK and RANKL control the expansion of mammary stem cells during pregnancy and the menstrual cycle (Asselin-Labat *et al*., 2010; Joshi *et al*., 2015). This is further supported by the fact that blocking RANKL systemically in pregnant mice results in a decreased capacity of mammary stem cells to form colonies in vitro (Joshi *et al*., 2015). Moreover, deleting RANK specifically in basal mammary epithelial cells recapitulates the lactation phenotype observed in full body RANK and RANKL knockout mice; by contrast, mammary gland development during pregnancy is not impaired when RANK is deleted specifically in luminal mammary epithelial cells (Schramek *et al*., 2010).

2.8.4 RANKL in Breast Cancer

In 2003 the Million Women Study and the Women's Health Initiative Study showed that women who received oestrogen plus progesterone hormone replacement therapy (known as combined HRT) have a significantly higher risk of developing breast cancer compared to women who only received oestrogen (Desantis *et al*., 2014).For increased breast cancer incidence, which was further confirmed by an extended post- intervention follow-up 10 year later (Manson *et al*., 2013). RANKL may be a useful biomarker to identify subgroups at high risk of breast cancer. Indeed, increased progesterone and RANKL serum levels stratify a subgroup of postmenopausal women, without known genetic predispositions, who exhibit a fivefold increased risk of developing breast cancer 12–24 months before cancer diagnosis (Kiechl *et al*., 2017). Similarly, higher concentrations of soluble RANKL are positively associated with an increased risk of oestrogen receptor positive ER+ but not ER- breast cancer (Sarink *et al*., 2017). Moreover, a recent clinical study revealed that RANK expression is increased in hormone receptor negative breast cancer and correlates with a worse recurrence-free survival and risk of bone metastasis (Vidula *et al*., 2017).

RANKL expression is regulated by progesterone in the mammary gland, and recent studies have revealed that RANKL/RANK is also involved in the development of sex hormone-driven breast cancer. In a hormone-induced mouse model the synthetic progestin, medroxy-progesterone acetate (MPA), drives proliferation of the mammary epithelium, whereas 7, 12-dimethylbenzanthracene (DMBA) administration causes gene mutations that together result in the formation of mammary adenocarcinomas (Vidula *et al*., 2017). Administration of MPA resulted in a 3000-fold up-regulation of RANKL in mammary epithelial cells (Schramek *et al*., 2010). Moreover, deletion of Rank as well as deletion of Ikkα, that encodes a downstream

regulator of the RANK signalling pathway, In mammary epithelial cells dramatically delayed MPA/DMBA induced mammary tumour development, indicating that the RANK/RANKL pathway relays signals through IKKα to drive progestin-driven mammary cancer (Park*et al*., 2017). In addition to coupling to mammary progenitors, RANK/RANKL confer resistance to γ-irradiation induced cell death in mammary epithelial cells, change cell adhesion, and regulate self-renewal capacity of tumour stem cells, all of which could contribute to breast cancer development (Park*et al*., 2017).

Importantly, selective pharmacological inhibition of RANKL using RANK-Fc could attenuate breast tumour progression not only in hormone and carcinogen driven mouse models but also in a transgenic spontaneous tumour model (Rao, *et al*., 2018), suggesting that RANKL inhibition could be a new weapon against breast cancer (Koch, 2011). Because RANK and RANKL control the onset of hormone induced mammary cancer, it offers a new and, based on its evolutionary role, rational avenue for therapeutic intervention. Ina multi-centre, randomized, double-blind, and placebo-controlled trial carried out in Austria and Sweden over 6years, it was found that breast cancer patients receiving aromatase inhibitor therapy and treated with denosumab had a significantly delayed onset for the first clinical fracture and an overall lower number of fractures without severe adverse events (Gnant *et al*., 2015). Therefore, targeting the RANKL/RANK pathway has already benefited thousands of breast cancer patients. Both studies provided strong population based evidence that progesterone is a crucial risk factor (Rao, *et al*., 2018).

2.9 Nuclear factor kappa-B (NF- κB)

The nuclear factor-κB (NF- κB) is a family of ubiquitously expressed transcription factors which consist of five mammalian members: p65 (relA), c-rel, relB, p50 (NF- κB1) and p52 (NF-κB2) (Chen and Greene, 2004). The family members are characterized by the presence

of a conserved 300 amino acid N-terminal Rel homology domain (RHD) (homologous to that encoded by the avian oncogene, vRel), which is responsible for dimerization, association with IκB inhibitory proteins, sequence specific DNA binding and translocation (Ghosh *et al*., 2012). The C-terminal regions of these proteins have domains responsible for either transcriptional activation (relA, c-rel and relB) or the inhibition of Rel protein activity (p105 and p100). The p105 and p100 proteins can be processed by proteolytic cleavage into p50 and p52, respectively. These proteins have Glycine rich regions (GRRs) which are important for this processing (Espinoza-Sánchez*et al*., 2019). The Rel family members are capable of forming different combinations of heterodimers and homodimers, the most common being the p65/p50 heterodimer which is often referred to as the NF-κB complex (Radhakrishnan and Kamalakaran, 2006). In resting cells NF-κB is present in the cytoplasm bound to one or more members of the IκB protein family (IκBα, IκBB, IκBγ, IκBε, Bcl-3, and the precursor Rel proteins p100 and p105). Various cell stimuli (e.g. TNFα, CD40 ligand, IL-1, LPS, TRANCE, EGF, phorbol esters, peroxides, ionizing radiation) induce cytoplasmic phosphorylation (via activation of the IκB kinase complex IKK) and subsequent proteasomal degradation of IκB inhibitory proteins, activating NF-κB for translocation into the nucleus where it binds promoter-specific κB consensus elements and regulates the transcription of NF-κB-dependent genes (Espinoza-Sánchez*et al*., 2019).

While phosphorylation and degradation of IκB inhibitory proteins are considered the rate-limiting if not obligate mechanisms by which NF-κB is activated, novel IKK-independent pathways leading to IκB proteasomal degradation as well as NF-κB phosphorylating kinases are now known that can also activate NF-κB (Espinoza-Sánchez*et al*., 2019). Most activated forms of NF-κB stimulate gene transcription, although specific NF-κB subunits lack transactivation domains; thus, activation and nuclear translocation of p50/p50 and p52/p52 homo-dimers result in repression of NF-κB dependent genes (Ghosh *et al*., 2012). Curiously,

when either the NF-κB p50 or p52 products of the p105 and p100 Rel precursor proteins are bound to the oncogenic and non-inhibitory IκB family member, Bcl-3, become transcriptionally competent and stimulate expression of NF-κB-dependent genes (Ghosh *et al*., 2012).

2.9.1 Nuclear factor kappa-Betta (NF- κB) and Breast cancer

The latest findings that NF-κB is required for normal mammary gland development, especially for the increased proliferation of epithelial cells during pregnancy, has strengthened the case for its possible role in mammary tumourigenesis (Sun*et al*., 2017). Several studies have reported increased NF-κB DNA-binding activity in both mammary carcinoma cell lines and primary human breast cancer tissues (Sun*et al*., 2017; Baldwin, 2012; Cogswell*et al*., 2000). Moreover, elevated IKK kinase activity has been seen in transformed breast cancer cell lines as well as in primary human breast cancer specimens, and inhibition of IKK activity decreased NF-κB activity in tumour cell lines (Romieu-Moure*et al*., 2001). Tumours induced in rat mammary using the carcinogen DMBA (7,12-dimethylbenzaanthracene) also display high levels of nuclear NF-κB binding activity (Baldwin, 2012). Increased NF-κB binding was detected in the mammary glands of rats treated with DMBA even before tumours were detectable, signifying that NF-κB activation plays an initial and critical role in chemical mammary carcinogenesis (Kim*et al*., 2000). The progression of rat mammary carcinoma cell line RM22-F5 from an oestrogen receptor (ER)-positive to an ER-negative state was found to go along with constitutive activation of NF-κB (Papademetrio *et al*., 2016).

A dominant negative mutation in IKKBblocked activation of NF-κB in ER-negative mouse mammary epithelial tumour cells, thus decreasing their tumourigenic potential while elevating their sensitivity to apoptosis-inducing anticancer drugs (Biswas *et al*., 2022). The

inhibitory effects of the transforming growth factor (TGF)-*B*1 on mammary tumour cell growth seem to be mediated via inhibition of the aberrantly activated NF-κB activity, while overexpression of c-Rel or RelA prevented this effect (Baldwin, 2012). However, it is clear that NF-κB activation is linked with promotion of cell growth in mammary tumours, and inhibition of NF-κB possibly offers an effective way to treat breast cancers(Papademetrio *et al*., 2016).

It is not clear, though, whether there is a difference in the composition of NF-κB dimers between normal mammary epithelial cells and mammary carcinomas. Despite the fact that normal mammary epithelial cells primarily express RelA and p50 and very little p52, high levels of NF-κB2 polypeptides (both p100 and p52) were observed in mammary carcinoma cell lines and primary tumours (Biswas *et al*., 2022; Cogswell*et al*., 2000). Bcl-3, a unique member of the IκB family that play a role as a co-activator of transcription, is greatly expressed in human breast tumour tissue, where it was found to be associated with p52:p52 homo-dimers to induce G1 to S phase transition by trans-activating the cyclin D1 gene (Westerheide *et al*., 2001). NF-κB2/p100 furthermore interacts with RelB in other cell types (Smith*et al*., 2014), and increased expression of RelB has been detected in primary breast carcinomas (Cogswell*et al*., 2000). Changes in the composition of NF-κB family members in tumour tissues suggest that the expression profile of NF-κB target genes may also be different from that of normal tissue. Apart from cyclin D1, a number of other genes were known to be NF-κB dependent in mammary tumours, including c-myc, MUC1 (transmembrane mucin glycoprotein overexpressed in human breast cancers), Ephrin A1 (membrane-bound ligand for the tyrosine kinase receptor EphA2, involved in the regulation of cell adhesion, cell proliferation, and tumour angiogenesis), Caveolin-1 (a member of the caveolin family, regulator of various intracellular signalling pathways) and others (Kim*et al*., 2000; Lagow and Carson, 2002; Deregowski *et al*., 2002).

Regardless of all the indications that suggest NF-κB to be implicated in breast cancer, it is still unclear whether constitutively activated NF-κB plays a relevant role in tumour formation. The mechanisms of NF-κB activation in mammary tumours are also not clear. As in other types of cancer, tumour cells originate from normal cells and often retain tissue-specific signalling pathways that may be somehow dys-regulated. It might be possible that the IKK*α*-NF-κB-cyclinD1 signalling pathway described above becomes aberrantly up-regulated during the oncogenic transformation process. In vitro studies have previously shown that ErbB2 and Ha-Ras can trigger signalling cascades that result in NF-κB activation during their involvement in cellular transformation (Papademetrio *et al*., 2016; Jo*et al*., 2000; Pianetti*et al*., 2001; Zhou*et al*., 2000). Studies with cell lines derived from mice carrying MMTV-driven neu (ErbB2), heregulin/NDF, TGF*α*, v-Ha-ras, and c-myc transgenes have revealed that certain oncogenes require and activate specific signal transduction pathways, such as Erk/MAP kinase, JNK/SAP kinase, PI3K/Akt kinase, protein kinase C or the Src related kinase pathways (Papademetrio *et al*., 2016). Between the five oncogenes assessed, heregulin led to highest activation of NF-κB via phosphorylation of EGFR and ErbB3 but not ErbB2/neu, while neu potentiated TNF*α* inducible NF-κB activation and c-myc potentiated TPA inducible NF-κB activation (Bhat-Nakshatri *et al*., 2002).

The potential importance of NF-κB in mammary tumourigenesis is further underscored by its ability to activate the cyclin D1 gene (Figure 2.5), another common marker for breast cancer. The cyclin D1 promoter was found to contain NF-κB binding sites, and in vitro experiments demonstrated its activation by NF-κB (Sun, 2017; Tyagi and Patro, 2019). There is also a good correlation between elevated nuclear NF-κB activity in breast tumours and higher levels of cyclin D1 mRNA (Cogswell*et al*., 2000). Importantly, transgenic overexpression of cyclin D1 in the mammary gland was shown to increase the incidence of mammary carcinomas (Tyagi and Patro, 2019), and ablation of cyclin D1 gene protects mice against breast cancers

induced by MMTV-neu or MMTV-v-Ha-ras (Yu *et al*., 2001). The cyclin D1 promoter contains binding sites for several transcription factors (Eto, 2000). Multiple signalling pathways have been shown to activate cyclin D1 in mammary epithelia, including MAPK/AP1, Wnt/*B*-catenin, Jak/Stat5, and src (Fig. 2). The crosstalk between these pathways is of great interest in understanding the mechanism underlying mammary epithelial transformation.

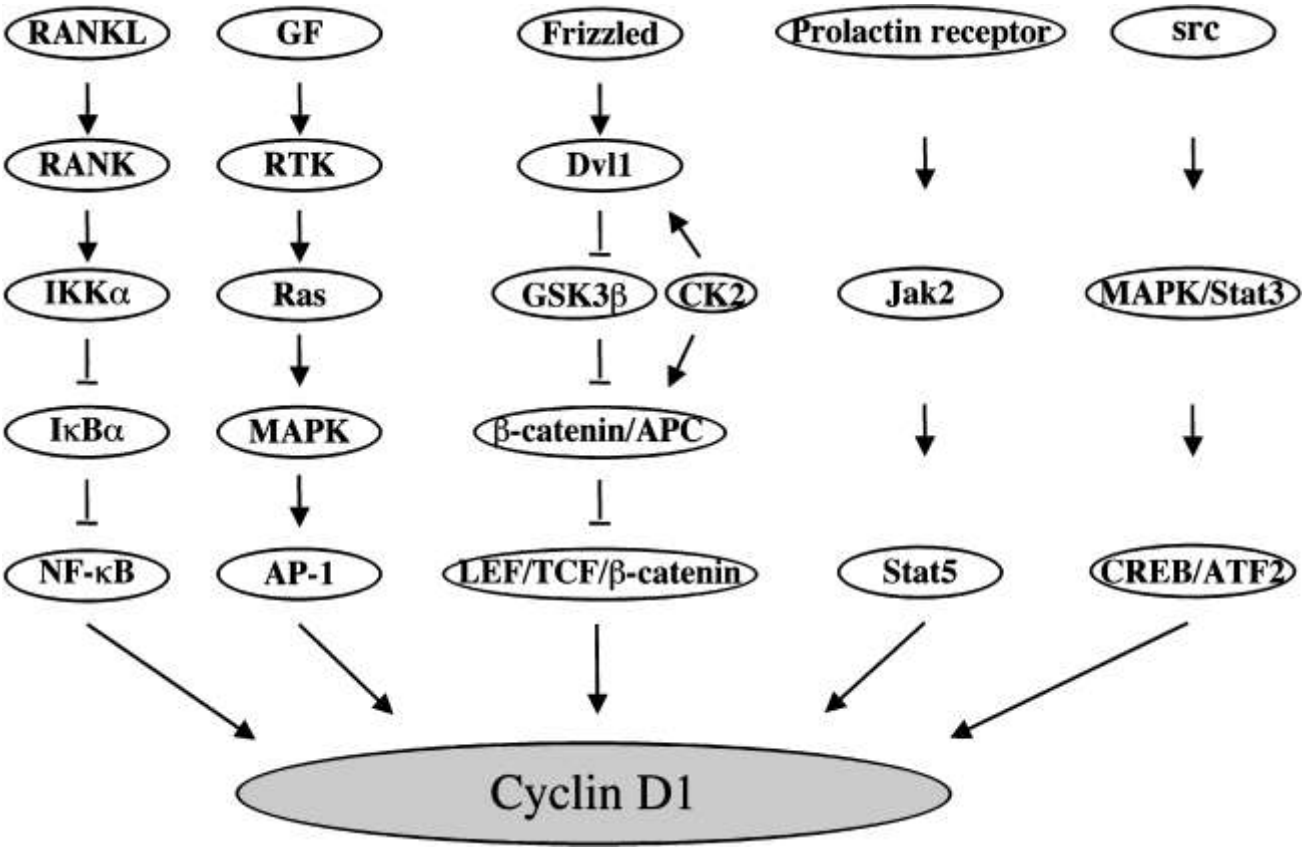

Figure 2. 5: Cyclin D1 gene is activated by multiple pathways in mammary epithelial cells

One pathway is initiated by binding of RANKL to its receptor RANK, which leads to activation of NF-κB via IKK*α*. IKK*α* activation is required for IκB*α* degradation and NF-κB (RelA: p50) translocation. This pathway is specifically activated during pregnancy by pregnancy hormones. The second pathway, which leads to activation of AP-1, is initiated by binding of growth factors to receptor tyrosine kinases (RTK). This signalling pathway is mediated through Ras and mitogen-activated protein kinase (MAPK) and JNK cascades. Wnt1 or Wnt10b bind to Frizzled to activate Dvl1 (mouse homolog of dishevelled) which inhibits the activity of GSK3*B*(glycogen synthase kinase 3*B*). Inactivation of GSK3*B*inhibits phosphorylation of *B*-catenin so that *B*-catenin cannot be degraded and translocate into the nucleus to activate cyclin D1 in a complex with LEF/TCF transcription factors. Other pathways include prolactin receptor/Jak/Stat pathway and src/ATF pathway, etc.

2.10 Oxidative stress and breast cancer

The generation of reactive oxygen and nitrogen species unrestrained, and subsequent oxidative stress, has been implicated in the pathogenesis of many chronic diseases, including cancer, diabetes, and cardiovascular disease, as well as aging in general (Sies*et al*., 2017; Flohé, 2020). Oxidative stress can be broadly defined as an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage (Sies, 2017). If the level of reactive species is high and overcomes the antioxidant defence mechanisms of the human body, oxidative damage can occur to lipids, proteins, or directly to DNA (Sies, 2017; Lee*et al*., 2017). DNA damage is hypothesized to play an important role in the initiation of carcinogenesis(Lee*et al*., 2017).

Oxidative stress mechanisms are also involved in the activation of cell signalling pathways, including tumour cell proliferation, increased tumour cell migration, and increased tumour cell pro-angiogenic factors, and play a key role in apoptosis, mechanisms that can impact both cancer progression and metastasis (Gorrini*et al*., 2013; Lee*et al*., 2017). Increased reactive oxygen species (ROS) and the resulting high oxidative stress are key characteristics of malignant tumours(Lee*et al*., 2017).

Biomarkers of oxidative stress have been investigated for their association with the development and progression of several cancer types, and in particular breast cancer, as oxidative stress mechanisms may be involved in several known breast cancer risk factors, including obesity and daily alcohol intake, and circulating oestrogen levels (Kangari *et al*., 2018).Breast cancer cells have been shown to be susceptible to oxidative damage and have high levels of oxidative stress, including protein damage, DNA damage, and lipid peroxidation. Furthermore, several breast cancer risk factors may alter levels of endogenous oxidative stress (Lee*et al*., 2017; Dorjgochoo*et al*., 2011).

Associations between oxidative stress biomarkers and breast cancer risk were inconsistent across studies, with evidence for both positive and inverse associations depending on the biomarkers evaluated and/or menopausal status. Two prospective cohort studies with measurement of oxidative stress biomarkers before breast cancer diagnosis reported inverse associations with breast cancer risk among premenopausal women only (Lee*et al*., 2017).Some studies have reported that higher levels of oxidative stress are associated with obesity and adipose tissue. The finding of higher levels of oxidative stress and increased risk of postmenopausal breast cancer could reflect the known obesity and postmenopausal breast cancer association (Munsell*et al*., 2014; Lee*et al*., 2017).

One key limitation that may contribute to the inconsistent results observed across studies is the timing of sample collection. Several studies collected samples after diagnosis of breast cancer, with some after surgery and/or during cancer treatment (such as chemotherapy). Levels of oxidative stress may change based on the presence and progression of the tumour itself and due to cancer treatments, including surgery, radiotherapy, and chemotherapy (Lee*et al*., 2017; Vera-Ramirez*et al*., 2011). Another limitation is that breast cancer has been shown to be an etiologically heterogeneous disease, with studies showing modification of known associations by tumour subtype (Lambertini*et al*., 2016; Munsell*et al*., 2014).

CHAPTER THREE

Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

All chemicals, reagents and assay kits used were of analytical and molecular grade and purchased from known reputable companies, RANKL ELISA Kit (Wuhan Fine Biotech Co., Ltd, China Batch No. H0313F033), NF-κB ELISA Kit (Wuhan Fine Biotech Co., Ltd, China. Batch No. H1950F033), 10% Trichloroacetic acid (TCA), phosphate buffer (0.2M, pH 8.0), Ellman's reagent (Sigma-Aldrich, Germany. Product NO. D-8130) 0.6% (w/v)Thiobarbituric acid (Sigma-Aldrich, Germany. Product NO. T-5500)

3.1.2 Equipment

Validated semi-structured questionnaire**,** plain tubes, Micropipette, cuvette, centrifuge (MPW-223e), water-bath (Thermo scientific, TSGP28), spectrophotometer (20D techmel & Techmel USA), Microtitre plate and reader (Wincom DNM-9602 China)

3.1.3 Subjects

All postmenopausal breast cancer patients aged 46 – 80 years with histologically confirmed breast cancer attending surgical outpatient clinic ABUTH Between June 2019 to December 2020 were recruited.

3.2 Methods

3.2.1 Study design

This study was cross-sectional designed to evaluate serum levels of RANKL, NF-κB and oxidative stress biomarkers among postmenopausal breast cancer women attending surgical outpatient clinic Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, Nigeria. Blood samples were collected from consenting women with histologically confirmed breast cancer. The flowchart of the study is shown in Figure 3.1.

3.2.2 Ethical clearance

Ethical approval (ABUTHZ/HREC/W41/2020) was obtained from the Health Research and Ethics Committee of the ABUTH, Zaria, in accordance with the Helsinki Declaration, and a voluntarily written and/or verbal informed consent was obtained from each participant before recruitment into the study

3.2.3 Exclusion criteria

1. Female breast cancer patients diagnosed with bone disorders, cardiovascular diseases, HIV, hypertension, diabetes mellitus, or other forms of cancers were excluded.

2. Also female breast cancer patients that are habitually alcoholics and cigarette smokers were excluded.

3. Female breast cancer patients with induced menopause and those aged 46 years but still menstruating were also excluded from the study

4. Female breast cancer patients outside the age bracket of 46-80 years were also excluded.

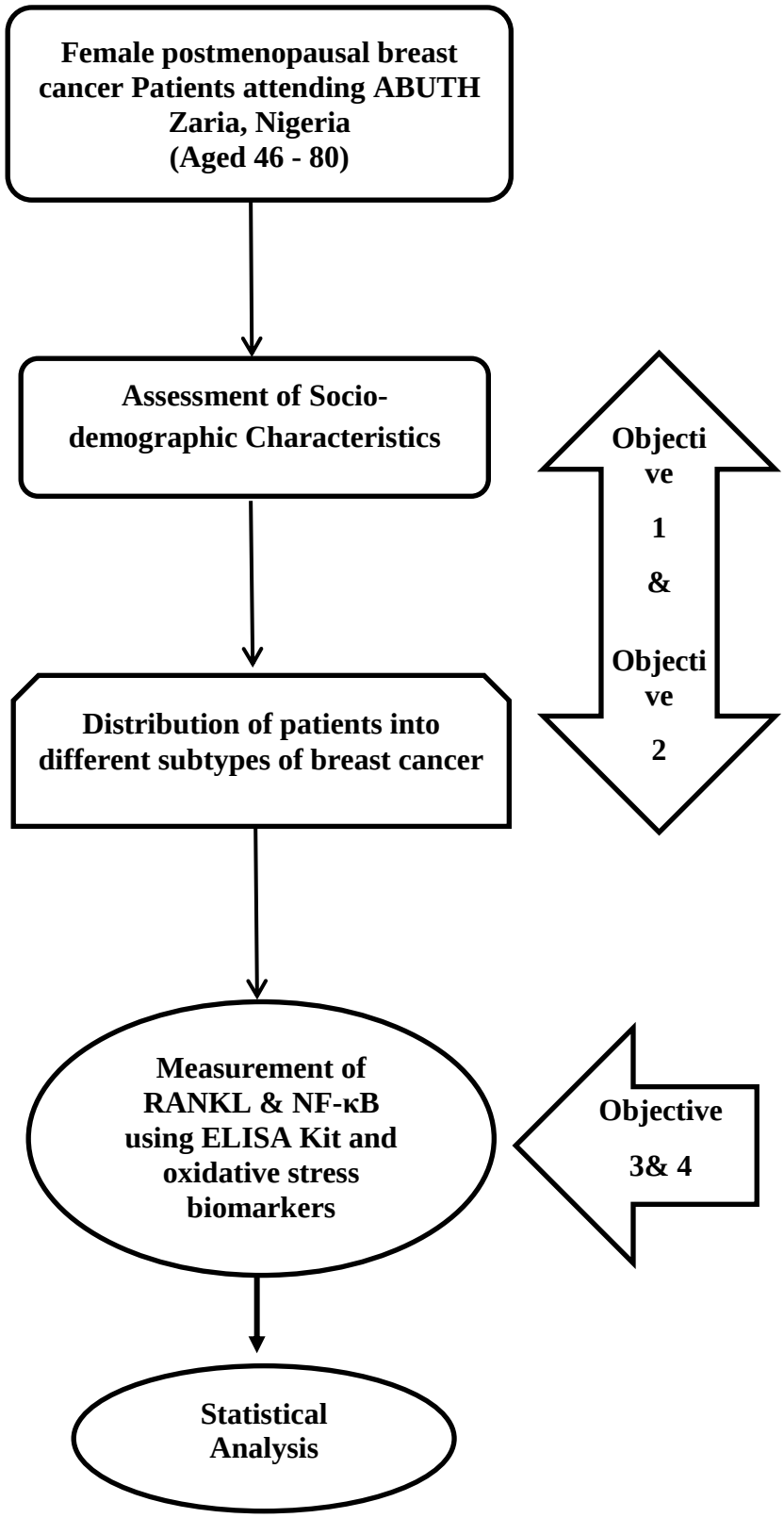

Figure 3. 1: Experimental flowchart of the study

3.2.4 Sample size

The sample size was calculated using the Dobson's formula

$$n = \frac{z^2 (p \times q)}{d^2}$$

Where n= sample size,

z= Confidence interval at 95% (1.96),

p= Prevalence rate (10.4%) as reported by Agbo *et al*. (2014),

q= 1 – p (1 – 0.104= 0.896),

d= Desired level of significance (0.05)

Substituting and adjusting for an attrition rate of 10%.

Sample size (n) = 158

3.2.5 Sampling technique

Convenience sampling was used for selection; targeting postmenopausal breast cancer patients attending surgical outpatient clinic, ABUTH, Zaria.

3.2.6 Data collection

3.2.6.1 Demographic data collection

A validated semi-structured questionnaire containing three section was administered to consented postmenopausal breast cancer women by interview so as to collect data on socio-demographic characteristics, and medical/clinical parameters (Appendix)

3.2.6.2 Patient Distribution

The selected breast cancer patients weredistributed into four different categories (luminal A, luminal B, HER2-enriched, triple negative) based on their immunohistochemistry results history. The patients' medical records including their pathology reports were used in accessing their clinical parameters.

3.2.6.3 Blood sample collection

About 5ml of blood sample from each participating patient was collected separately into sterilized dry plain tubes by a trained phlebotomist using vein puncture and allowed to clot for about 30 minutes. It was then centrifuged at 3500×g100 for 15 minutes and the serum obtained was stored in the freezer at -20° until further analysis.

3.2.6.4 Measurement of Serum RANKL concentration

RANKL concentration was determined in serum samples using a commercially available ELISA Kit (Wuhan Fine Biotech Co., Ltd, China Batch No. H0313F033) according to Manufacturer's instructions.

The assay utilizes "sandwich" ELISA technique. All reagents and samples were allowed to equilibrate to room temperature (18-26^{0}C). The positions of STD/SAMPLE/CTRL (Standards/Sample/Control) were marked on a protocol sheet. Each microtiter well was washed 2 times by dispensing 250µL of diluted Wash buffer. After the final washing step the residual buffer was removed by tapping the plate on absorbent paper. Exactly 100µl of the prepared standard was allotted into the standard wells while 100µl of sample dilution buffer was added into the blank well and 100µl of the sample were properly added into the test sample wells. The plate was sealed with a cover and incubated at 37°C for 90 minutes. After incubation the content of the plate were discarded and washed two times with wash buffer, and then 100µl of biotin-labelled antibody was added into the wells, the plate was then covered and incubated for 60 minutes at 37°C. Later the content of the plate was discarded and washed three times with a wash buffer, then 100µl of HRP-streptavidin conjugate

(SABC) working solution was pipetted into each well, which is then covered and incubated at 37°C for 30 minutes. After the incubation the plate was washed with a wash buffer 5 times, allowing the wash buffer to stay into the well for 2 minute each time. After which 90µl of TMB substrate was added into each well, the plate was sealed and incubated at 37°C in the dark 15 minutes. Then 50µl of acidic stop solution was added into each well. The colour changes from blue to yellow immediately after the addition of the substrate. The absorbance was read in a micro plate reader at 450nm immediately after adding the stop solution.

3.2.6.5 Measurement of Serum NF-κB concentration

Serum Human phosphorylated nuclear factor kappa-B (NF-κB) concentration was determined using commercially available ELISA Kit (Wuhan Fine Biotech Co., Ltd, China. Batch No. H1950F033)

The assay utilizes "sandwich" ELISA technique. All reagents and samples were allowed to equilibrate to room temperature (18-26^{0}C). The positions of STD/SAMPLE/CTRL (Standards/Sample/Control) were marked on a protocol sheet. Each microtiter well was washed 2 times by dispensing 250µL of diluted Wash buffer. After the final washing step the residual buffer was removed by tapping the plate on absorbent paper. Exactly 100µl of the prepared standard was allotted into the standard wells while 100µl of sample dilution buffer was added into the blank well and 100µl of the sample were properly added into the test sample wells. The plate was sealed with a cover and incubated at 37°C for 90 minutes. After incubation the content of the plate were discarded and washed two times with wash buffer, and then 100µl of biotin-labelled antibody was added into the wells, the plate was then covered and incubated for 60 minutes at 37°C. Later the content of the plate was discarded and washed three times with a wash buffer, then 100µl of HRP-streptavidin conjugate (SABC) working solution was pipetted into each well, which is then covered and incubated at

37°C for 30 minutes. After the incubation the plate was washed with wash buffer 5 times, allowing the wash buffer to stay into the well for 2 minutes each time. After which 90µl of TMB substrate was added into each well, the plate was sealed and incubated at 37°C in the dark for 15 minutes. Then 50µl of acidic stop solution was added into each well. The colour changes from blue to yellow immediately after the addition of the substrate. The absorbance was read in a micro plate reader at 450nm immediately after adding the stop solution.

3.2.6.6 Determination of serum reduced glutathione

Reduced glutathione (GSH) concentration was determined according to Ellman (1959) as described by (Rukkumani, Aruna, Varma, Rajasekaran, & Pad-, 2004).

The assay is based on the reaction of 5,5- dithiobisnitrobenzoic acid (DNTB) and reduced Glutathione (GSH). To 150µl of the sample, 1.5ml of 10% trichloroacetic acid (TCA) was added and then centrifuged at 1500 ×g100 for 5 minutes. After which 1000µl of the supernatant was treated with 500µl of Ellman's reagent and then 3ml of phosphate buffer (0.2M, pH 8.0) was added. The absorbance of the mixture was read at 412nm using a spectrophotometer. The concentration of GSH was obtained from the standard curve (Appendix).

3.2.6.7 Determination of serum malondialdehyde

Lipid peroxidation is evidenced by formation of TBARS which was measured using the modified method of (Niehaus & Samuelsson, 1968) and described by (Akanji, Adeyemi, Oguntoye, & Sulyman, 2009).

Lipid peroxidation generates peroxide intermediates which upon cleavage release malondialdehyde (MDA), a product which reacts with thiobarbituric acid (TBA), forming a

MDA-TBA adduct that absorbs strongly at 535nm. Exactly 150µl of the samples was treated with TBA-TCA-HCl reagent (1:1:1 ratio) which was then placed in a water bath at 90°C for 60 minutes, later the mixture was cooled and centrifuged at 3000 rpm for 5 minutes and the absorbance of the pink supernatant (TBA-malondialdehyde complex) was then read at 535nm. Malondialdehyde formed was then calculated using the molar extinction coefficient of $1.56 \times 10^{-5} cm^{-1} M^{-1}$

3.3Dataanalysis

Where appropriate, results were presented as the mean ± SD. Data was analysed using a statistical software package (SPSS for windows, version 21, IBM Corporation, NY, USA). Statistical difference between means was analysed using one-way ANOVA and Duncan post-hoc test was used to ascertain the degree of significance, while association studies and relationships were analysed using Chi-square test and correlation analysis respectively. Results are significantly different at $P \leq 0.05$.

CHAPTER FOUR

Socio-demographic characteristics	Frequencies (n = 60)	Percentage (%)

RESULTS

4.1. Socio-demographic Characteristics ofPostmenopausal Breast Cancer Patients attending Surgical Outpatient Clinic ABUTH, Zaria

The socio-demographic characteristics of the histologically confirmed breast cancer patients showed that the majority (38.3%) of the breast cancer patients were within the age group of 45 – 50 years with mean age of 54.7±6.7 years while 26.67% were within the age group of 51 – 55 years. Among the breast cancer patients, 76.6% were married while 10% were widowed. Forty percent of respondents had tertiary education followed by secondary education with 30%. Majority of the breast cancer patients (55%) were of Hausa ethnic group and 60% were unemployed (Table 4.1).

Marital Status			
	Single	4	6.7
	Married	46	76.6
	Divorced	4	6.7
	Widowed	6	10.0
Ethnicity			
	Hausa	33	55.0
	Yoruba	8	13.3
	Igbo	7	11.7
	Others	12	20.0
Occupation			
	Public Servant	14	23.3
	Unemployed	36	60.0
	Business Women	9	15.0
	Others	1	1.7
Educational			
	Primary	8	13.3
	Secondary	18	30.0
	Qur'anic	10	16.7
	Tertiary	24	40.0
Age			
	45-50	23	38.3
	51-55	16	26.7
	56-60	10	16.7
	61-65	8	13.3
	≥66	3	5.0

Table 4. 1: Socio-Demographic Characteristics ofPostmenopausal Breast Cancer Patients attending Surgical Outpatient Clinic ABUTH, Zaria

4.2.Distribution of Breast Cancer among Postmenopausal Breast Cancers Patients attending Surgical Outpatient Clinic ABUTH, Zaria

Breast cancer cases were classified into different subtypes based on the immunohistochemistry result (Table 4.2). Triple negative breast cancer (TNBC) was the most frequent accounting for 40% followed by Luminal A (31.7%), while HER2-enriched and Luminal B were 20% and 8.3% respectively. Hence 40% of the cases were ER-positive (Luminal A and Luminal B), whereas 60% are found to be ER-negative (HER2-enriched and triple negative). The result also showed that 100% had invasive carcinoma, no specific type NST-histological type and most of the patients presented at latter stages of the disease; stage III (53.3%) and stage IV (28.3%) (Table 4.2).

Table 4. 2: Distributionof Breast Cancer among Postmenopausal Breast Cancer Patients attending Surgical Outpatient ClinicABUTH, Zaria

Classification			Frequency (n=60)	Percentage (%)
Molecular	Breast cancer subtypes	Luminal A*	19	31.7
		Luminal B*	5	8.3
		**HER2-enriched	12	20.0
		**Triple Negative	24	40.0
Histopathology	Histology types	Invasive carcinoma	60	100.0
Staging	Breast cancer stages	Stage I	2	3.3
		Stage II	9	15.0
		Stage III	32	53.3
		Stage IV	17	28.4

Luminal A: (ER+, PR+, HER2-),Luminal B: (ER+, PR+, HER2+), HER2-enriched: (ER-, PR-, HER2+),Triple negative: (ER-, PR-, HER2-)

*Oestrogen receptor positive, **Oestrogen receptor negative

4.3.Association between Breast Cancer and Possible Risk Factors among Postmenopausal Breast Cancer Patients attending Surgical OutpatientClinic ABUTH, Zaria

Association between possible risk factors and breast cancer subtypes showed significant association (χ^2= 20.219, *P*= 0.017) between ethnicity and breast cancer subtypes (Table 4.3). However, the results showed no association (P>0.05) between breast cancer and marital status, age at menarche, age, alcohol consumption, birth control pills, pregnancy and body mass index (Table 4.3). The association between possible risk factors and breast cancer severity indicated significant association between breast cancer severity and pregnancy (χ^2= 25.671, *P*= 0.012) as well as breast cancer severity and body mass index (χ^2= 25.278, *P*= 0.001), between breast cancer severity and marital status (χ^2= 18.992, *P*= 0.025) (Table 4.4). In addition there was no significant association (*P*>0.05) between breast cancer severity and age at menarche, age, alcohol consumption as well as the use of birth control pills (Table 4.4).

Table 4. 3: Association between Possible Risk Factors and Breast Cancer Subtypes among Postmenopausal Breast Cancer Patients attending Surgical Outpatient ClinicABUTH, Zaria

Variables		LA N (%)	LB N (%)	HER2 N (%)	TN N (%)	χ^2-values	*P*-values
Marital Status	Single	2(50.0)	1(25.0)	0 (0.0)	1(25.0)	9.499	0.393
	Married	14(30.4)	4(8.7)	9(19.6)	19(41.3)		
	Divorced	0 (0.0)	0(0.0)	0 (0.0)	4(100.0)		
	Widowed	1(16.7)	1(16.7)	2(33.3)	2(33.3)		
Ethnicity	Hausa	13(39.4)	1(3.0)	4(12.1)	15(45.5)	20.219	0.017*
	Yoruba	0(0.0)	1(12.5)	4(50.0)	3(37.5)		
	Igbo	1(14.3)	3(42.9)	0(0.0)	3(42.8)		
	Others	3(25.0)	1(8.3)	3(25.0)	5(41.7)		
Age	45-50	7(30.43)	2(8.7)	4(17.4)	10(43.5)	12.822	0.382
	51-55	6(37.5)	1(6.3)	0(00.0)	9(56.2)		
	56-60	3(30.0)	1(10.0)	4(40.0)	2(20.0)		
	61-65	1(12.5)	2(25.0)	2(25.0)	3(37.5)		
	≥66	0(00.0)	0(00.0)	1(33.3)	2(66.7)		
Age at menarche	11-13	10(47.1)	2(11.8)	4(23.5)	3(17.6)	6.870	0.371
	14-16	3(22.2)	2(5.6)	5(16.7)	13(55.5)		
	≥17	0(00.0)	0(00.0)	0(00.0)	2(100.0)		
Birth control pills	Yes	5(21.7)	1(4.4)	5(21.7)	12(52.2)	2.673	0.445
	No	12(32.5)	5(13.5)	6(16.2)	14(37.8)		
Alcohol consumption	Yes	0 (0.0)	1(50.0)	0(0.0)	1(50.0)	4.297	0.231
	No	17(29.3)	5(8.6)	11(19.0)	25(43.1)		
Pregnancy	0	2(50.0)	0(0.0)	1(25.0)	1(25.0)	9.728	0.640
	1-2	5(45.5)	2(18.2)	1(9.0)	3(27.3)		
	3-4	4(21.1)	2(10.5)	2(10.5)	11(57.9)		
	5-6	3(21.4)	2(14.3)	3(21.4)	6(42.9)		
	≥7	3(25.0)	0 (0.0)	4(33.3)	5(41.7)		
BMI	Underweight	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4.300	0.231
	Normal	5(41.7)	1(8.3)	2(16.7)	4(33.3)		
	Overweight/obese	1(7.7)	2(15.4)	2(15.4)	8(61.5)		

Results are significant at $P<0.05$ (Chi-square test)

LA = Luminal A, LB = Luminal B, TN = triple negative, HER2 = human epidermal growth factor receptor 2

Table 4. 4: Association between Possible Risk Factors of Breast Cancer and Breast Cancer stagesamong Postmenopausal Breast Cancer Patients attending Surgical Outpatient

Possible Risk factors		Stage I N (%)	Stage II N (%)	Stage III N (%)	Stage IV N (%)	χ^2-values	*P*-values
Marital status	Single	0(0.0)	2(50.0)	2(50.0)	0(0.0)	18.992	0.025*
	Married	2(4.3)	5(10.9)	29(63.1)	10(21.7)		
	Divorced	0(0.0)	0(0.0)	1(25.0)	3(75.0)		
	Widowed	0(0.0)	2(33.3)	0(0.0)	4(66.7)		
Ethnicity	Hausa	1(3.0)	5(15.2)	15(45.5)	12(36.3)	11.818	0.224
	Yoruba	1(12.5)	1(12.5)	5(62.5)	1(12.5)		
	Igbo	0(0.0)	3(42.9)	3(42.9)	1(14.2)		
	Others	0(0.0)	0(0.0)	9(75.0)	3(25.0)		
Age at menarche	11-12	1(6.0)	3(17.6)	10(58.8)	3(17.6)	9.258	0.414
	13-14	0(0.00)	1(5.6)	11(61.1)	6(33.3)		
	15-16	1(14.2)	2(28.6)	2(28.6)	2(28.6)		
	≥17	0(0.00)	1(50.0)	0(0.00)	1(50.0)		
Age	45-50	1(4.3)	2(8.6)	14(61.0)	6(26.1)	14.973	0.243
	51-55	1(6.2)	1(6.2)	11(68.8)	3(18.8)		
	56-60	0(00.0)	2(20.0)	6(60.0)	2(20.0)		
	61-65	0(00.0)	3(37.5)	1(12.5)	4(50.0)		
	≥66	0(00.0)	1(33.3)	0(00.0)	2(66.7)		
Birth Control	Yes	1(4.4)	4(17.4)	11(47.8)	7(30.4)	1.521	0.677
	No	1(2.7)	10(27.0)	19(51.4)	7(18.9)		
Alcohol consumption	Yes	0 (0.0)	1(50.0)	1(50.0)	0(0.0)	1.182	0.757
	No	2(3.4)	13(22.4)	29(50.0)	14(24.1)		
Pregnancy	0	0(0.0)	3(75.0)	1(25.0)	0(0.0)	25.671	0.012*
	1-2	0(0.0)	1(9.1)	10(90.9)	0(0.0)		
	3-4	1(5.2)	4(21.1)	8(42.1)	6(31.6)		
	5-6	0(0.0)	6(42.9)	6(42.9)	2(14.2)		
	≥7	1(8.3)	0 (0.0)	5(41.7)	6(50.0)		
BMI	Underweight	0(0.0)	0(0.0)	0(0.0)	0(0.0)	25.278	0.001*
	Normal	0(0.0)	2(16.7)	8(66.6)	2(16.7)		
	Overweight	0(0.0)	3(25.0)	7(58.3)	2(16.7)		
	Obese	1(100.0)	0(0.0)	0(0.0)	0(0.0)		

ClinicABUTH, Zaria

Results are significant at $P<0.05$ (Chi-square test)

4.4. Serum Levels of Pro-inflammatory Markers (RANKL and NF-κB) among Postmenopausal Breast Cancer Patient attending Surgical Outpatient ClinicABUTH, Zaria

The serum levels of RANKL and NF-κB within the different molecular subtypes of breast cancer showed that the serum concentrations of RANKL were significantly (P<0.05) higher in Luminal A and Luminal B subtypes of when compared to apparently healthy controls (Figure 4.1). Also the concentration of RANKL was significantly (P<0.05) higher in HER2-enriched and triple negative subtypes of breast cancer when compared to the apparently healthy control group. Although, there was no significant (P>0.05) difference between luminal A subtypes and luminal B subtype as well as between HER2-enriched subtype and triple negative subtype of breast cancer. Generally, RANKL concentration is significantly (P<0.05) higher in breast cancer patients when compared to the apparently healthy individuals (Figure 4.1).

NF-κB concentration across the different subtypes of breast cancer, was significantly (P<0.05) higher in triple negative and HER2-enriched subtypes as compared to apparently healthy control (Figure 4.1). The data also revealed that NF-κB concentration was significantly (P<0.05) higher in luminal A and luminal B subtypes of breast cancer when compared with apparently healthy control. Similarly, NF-κB concentration was significantly (P<0.05) higher in all breast cancer patients when compared to apparently healthy controls (Figure 4.1).

The serum concentration of RANKL and NF-κB across different stages of breast cancer severity demonstrated a significant (P<0.05) increase in RANKL across stage I to stage IV (Figure 4.2). However, there was no significant (P>0.05) difference between stage II and stage III as well as between stage III and stage IV. The result also revealed a significant

(P<0.05) increase in NF-κB concentration from stage I through stage IV. However, the increase was not significant (P>0.05) with respect to stages I and II as well as between stages II, III and IV (Figure 4.2).

The serum RANKL and NF-κB concentrations across different chemotherapy courses taken by the patients showed a slight decrease in RANKL concentration from 1st course through 6th course though the decrease was not significant (P>0.05) (Figure 4.3). The result also showed a significant (P<0.05) decrease in NF-κB concentration in 3rd course when compared to 2nd course of chemotherapy, but there was no significant (P>0.05) difference with 1st course, 4th course, 5th course and 6th course of the chemotherapy (Figure 4.3).

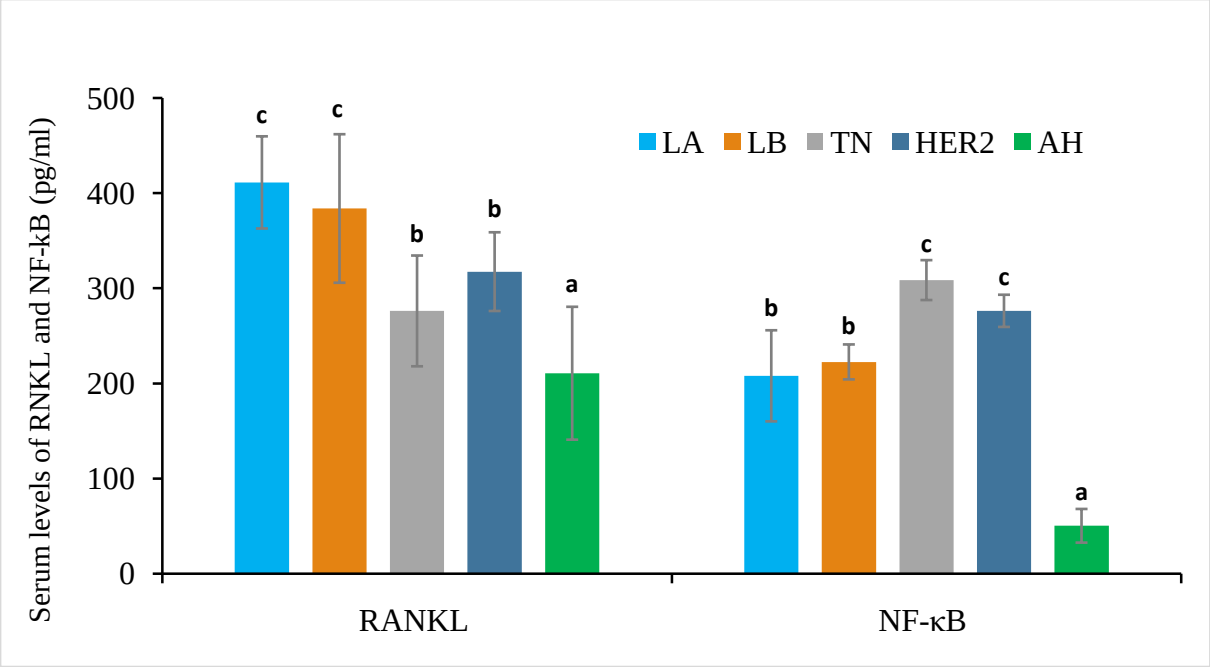

Figure 4. 1: Serum levels of pro-inflammatory markers (RANKL and NF-κB) across breast cancer subtypes among postmenopausal breast cancer patient attending Surgical Outpatient Clinic ABUTH, Zaria

LA = Luminal A, LB= Luminal B, TN = Triple negative, HER2 = Human epidermal growth factor receptor 2, AH = apparently healthy, NF-κB= Nuclear transcription factor kappa-B, RANKL = Receptor activator of nuclear factor kappa-B ligand

Bars with different alphabets are significantly different from each other using one-way ANOVA and Duncan multiple range test ($P<0.05$)

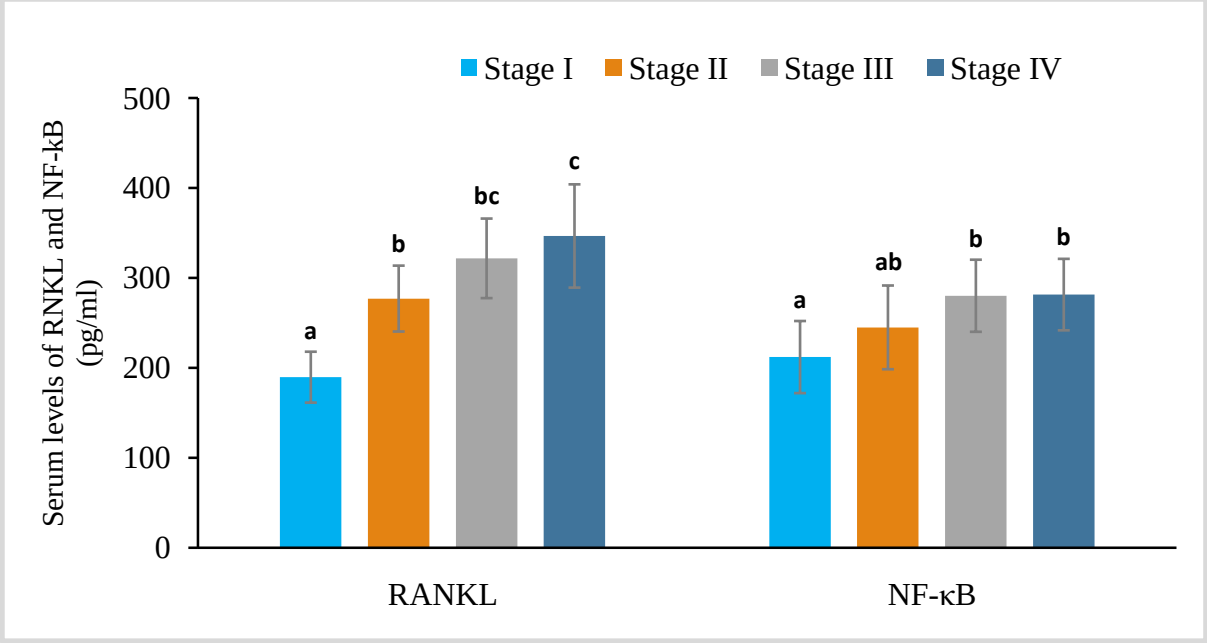

Figure 4. 2: Serum levels of pro-inflammatory markers (RANKL and NF-kB) across breast cancer stages of severity among postmenopausal breast cancer patient attending Surgical Outpatient Clinic ABUTH, Zaria

NF-κB= Nuclear transcription factor kappa-B, RANKL= Receptor activator of nuclear factor kappa-B ligand

Bars with different alphabets are significantly different from each other using one-way ANOVA and Duncan multiple range test ($P<0.05$)

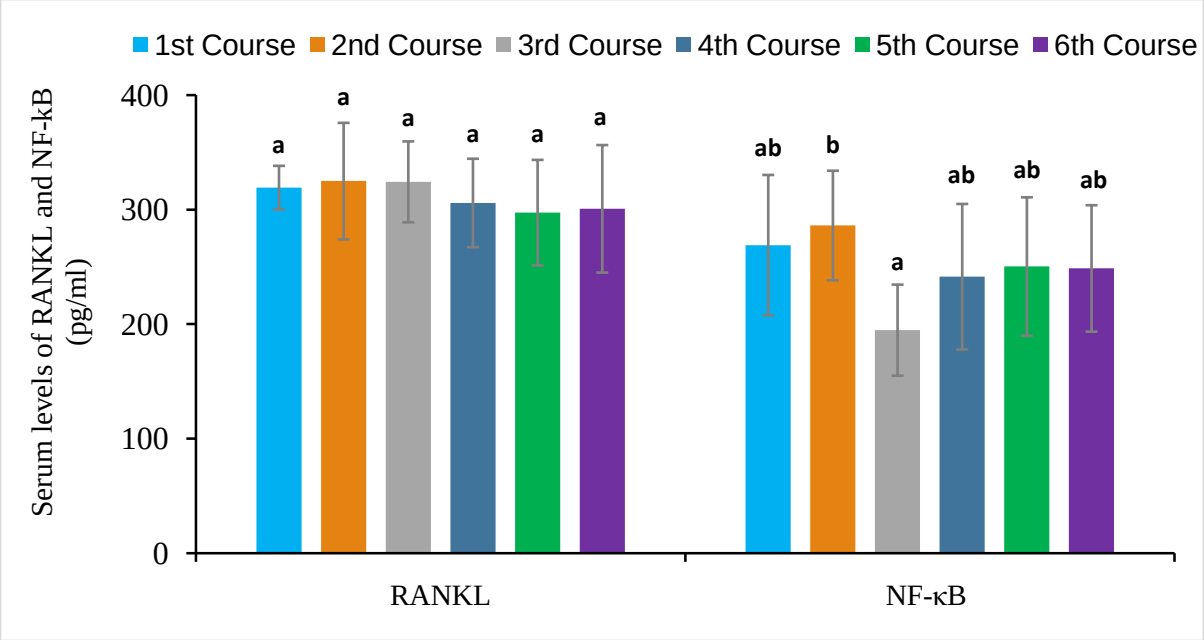

Figure 4. 3: Serum levels of pro-inflammatory markers (RANKL and NF-κB) across different chemotherapy courses among postmenopausal breast cancer patient attending Surgical Outpatient Clinic ABUTH, Zaria

NF-κβ= Nuclear transcription factor kappa-B, RANKL = Receptor activator of nuclear factor kappa-B ligand

Bars with different alphabets are significantly different from each other using one-way ANOVA and Duncan multiple range test ($P<0.05$)

4.5. Associations between Serum RANKL & NF-κB levels and Possible Risk Factors among Postmenopausal Breast Cancer Patients attending Surgical Outpatients ClinicABUTH, Zaria

The association between possible risk factors and serum concentration of RANKL and NF-κB were tested using Chi-square. The result indicated no association ($P>0.05$) between serum RANKL and the possible risk factors (Table 4.5). Moreover, there is no association between NF-κB concentration and possible risk factors (age, pregnancy, birth control pills, alcohol consumption, and age at menarche, ethnicity, education level, occupation and marital status).

Table 4. 5: Association between Possible Risk Factors and Serum levels of RANKL & NF-κB among Postmenopausal Breast Cancer Patients attending Surgical Outpatient ClinicABUTH, Zaria

Possible risk factors	RANKL(pg/ml)		NF-κB (pg/ml)	
	χ^2-values	*P*-values	χ^2-values	*P*-values
Age	3.704	0.157	1.151	0.562
Pregnancy	2.355	0.671	2.233	0.693
Birth Control	0.255	0.613	2.090	0.148
Alcohol consumption	0.500	0.479	0.182	0.669
Age at menarche	0.993	0.803	0.684	0.711
Ethnicity	3.949	0.267	1.906	0.592
Education level	5.910	0.116	6.205	0.102
occupation	4.900	0.086	2.015	0.365
Marital status	1.147	0.766	1.736	0.420

NF-κB= Nuclear transcription factor kappa-B, RANKL= Receptor activator of nuclear factor kappa-B ligand

4.6. Serum Levels of Some Oxidative Stress Biomarkers among Postmenopausal Breast Cancer Patientsattending Surgical Outpatient Clinic ABUTH, Zaria

The serum concentration of some oxidative stress markers (GHS and MDA) were presented in Figure 4.4. The results showed no significant (P>0.05) difference among the four subtypes of breast cancer with respect to GSH concentration, but it is significantly (P<0.05) higher in breast cancer patients when compared to apparently healthy individuals. Furthermore, the serum MDA concentration was significantly (P<0.05) higher in Luminal A subtype when compared to HER2-enriched. Although higher Luminal A is not significant (P>0.05) when compared to triple negative as well as Luminal B subtypes of breast cancer. Serum MDA concentrations were significantly (P<0.05) higher among breast cancer patients when compared to apparently healthy individuals (Figure 4.4).

Serum oxidative stress markers across stages of breast cancer severity was presented in (Figure 4.5) The result showed an increase in MDA concentration from stage I to stage IV. However, stages III and IV have significantly (P<0.05) higher MDA level compared to stage I and II. Serum GSH level was decreased from stage I to IV, with Stages III and IV significantly (P<0.05) lower compared to stages I and II.

Figure 4.6.Shows oxidative stress levels across different chemotherapy cycles. There was no significant (P>0.05) difference in MDA concentration across the different chemotherapy courses. GSH concentration decreases from 1^{st} to 6^{th} course. However, the reduction in GSH was significantly (P<0.05) lower in the 6^{th} course compared to 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} courses. It is also significantly (P<0.05) lower in the 4^{th} and 5^{th} compared to 1^{st}, 2^{nd}, and 3^{rd} courses.

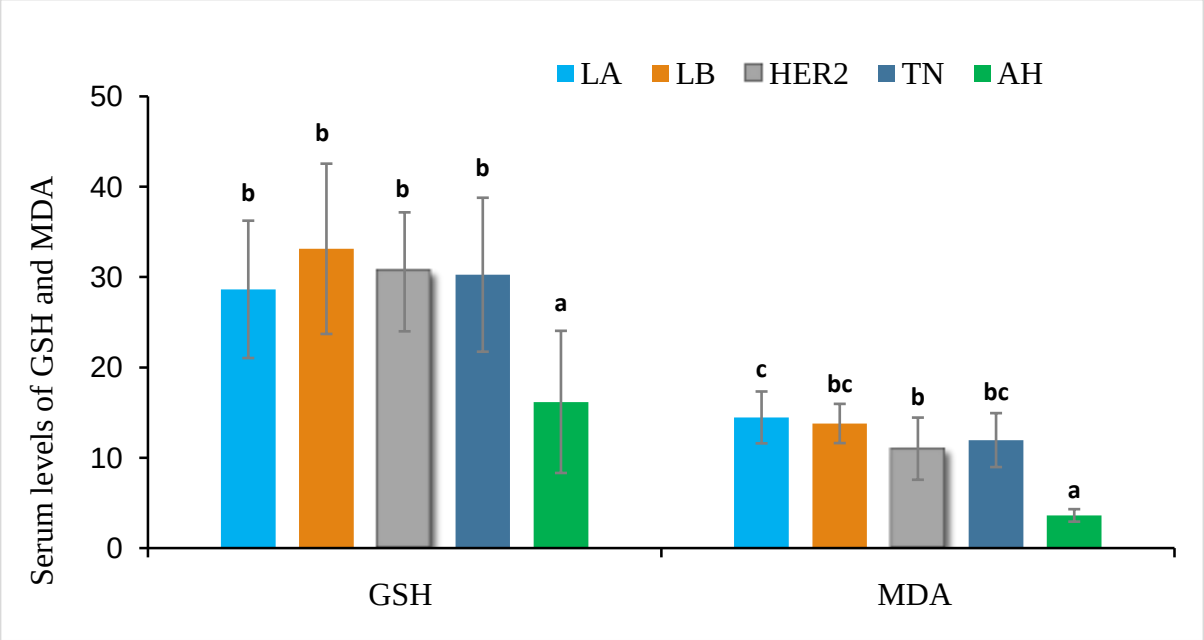

Figure 4. 4: Serum Levels of some Oxidative Stress Biomarkers across breast cancer subtypes among postmenopausal Breast Cancer Patients attending Surgical Outpatient Clinic ABUTH, Zaria

LA = Luminal A, LB = Luminal B, TN = Triple negative, HER2 = Human epidermal growth factor receptor 2, AH = apparently healthy, MDA(µM)= Malondialdehyde, GSH(µg/ml) = Reduced glutathione

Bars with different alphabets are significantly different from each other using one-way ANOVA and Duncan multiple range test ($P<0.05$)

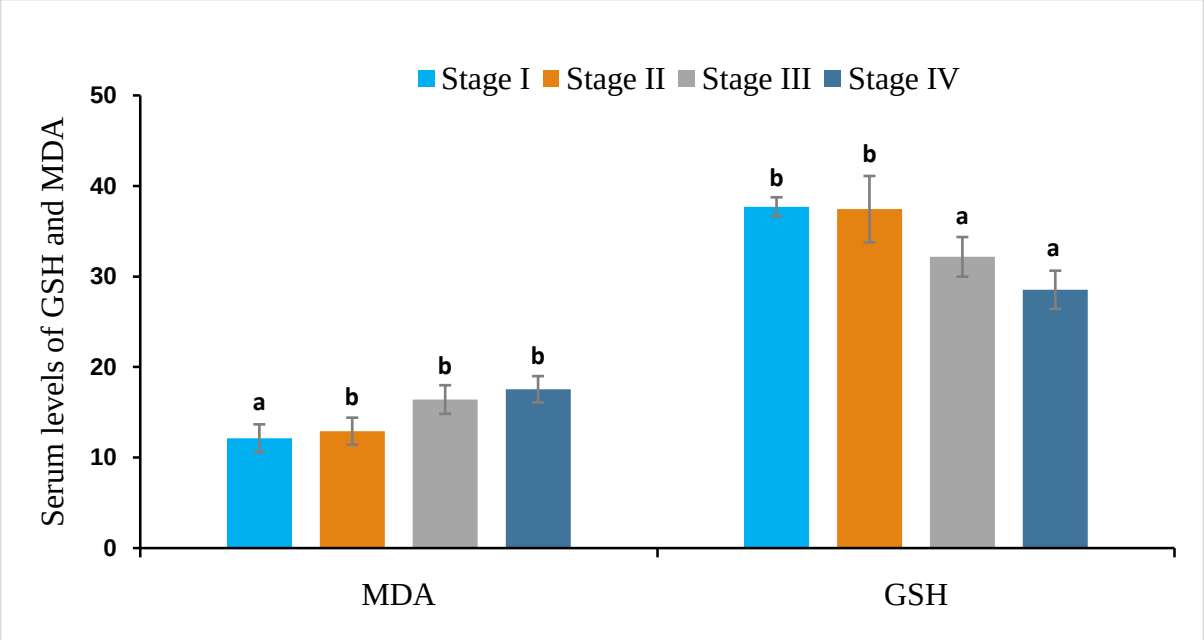

Figure 4. 5: Serum Levels of some Oxidative Stress Biomarkers across breast cancer stages of severity among postmenopausal Breast Cancer Patients attending Surgical Outpatient Clinic ABUTH, Zaria

MDA (µM)= Malondialdehyde,GSH (µg/ml)=Reduced glutathione

Bars with different alphabets are significantly different from each other using one-way ANOVA and Duncan multiple range test ($P<0.05$)

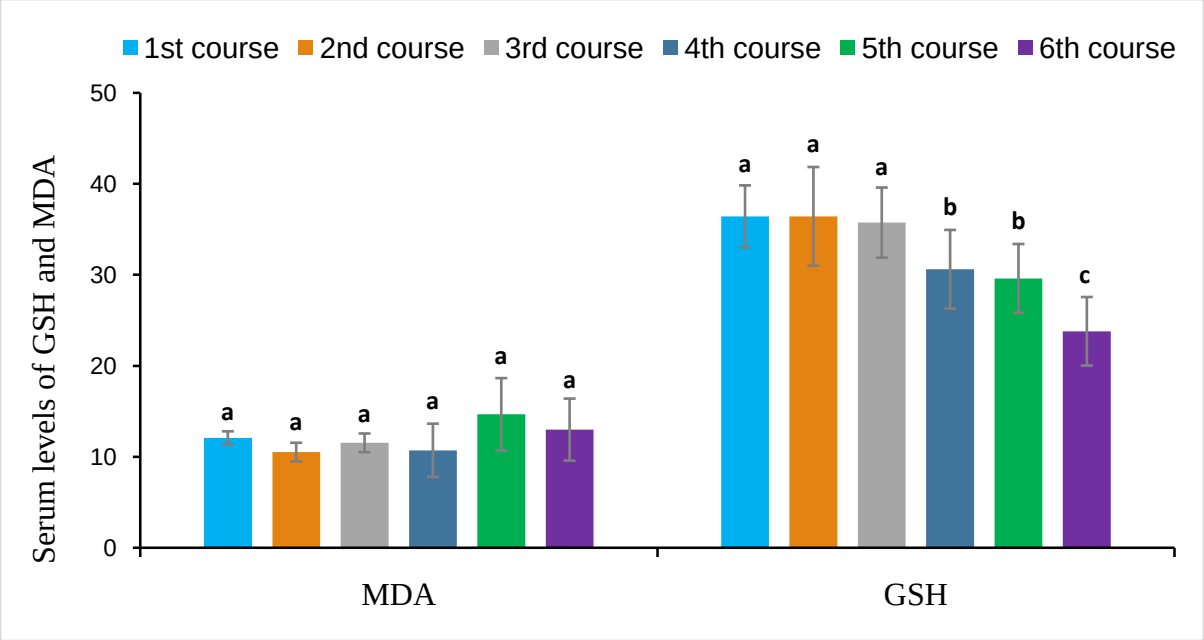

Figure 4. 6: Serum Levels of some Oxidative Stress Biomarkers across different chemotherapy courses among postmenopausal Breast Cancer Patients attending Surgical Outpatient Clinic ABUTH, Zaria

MDA (µM)= Malondialdehyde,GSH (µg/ml)= Reduced glutathione

Bars with different alphabets are significantly different from each other using one-way ANOVA and Duncan multiple range test ($P<0.05$)

4.7. Relationship between Oxidative Stress Biomarkers and Inflammatory Markers among Postmenopausal Breast Cancer Patients Attending Surgical Outpatient Clinic ABUTH, Zaria

The relationship between some oxidative stress biomarkers (GSH & MDA) and the pro-inflammatory biomarkers (RANKL & NF-κB) of the breast cancer patients were tested using Pearson's correlation (Table 6). The results showed that there were significant positive correlations between NF-κB and RANKL (r = 0.916; *P*=0.001), NF-κB and GSH (r = 0.695; *P*=0.001) and NF-κB and MDA (r = 0.834; *P*=0.001). In addition, significant positive correlations were also observed between the level of RANKL and GSH (r = 0.842; *P*=0.001) and between RANKL and MDA (r = 0.939; *P*=0.001). There was also a significant positive relationship between serum GSH and MDA (r = 0.860; *P*=0.001).

Table 4. 6: Relationship between levels of Oxidative Stress Biomarkers and Pro-inflammatory Markers (RANKL & NF-κB) among Postmenopausal Breast Cancer Patients

		NF-κB	RANKL	GSH	MDA
NF-κB	Pearson Correlation	1	0.916**	0.695**	0.834**
	Sig. (2-tailed)		0.001	0.001	0.001
RANKL	Pearson Correlation		1	0.842**	0.939**
	Sig. (2-tailed)			0.001	0.001
GSH	Pearson Correlation			1	0.860**
	Sig. (2-tailed)				0.001
MDA	Pearson Correlation				1
	Sig. (2-tailed)				

**. Correlation is significant at the 0.01 level (2-tailed). (Pearson/Spearman correlation)

attending Surgical Outpatient Clinic ABUTH, Zaria

GSH = Reducedglutathione, MDA = Malondialdehyde,NF-κB= Nuclear transcription factor kappa-BRANKL = receptor activator of nuclear factor kappa-B ligand

CHAPTER FIVE

DISCUSSION

Breast cancer incidence and mortality is characterized by variations in disease pathogenesis and treatment outcomes across racial and ethnic boundaries, pointing to a multifactorial basis for the disease (Yedjou *et al*., 2019). A number of factors could be responsible for the high mortality rate among breast cancer patients in West Africa and Nigeria in particular, necessitating research into socio-demographic patterns, breast cancer phenotypes and possible biochemical alterations in these patients (Azubuike *et al*., 2018). The modal age of the respondents from this study was 45 - 50 years, while the mean age was 54.7 ± 6.7 years and is similar to the age of breast cancer patients observed by a study carried out in South-West Nigeria, as the peak age of developing breast cancer in Nigeria is between 35 – 45 years (Olaogun *et al*., 2020). The current study also indicates that the majority 55% and 60% of the patients were of Hausa ethnic group and unemployed respectively, this might be because Hausa is the dominant ethnic group within the study location and less opportunity of securing a job in the study area (Agbo *et al*., 2014).

The immunohistochemistry data showed that triple-negative breast cancer (TNBC) was the most frequent in the present study. The 40% frequency of triple-negative subtype established in the present study is lower than the 46.6% reported in Kano (Usman *et al*., 2019), it is also less than the 52.6% in Maiduguri (Minoza *et al*., 2016) and the 87% in Lagos (Makanjuola *et al*., 2014) highlighting the dominance of this subtype in Nigeria and also agrees with previous studies which showed that women of African ancestry are more predisposed to the triple negative breast cancer phenotype. The consequence of this for Nigerian women with the disease and managing oncologists is the non-suitability of these women for neither hormonal therapy nor adjuvant therapy (Shimelis *et al*., 2018). However, just as reported by

Tischkowitz *et al*. triple-negative tumours were as well the major molecular subtype regardless of age group of patients or histological entity (Tischkowitz *et al*., 2007). Moreover, triple-negative tumours have also been associated with BRCA1 mutation (Zepeda- Castilla *et al*., 2008). This may clarify the poor outcome noted by Dietze *et al.* among African women with carcinoma of the breast (Dietze *et al*., 2015). However, reports from two separate works in Ilorin by Adeniji and co-workers showed two different variants of triple- negative cancer;basal-like (ER-ve, PR-ve, HER2-ve, CK5/6+ve, and/or EGFR+) and unclassified (negative for all five markers) respectively, constituting 25% and 24% of all breast carcinomas (Adeniji *et al*., 2010; Adeniji *et al*., 2016). In another study carried out in ABUTH Zaria with a larger sample size indicates triple negative breast cancer to be the least subtype with 13.48% (89) of the 660 breast carcinoma cases diagnose between January 2009 to December 2016 (Liman *et al*., 2020). This contradict the general perception that black women were more prone to triple negative breast cancer phenotype.

HER2-enriched was the second most common subtype of the oestrogen receptor negative breast cancer cases with 20% of the cases in the index study. Studies in Sub-Saharan Africa have documented a high proportion of HER2-enriched cases (Adebamowo *et al*., 2008; Banjo *et al*., 2008; Yarney *et al*., 2008). However, Adebamowo *et al*.in a previous study reported HER2-enriched as the third most common (Adebamowo *et al*., 2008). In contrast, a lower frequency (15%) was reported (Huo *et al*., 2009). This subtype of tumour has an implication in that the tumours may be more responsive to anti-EGFR-targeted therapy (Trastuzumab). A 52% increase in disease-free survival and a 33% reduction in risk of death has been shown to be linked with adjuvant trastuzumab therapy (Romond *et al*., 2005). Though this may be positive, a high risk of early and frequent relapse was observed among patients on this therapy. Therefore, just as observed even in developed countries, high cost of this drug may limit its access due to less readily available resources in Nigeria (Romond *et al*., 2005).

The oestrogen receptor positive breast cancer cases are composed of LUMA and LUMB tumours. In the present study, 40% of the cases were oestrogen receptor positive. Though higher than 35.5% reported in Kano (Usman *et al*., 2019) and 2.1% and 11.1% reported in Lagos (Makanjuola *et al*., 2014) as well as (Banjo *et al*., 2008) in Ogun, respectively. This wide range variation in oestrogen receptor positivity rate is further emphasized by results from outside Nigeria comprising Saudi Arabia (74.8%), (Elkablawy *et al*., 2015) USA (58%), (Carey *et al*., 2006) and Egypt (55.1%) (El-Hawary *et al*., 2012). LUMA (31.7%) tumours were more frequent than LUMB (8.3%) in the present study reflecting a similar report. (Minoza *et al.*, 2016) also in Northern Nigeria as well as Seshi *et al.*in Cote d'voire (Seshi *et al*., 2015). It nevertheless contrasts with a higher frequency of LUMB reported in Morocco. (El Fatemi *et al*., 2012). LUMB subtypes have been reported to have a poorer prognosis than LUMA subtype, thus, oestrogen receptor positive tumours have little response to chemotherapy (Rastelli and Crispino, 2008). In northern Nigerian women this has an implication, even though the supports use of anti-oestrogens (tamoxifen), compensated the relative poorer response of oestrogen receptor positive tumours to chemotherapy in addition to high risk increase in resistance to anti-oestrogens (Titiloye *et al*., 2013).

The present study indicates invasive carcinoma (NST) to be the most common histological type like in other studies in Nigeria. Yet, the percentage (83.3%) was slightly higher than what was recorded in other parts of the North, 78.8% in Gombe and 82.6% in Maiduguri. This result perhaps indicates that late presentation is the custom in Nigeria; poor acceptance of breast screening strategies, particularly mammography, and as described by Odusanya and Tayo inadequate awareness of breast cancer among the Nigerian populace (Odusanya and Tayom, 2001). The present study also shows that majority of the patients were observed to be in stages III and IV, suggesting that the cancer was aggressive and possibly metastasized which makes it more difficult to manage, this also agrees with the aggressive pattern of breast

cancer pathogenesis observed in previous studies (Ali-Gombe *et al*., 2021; Olaogun *et al*., 2020).

Interestingly, the data from our study showed no association between breast cancer and marital status, age at menarche, and body mass index which are established risk factors for breast cancer predisposition (Kamińska *et al*., 2015). This might be due to the fact that some of these risk factors are modifiable. We also found no association between history of alcohol consumption, use of birth control pills and breast cancer risk which disagrees with a previously reported study carried out among breast cancer patients in West Africa (Qian *et al*., 2014). This could be due to the lifestyle of the women in the area, as most of them are from local communities and are mostly not exposed to alcohol use as well as birth control pills for personal and religious reasons.

Moreover, the serum level of Receptor Activator of Nuclear factor Kappa beta Ligand (RANKL) among the breast cancer patients was significantly higher when compared to apparently healthy control subjects, agreeing with previous studies which showed that RANKL plays significant roles in mammary epithelial cell proliferation and is dysregulated during breast cancer (Gonzalez-Suarez *et al*., 2010). The data also showed that among the patients, RANKL was higher in patients with triple negative phenotype compare to apparently control subject which is reportedly associated with worse clinical outcomes and bone metastasis in breast cancer patients with higher RANKL activity (Owen, Ye, Sanders, Mason, & Jiang, 2013). Also, serum NF-kB levels were significantly higher in breast cancer patients when compared to apparently healthy control, agreeing with the observation that NF-kB activity correlates with increased disease severity among breast cancer patients (Espinoza-Sánchez *et al*., 2019).

The result also showed NF-kB to be higher in oestrogen receptor negative, this is because downregulation of oestrogen receptor in breast cancer leads to activation of NF-kB which results to overexpression of human epidermal growth factor receptor (HER-2) through epidermal growth factor receptor (EGFR) and mitogen activated protein kinase (MAPK) pathways (Smith*et al*., 2014),. The serum levels of both RANKL and NF-kB were found to correlate with disease severity and were higher in patients at later stages of the disease, further suggesting that these proteins could have prognostic potential.

Oxidative stress is implicated in breast cancer pathogenesis (Roque *et al*., 2015), and our data showed significantly lower level of reduced glutathione (GSH) and higher level of malondialdehyde (MDA) in breast cancer patients when compared to apparently healthy control subjects which agrees with previous studies (Muhammed *et al*., 2019; Malik *et al*., 2017). MDA was more elevated while GSH was more depleted in stage III and IV patients suggesting that oxidative stress correlated with disease progression which is similar to previously reported studies (Sadati *et al*., 2016).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Breast cancer still remains the leading cause of cancer related death across the Sub-Sahara Africa especially Nigeria even with advances made in t its management, this might due to the poor health facilities and cost of its treatment. Another contributing factor is the poor acceptance of breast cancer screening strategies particularly mammography. Finding a cost effective biomarkers for screening/diagnosis as well as monitoring of the disease is still one of the many challenges facing modern scientific research. This study for the first time, access the serum levels of RANKL, NF-kB and some oxidative stress biomarkers among breast cancer patients.

The study demonstrate that majority of the patients were married, unemployed and of Hausa ethnic group.The most common clinical parameters of the patientswere triple negative breast cancer (TNBC) molecular subtype followed by luminal A (LUM-A) subtypes. However, invasive NST is the common histological type among the patients, with the majority of the patients presented at later stages (stage III and IV) of the disease.

The serum levels of RANKL and NF-kB were elevated in the patients and were higher as the disease progressed pointing to the prognostic potential of these markers. Malondialdehyde was elevated while glutathione was depleted in the patients and was more evident as the disease progressed suggesting oxidative stress among the patients.

In general, the study point out that there is increase in serum levels of RANKL, NFKB and oxidative stress biomarkers among postmenopausal breast cancer patients attending Ahmadu University Teaching Hospital Zaria

The limitation of the current study is that; the time or period by which the study was conducted is too short as the programme is time bound and there was small number of samples as a result.

6.2 Recommendations

i. The results showed that RANKL and NF-κBserum levels were higher among the postmenopausal breast cancer patients, necessitating further research on their possible clinical utilities.

ii. A more structured longitudinal study with large number of sample is needed to further ascertain the level of these markers within the northern part of this country.

iii. A study should be conducted to ascertain the serum levels of these biomarkers among pre-menopausal patients with breast cancer.

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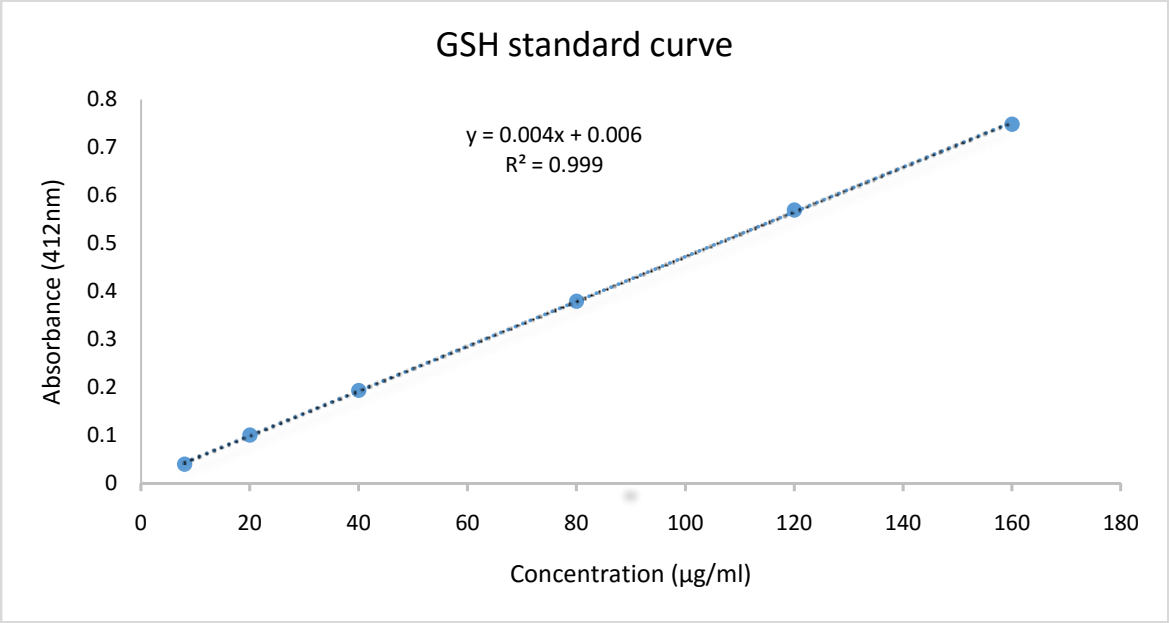
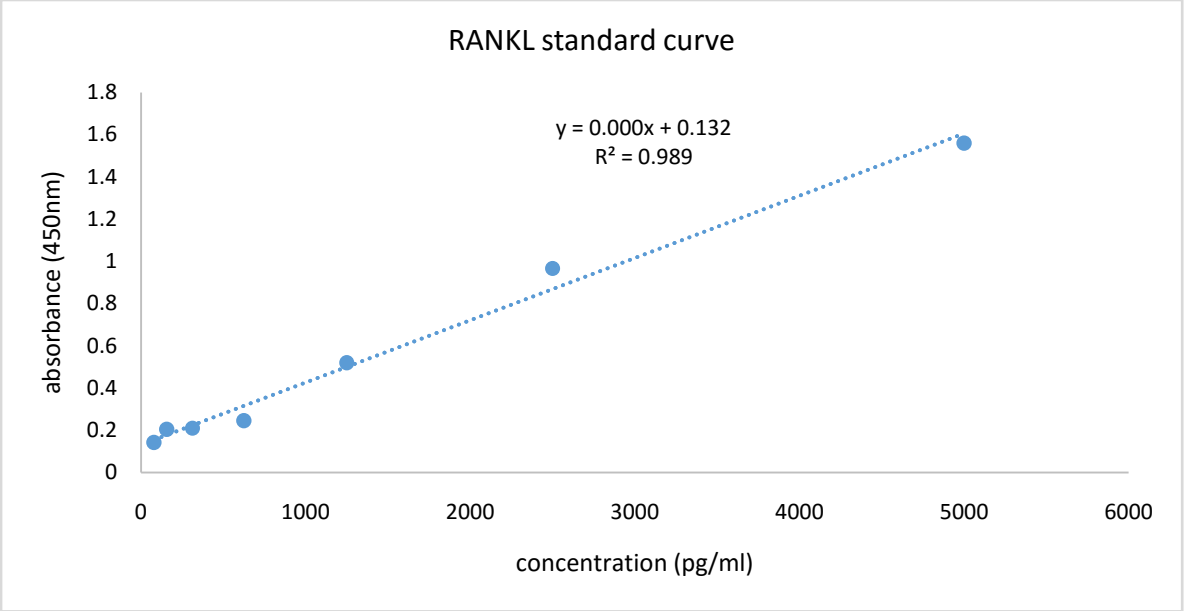
APPENDICES

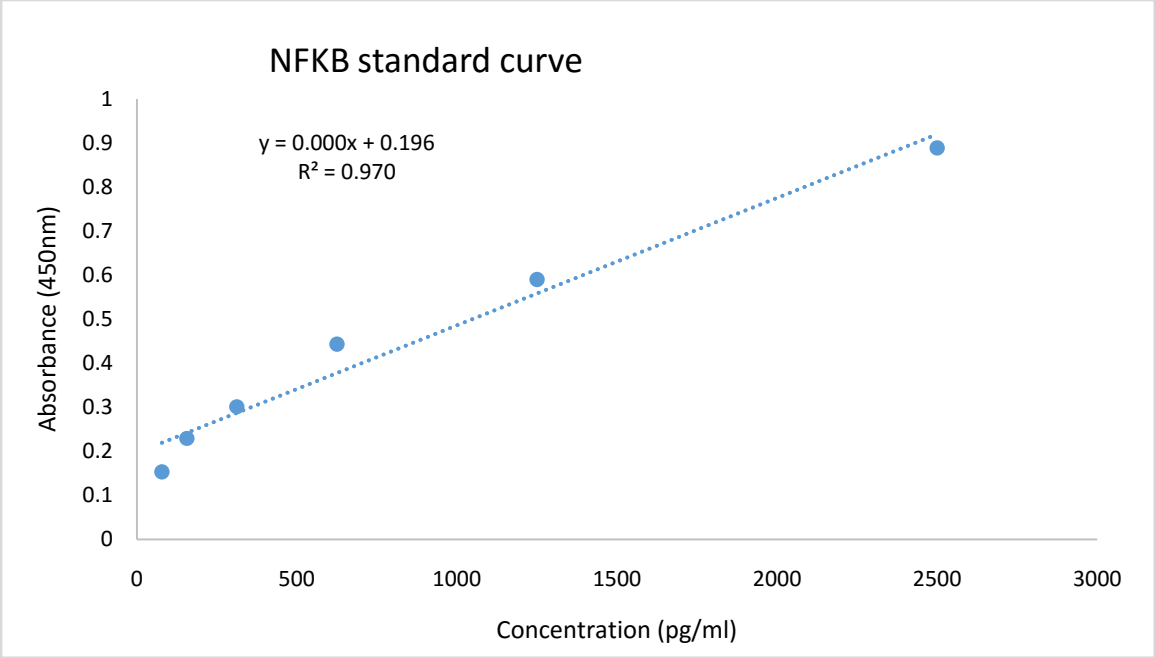
Appendix 1: Reduced Glutathione standard curve



Appendix 2: RANKL standard curve



Appendix 3: NF-kβ standard curve



Appendix4: Questionnaire

Questionnaire on socio-demographic characteristics among postmenopausal breast cancer patients of different subtypes attending Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, Nigeria.

I am **ABUBAKAR ABDULLAHI MARU** with registration number **P17LSBC8014**, an MSc student from Biochemistry Department, Ahmadu Bello University Zaria, Nigeria. I wish to request for permission to use your patients as respondents for my MSc research project titled **'Evaluation of serum levels of RANKL, NF-kB and oxidative stress biomarkers among postmenopausal patients with breast cancer attending Ahmadu Bello University Teaching Hospital Zaria, Nigeria'**

This questionnaire is to be used for gathering information on socio-demographic factors among female postmenopausal breast cancer patients and apparently healthy postmenopausal women. The questionnaire is to be filled by ticking the appropriate option and filling in the blank space where necessary. Your response will be confidential as only the researchers involve in this study will see your response. Your participation in this study is also voluntary and you also do not have to answer any question that makes you uncomfortable

Questionnaire serial number: _____ **Hospital No.:** _____

Name (initials): _____

Section A (To be filled by the female participant)

1. Age (yrs):_____ Weight (Kg):_____
2. Marital status: Single [ ] married [ ] divorced [ ] widowed [ ]
3. Ethnicity: Hausa [ ] Yoruba [ ] Igbo [ ] Others……..……
4. Occupation (please specify the nature of the job):
   Public servant [ ] Unemployed [ ] Business woman [ ]
   Farmer [ ] Other ……..……..
5. Highest education attainment:
   No education [ ] Primary education [ ] Qur'anic education [ ]
   Secondary education [ ] Tertiary education [ ] others ……..…

Section B (To be filled by the female participant)

6. How often do you go for medical check-up?
   Once in a month [ ], Twice in a month [ ], others …….……..

7. Have you been diagnosed with any kind of disease before? Yes [ ] No [ ]
   If yes which type? Cancer [ ] Cardiovascular [ ] Diabetes [ ]
   HIV [ ] Others……..…
8. Have you used any birth control pills before? Yes [ ] No [ ]
   If yes, for how long? One month [ ], Two months [ ], Three months [ ], Others …...….
9. Have you been pregnant before? Yes [ ] No [ ]
   If yes, how many times? One [ ], Two [ ], Three [ ], others ……..
10. Do you smoke cigarette? Yes [ ] No [ ]
    If yes, for how long? _____
    If stopped, when did you stop? _____
11. Do you drink alcohol? Yes [ ] No [ ]
    If yes, for how long and what quantity? _____

Section C (To be obtained from medical record)

12. Do you have breast cancer? Yes [ ] No [ ]
    If yes, when were you diagnosed? _____
13. What is the breast cancer subtype of the patient?
    Luminal A (ER+, PR±, HER2-) [ ]
    Luminal B (ER+, PR±, HER2+) [ ]
    HER2 enriched (ER-, PR-, HER2+) [ ]
    Triple –ve (ER-, PR-, HER2-) [ ]
14. At what stage was the breast cancer diagnosed in the patient (Manchester staging)?
    Stage 1 [ ] Stage 2 [ ] Stage 3 [ ] Stage 4 [ ]
15. Are you currently on any chemotherapy? Yes [ ] No [ ]
    If yes (please specify) _____
    For how long? 1st course [ ] 2nd course [ ] 3rd course [ ] 4th course [ ]
    5th course [ ] 6th course [ ] other (specify) ……

Appendix 5: Ethical Clearance

HEALTH RESEARCH ETHICS COMMITTEE
AHMADU BELLO UNIVERSITY TEACHING HOSPITAL SHIKA,
ZARIA, NIGERIA

E-mail: abuthshika@yahoo.com *website:* www.abuth.org

Chairman of Board: **ALH. AHMADU RUFAI MACHIKA**
Chief Medical Director: **PROF. HAMIDU A.U.,** MBBS, FWACS, MVUS (USA)
Chairman, Medical Advisory Committee: **PROF. ADAMU AHMED,** MBBS, (ABU), LLB. (ABU), BL, FWACS, FICS, FACS.
Director of Administration: **ALH. ABDULRAHEEM SALLAU,** BA (Pub. Admin) PGDPA, MPA, (ABU) AHAN, ACIPM
Ag. Chairperson: **HREC Prof. Aisha I. Mamman** MBBS, FMCPath

NHREC/10/12/2015 **D-U-N-S NUMBER: 954524802**

ABUTH/HREC/ CL/05 **20th January, 2020**

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

Evaluation of RANKL and Nf-KB levels among Postmenopausal Breast Cancer Patients of different subtypes attending Ahmadu Bello University Teaching Hospital, Zaria.

ABUTH Ethics Committee assigned number: - ABUTHZ/HREC/W41/2020

Name of the principal Investigator: - Abubakar Abdullahi Maru.
Address of the Principal Investigator: - Dept. of Biochemistry ABU, Zaria
Date of receipt of valid application: - 23rd December, 2019

Date of meeting when final determination On ethical approval was made: - 7th January, 2020

This is to inform you that the research described in the submitted protocol, the consent forms and other participant information materials have been reviewed and ***given full approval by the Health Research Ethics Committee.***

Please note: this approval dates from **20th January, 2020 - 20th January, 2021**

No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: **http://www.nhrec.net.**

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.

21st January 2020

Prof. Aisha I. Mamman *MBBS, FMCPath*
Chairperson

Appendix 6: Supplementary Table 1

Table 1: Determinants of Serum RANKL Concentration among Postmenopausal Breast Cancer Patients Attending Surgical Outpatient ClinicABUTH, Zaria by multiple regression analysis

Variables	**B**	**Std. Error**	**Beta**	***P*-value**
Age	-28.662	32.658	0-.384	0.420
BMI	11.082	17.058	0.355	0.545
Education level	1.181	36.033	0.021	0.975
Breast cancer subtypes	-3.247	41.493	-0.058	0.941
Breast cancer severity	53.147	57.237	0.490	0.396
Birth control pills	72.937	74.324	0.482	0.371
Alcohol consumption	130.061	125.221	0.599	0.347
Age menarche	-39.710	50.380	-0.266	0.466
Chemotherapy courses	-6.382	15.496	-0.170	0.697
Pregnancy	13.760	18.767	0.269	0.496
Marital status	173.793	213.842	0.583	0.453
Ethnicity	38.517	37.794	0.646	0.355
Occupation	-21.702	44.369	-0.241	0.645

R^2=0.704; **adjusted R^2**=-0.065; **F**=0.9 (**df.** 13); ***P*value** = 0.590

Results are significant at $P<0.05$

Appendix 6: Supplementary Table 2

Table 2: Determinants of Serum NF-κB Concentration among Postmenopausal Breast Cancer Patients Attending Surgical Outpatient ClinicABUTH, Zaria by multiple regression analysis

Variables	**B**	**Std. Error**	**Beta**	***P*-value**
Age	16.161	25.992	0.240	0.598
BMI	-8.647	13.497	-0.352	0.587
Education level	0.816	44.569	0.018	0.987
Breast cancer subtypes	2.521	40.102	0.056	0.956
Breast cancer severity	-30.149	51.987	-0.336	0.621
Birth control pills	-25.500	96.080	-0.194	0.816
Alcohol consumption	-74.598	163.508	-0.433	0.693
Age at menarche	-11.779	56.524	-0.089	0.854
Chemotherapy courses	9.054	15.947	0.292	0.627
Pregnancy	-15.491	16.765	-0.381	0.453
Marital status	-139.542	236.839	-0.593	0.615
Ethnicity	-51.273	41.721	-1.080	0.344
Occupation	-4.679	47.010	-0.050	0.930

R^2=0.898; **adjusted R^2**=0.234; **F**=1.34 (**df**. 13); ***P* value** = 0.504

Results are significant at *P*<0.05