BARRIERS TO EARLY DIAGNOSIS, TUMOUR NECROSIS FACTOR AND RECEPTOR GENETIC VARIANTS AS POSSIBLE PREDICTORS FOR BREAST CANCER AMONG NIGERIAN WOMEN

BY

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DEDICATION

This work is dedicated to my parents

Mr. and Mrs.Sma Alamukii.

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ABSTRACT

Breast cancer is a major public health concern and early diagnosis is important in the treatment of the disease. In Nigeria, there has been an increase in breast cancer-related deaths, mostly because of delayed diagnosis due to reliance on clinical manifestation. Previous studies in Nigeria, identified mutant genes associated with breast cancer, but with limited information on Tumour Necrosis Factor-alpha (*TNF-α*) gene. The TNF-α is a cytokine that plays a significant role in initiation and progression of breast cancer. Therefore, this study was designed to determine the epidemiological factors affecting early diagnosis of breast cancer and identify key genetic variants of *TNF-α* and its receptor as potential predictors for breast cancer in Nigerian women.

This study was carried out at the University College Hospital, Ibadan, Nigeria from May 2017 to July 2021. Interviews were conducted with 25 breast cancer patients and 10 health workers using purposive sampling techniques. In the case-control quantitative study, 100 cases and 100 controls were recruited by randomised sampling. Socio-demographics were documented, and blood samples collected from each participant. The *TNF-α* and its receptor levels were quantified by ELISA, and *TNF-α (488 G/A, 238 G/A, 308 G/A, 859 C/T, 1032 C/T)* and its receptor *(TNFR1A+IV56+10 -G /A)* alleles were genotyped by allele specific PCR. Sequencing of *TNF-α* isolates was done using a nanopore sequencer. Interviews were transcribed and analysed using the thematic narrative. Descriptive statistics, unpaired t-Test, ANOVA and Fisher's exact test were used to analyse results with odd ratios at $\alpha_{0.05}$.

Breast self-examination, a post-symptomatic diagnostic procedure, emerged as the major factor (88.0%) preventing effective early diagnosis of breast cancer in health facilities. Other factors included inadequate awareness, cost of diagnosis, health insurance scheme, alternative medicine and religious belief. The age of breast cancer patients was 45.81±10.66 years and most of the participants (96.0%) had no family history of breast cancer. Eighty percent of cases never used birth control, while 95.0% had never taken fertility hormone pills. At diagnosis, 58.0% of cases presented with Grade 2 tumour. The *TNF-α* level was significantly lower in cases compared to controls and correlated with tumour grades (R^2=0.12). Soluble-*TNF-α* receptor levels were not significantly different between cases and controls. Five alleles showed a significant

association with breast cancer: *TNF-α 488G* (OR=0.24, 95% CI= 0.08-0.74), *TNF-α 380G* (OR= 0.51, 95%CI= 0.51-0.93), *TNF-α 308A*, OR = 0.33, 95%CI=0.14-0.78), and *TNFR1A+IV56+10-G* (OR= 0.35, 95% CI= 0.19-0.68) *TNF-α 1032C* (OR= 2.08 CI= 1.18-3.65). Other alleles: *TNF-α488A, 238G/A, 859C/T, 380A, 1032T and TNFR1A+IV56+10-A* showed no association with breast cancer. *TNF-α 488G, 308A* and *1032C* were associated with TNF-α levels in cases, while *TNF-α 488G, 238A* and *1032T* were associated with TNF-α levels in controls. The presence of single nucleotide polymorphisms of *TNF-α* was confirmed through sequence alignment.

Occurrence of breast cancer among Nigerian women is mostly sporadic and reliance on breast self-examination appears to be ineffective for early diagnosis. The Tumour Necrosis Factor-alpha gene variants (*TNF-α 488G, 308A, and 1032C)* might be predictors for breast cancer among Nigerian women.

Keywords: Breast cancer, Tumour Necrosis Factor-*alpha* (*TNF-α*), Single nucleotide polymorphisms, Soluble -Tumour Necrosis Factor Receptor 1, cancer in Nigerian women.

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LIST OF ABBREVIATIONS

A - adenine

ADCC- Antibody-dependent cell-mediated cytotoxicity

BRECAN- Breast Cancer Associations of Nigeria

cdk- Cyclin dependant kinase

conc- concentration

C- cytosine

DCIS- Ductal Carcinoma *in Situ*

DNA- deoxyribonucleic acid

dNTP- deoxynucleotide triphosphate

EDTA- ethylene diamine tetra-acetic acid

ELISA- Enzyme Linked Immunosorbent Assay

ER- oestrogen Receptor

EOBC- early-onset breast cancer

GF- Growth Factor

G-guanine

HER-2- Human Epidermal Growth Factor Receptor 2

IBC- Inflammatory Breast Cancer

mins- minutes

NCBI-BLAST-National Centre for Biotechnology Information-Basic Local Alignment Search Tool

ROS- Reactive oxygen species

rpm- revolution per minute

RTK- Receptor Tyrosine Kinase

PCR- Polymerase Chain Reaction

PI3K- Phosphatidylinositol 3-kinase

PLCγ- phospholipase Cγ

PR- Progesterone Receptor

SDG-Sustainable Development Goal

sec- seconds

SNP- Single Nucleotide Polymorphism

Src- non-receptor protein tyrosine kinase

STATs- Signal Transducers and Activators of Transcription

T- thymine

TME- tumour microenvironment

TNBC- Triple-negative Breast cancer

TNF-α- Tumour Necrosis Factor- alpha

TNFR1- Tumour Necrosis Factor Receptor 1

WHO-World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

As one of the leading causes of death among women, breast cancer is a serious public health concern (Akinde *et al.,* 2015; Sung *et al.,* 2021). According to Uchendu, (2020) breast cancer was top on the list of cancers among women and the commonest cancer when both sexes were combined in South-western Nigeria. The frequency of breast cancer varies in different age groups, with the highest prevalence seen in the 26–45 years old group (Adetifa and Ojikutu, 2011; Olaogun *et al.,* 2020) . This signifies that breast cancer development is concurrent with female biological development. Breast cancer is caused by a complex interplay between hereditary and environmental factors (Xu *et al*., 2014). The genetic factors include candidate genes and gene variants such as single nucleotide polymorphism (SNP), microsatellite markers, copy number variations, and other genetic markers that may influence the genetic stability, chromatin structure and transcriptional activity (Talseth-Palmer and Scott, 2011).

Single Nucleotide Polymorphisms (SNPs) are single nucleotide alterations in genes that can influence a change in the expression of a particular protein. These are the most prevalent genetic variation that affect repair of DNA mismatch, regulation of cell cycle, cell metabolism, and immunity in people and are connected with higher possibility of developing cancer (Deng *et al.,* 2017). The SNPs can be found in the promoter, exons, introns, 5′- and 3′ UTRs, among other areas of genes. Their manifestation in various genes have been implicated to influence the occurrence of breast cancer (Li *et al*., 2014). Many malignancies have been associated with SNPs in genes of some cytokines, including those for Tumor Necrosis Factor Alpha (TNF-α) and Interleukins (Marsh *et al.,* 2003; Aznag *et al.,* 2020). TNF-α is a significant pro-inflammatory cytokine that was formerly believed to have an anti-carcinogenic impact but was later discovered to be involved in carcinogenesis (Azmy *et al.,* 2004). It has been widely explored for

genetic variation with 16 identified polymorphisms in and around *TNF-α* promoter region, including 5 microsatellites, and some of them have been linked with various diseases (Marsh *et al.,* 2003; Sargen *et al.,* 2006).

Tumor Necrosis Factor Alpha SNPs have been linked with the possibility of having bladder cancer *(Marsh et al.,* 2003; Lu *et al.,* 2016). The *TNF-α 238G* was also found to be linked to high TNF-α production (Azmy *et al.,* 2004). It has been observed that this polymorphic form has an apparent protective role against a range of tumours (Azmy *et al.,* 2004). Ahmad *et al.,* (2020) in an Indian population, indicated a link between the *TNF-α 308* variant and breast cancer, while Xu *et al*. (2014) observed a link between SNPs of *TNF receptor 1A* (TNFRSF1A) gene and sporadic breast cancer among Northeast Chinese Han women. No link was observed between *TNF-238 & 308* and breast cancer susceptibility among a Caucasian population (Azmy *et al.,* 2004). These findings point to the significance of conducting studies on the levels of specific polymorphic types of *TNF-α* and their association with breast cancer in different countries.

Unpublished data from our group (M.Sc thesis, 2015. Unpublished) showed that among a cohort of Nigerian women, T*NF-1032C and 859T* exhibited a positive relationship with breast cancer while *TNF- 488A, 308G, and 380A* showed a negative relationship with breast cancer. It therefore appears that SNPs in *TNF-α gene* seems to be significant in the occurrence of breast cancer among patients in Nigeria. TNF-α receptors, TNFRSF1A and TNFRSF1B, has also been shown to be important in tumour growth and spread (Xu *et al*., 2014). Xu *et al*., (2014) showed that some TNF receptors SNPs might be linked to breast cancer occurrence. This cytokine, TNF-α is also a current immunotherapy target in cancer treatment research, thus, making it an important protein to study (Mercogliano *et al.,* 2020; Mercogliano *et al.*, 2021).

The most prevalent malignancy in Nigeria is breast cancer, liable for around 24.0% of all cancer-associated deaths (Ntekim *et al.,* 2022). Its prevalence has steadily increased over time with poor prognosis observed among patients (Akinde *et al.,* 2015; Sung *et al.,* 2021).The highest proportion of women who survive breast cancer are found in developed nations like Sweden, and Japan (about >80%), followed by middle-income countries (about 60%), and low-income nations (approximately 40%) (Francies *et al.,*

2020) . The lower survival rate in nations like Nigeria is brought on by a dearth of early detection programmes, a lack of facilities for diagnosis and treatment, and a large percentage of women who seek care when their illnesses are already advanced. Early diagnosis is the key to breast cancer survival, and patients who present with an advanced stage of the disease run a risk of poor prognosis (Ayoade *et al.*, 2014)

It is essential to determine the variables that limit timely diagnosis of breast cancer in Nigeria to increase patients' survival rates. In an effort to reduce the frequency of women being diagnosed with progressive stage of breast cancer in hospitals, some government and non-governmental organizations have focused their attention on the creation of awareness for women's self-breast examinations among other early detection programmes such as clinical breast examination and mammography. Nevertheless, the presence and activities of these organizations have not stopped women presenting with late-stage breast cancer at health care centres (Awofeso *et al.*, 2018; McKenzie *et al.*, 2018). Therefore, it is crucial to assess the existing awareness programme in Nigeria in order to comprehend why no appreciable change has been noted in the fraction of women coming up with an advanced breast cancer diagnosis in Nigerian hospitals.

In addition to awareness impact, there might be other factors that could hamper early diagnosis of breast cancer; which may include social/cultural, financial, personal, and structural barriers (Anderson and Tsu, 2008). Pruitt *et al*. (2015) identified several social hurdles to breast cancer diagnosis and treatment in Nigeria. These helped to explain why some patients delayed seeking medical attention and eventually present with progressive stages of the disease. These social hurdles included fear, the cost of a diagnosis, poor health-seeking behaviours, ignorance, and religious beliefs (Pruitt *et al.*, 2015; Akuoko *et al.*, 2017; Ilaboya *et al.*, 2018). Besides these, there are other barriers, such as structural barriers and inadequate health policies on cancer and other non-communicable diseases, which can affect timely detection of breast cancer in Nigeria. Structural barriers to health care in general, including infrastructure, transportation, appointment waiting time, clinics, and co-payments, can also affect breast cancer patients (Ilaboya *et al.*, 2018). Structural barriers to delayed breast cancer diagnosis in Nigeria have not been completely identified.

1.2 Statement of the Problem

According to Akinde *et al.,* (2015), there are roughly 10,000 cancer-related fatalities and 250,000 new cases reported per year in Nigeria. Deaths due to cancer of the breast in nations like Nigeria were linked to late clinical manifestation and inadequate facilities for detection and treatment *(*Afolayan *et al.,* 2012; Awofeso *et al.,* 2018). Most patients present with advanced stage of breast cancer at diagnosis with lumps ranging from 2 to 16cm in size (Ayoade *et al.,* 2014; Olaogun *et al.,* 2020). Poor prognosis experienced in Nigeria was largely due to late diagnosis (McKenzie *et al.,* 2018). Predictive diagnosis is therefore vital to determine the possible outcome of breast cancer. Hence, the need for research into new and effective genetic markers for the timely identification of risk for breast cancer, which can help in the prevention and improved treatment among women in Nigeria. The best-known genes connected with the risk of hereditary breast cancer are the tumour suppressor genes: BReastCAncer genes 1 and 2 (*BRCA1 & 2*) (Petrucelli *et al.,* 2022). However, the prevalence of the mutated form of these genes is generally low among women with breast cancer and differs within populations (Petrucelli *et al.,* 2022). Only 7.1 % and 3.9% of the 434 cohort of Nigerian patients with breast cancer evaluated in an investigation by Fackenthal *et al.,* (2012) had deleterious mutations in the BRCA 1 and 2 genes, respectively. This result was confirmed with a larger sample size by Zheng *et al.,* (2018). These data raised the possibility of the involvement of some other genetic factor(s) that might be responsible for the occurrence of the disease in the observed patients.

1.3 Justification of the study

The WHO Breast Cancer Global Initiative framework has recommended that countries focus on early detection programs for breast cancer, aiming to diagnose and treat at least 60% of cases as early-stage disease. As part of the Sustainable Development Goal (SDG) for good health for all, there is an opportunity to improve breast cancer management policies in Nigeria. This can be achieved by understanding the structural obstacles that hinder timely detection of breast cancer. Therefore, it is necessary to assess all barriers to timely breast cancer diagnosis and comprehend how these obstacles interact within the healthcare delivery and utilization system in Nigeria.

Moreover, this approach will advance our knowledge and comprehension of the factors contributing to the poor prognosis among breast cancer patients in Nigeria. It will also shed light on how these factors can be controlled to enhance timely detection and prognosis of breast cancer in the country. Despite the increasing awareness about breast cancer through the work of non-governmental organizations, a significant number of patients in Nigeria still present with late-stage breast cancer at healthcare centres (Awofeso *et al.*, 2018). This highlights the urgency to examine the current situation and understand why late-stage diagnoses of the disease persist in Nigeria.

Additionally, no study in Nigeria has investigated the association between *TNF-α* and receptor SNPs with breast cancer among Nigerian women. It is anticipated that identifying novel genetic markers for early or predictive diagnosis could enhance breast cancer care in Nigeria.

1.4 Aim and Objectives:

The aim of this study is to the determine the epidemiological factors affecting early diagnosis of breast cancer in Nigeria and to identify key genetic variants of TNF-α cytokine that can be used as possible predictors for breast cancer in Nigeria.

1.5 Specific Objectives:

The specific objectives are to:

1. investigate factors preventing early diagnosis of breast cancer in Nigeria.
2. describe the socio-demographic of breast cancer patients.
3. measure the levels of TNF-α and TNF-α receptors in plasma samples of breast cancer and control patients.
4. determine the distributions of *TNF-α* SNPs and *TNF-α* receptor SNPs found in breast cancer and control patients.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Cancer

Cancer comprises of illnesses that arise from the uncontrolled division of cells. Occurrence of these diseases can be in any part of the body, and there are various types of cancer, with distinct mode of diagnosis and treatment. Cancer is a complicated collection of diseases with many different manifestations that can be brought on by a mix of a person's genetic makeup and environmental influences. Retinoblastoma, ovarian cancer, leukemia, and breast cancer are just a few of the more than 200 different forms of cancer. Although, cancer typically manifests as a tumour made up of a mass of cells, cancer is a disturbance of the cells, and the apparent tumour is the final result of the culmination of numerous changes that may have taken years to develop. The process by which cancer develops in an individual is termed tumorigenesis or carcinogenesis.

2.1.1Tumorigenesis and Cancer Biology

Tumorigenesis is the process by which malignant characteristics, such as dedifferentiation, accelerated cell division, metastasis, circumvention of immune surveillance, and metabolism dysregulation, develop in normally occurring cells (Cao, 2017). Tumour cells significantly alter the cellular, molecular and physical characteristics of their host tissues to enable growth and progression (Anderson and Simon, 2020). A normal process of cell division and checkpoints that detect mistakes in DNA replication, chromosome segregation, and DNA damage brought on by endogenous or external agents are employed to monitor normal cell cycle progression. The cell cycle is stopped when these checkpoints are activated, allowing cells to correct any flaws and stop them from affecting new daughter cells.

There are proteins that interact with one another and carefully control the cell division in normal cells at several checkpoints. Checkpoints at each stage of the cell cycle ensure the cycle is successfully completed and prevent DNA that has not been fully copied from being transferred on to daughter cells. Cyclin-dependent kinases (CDKs) are the main proteins of this regulatory system and they are responsible for promoting advancement through the various cell cycle stages by phosphorylating and activating other downstream kinases (Fig. 2.1). Cyclins, are activating components that are synthesized and destroyed in a cell cycle-reliant manner and are necessary for CDK function. The CDK inhibitors further tighten the control over cyclin-CDK complexes (Wang, 2021).

In healthy cells, activation of p53, a transcription factor, can cause cell cycle seizure and apoptosis by inducing the production of cell cycle inhibitors, which prevent cell division until any damage has been revamped, or by triggering cell death, if the genomic damage is too much. Over 50% of all human tumours have abnormal p53 signaling. Cancer cells survive longer when the p53 protein is inactive because it is unable to signal and activate the cell's apoptotic machinery (Coskun *et al.*, 2022).

Likewise, proteins called growth factors (GFs) are also necessary for the physiological process of growth control, which strives to maintain tissue homeostasis. They transmit signals of cell development between cells. Through signaling pathways, certain growth factor receptors (GFRs) on the cell surface recognize these signals, which they then transfer to activate target molecules that promote proliferation. Receptor tyrosine kinases (RTKs) are a particular subset of GF receptors with tyrosine kinase action. The RTK is crucial in the control of the cell division process and there are 20 families of RTKs. The most commonly investigated RTKs in cell cycle research are the insulin receptor, epidermal growth factor receptor, platelet-derived growth factor receptor, and nerve growth factor receptor families. The cell division process is driven by GFs via activating RTKs and downstream signalling pathways that regulate cyclin-Cdk complexes. (Fig 2.2) (Stone *et al.*, 2022).

The following actions describe normal cell proliferation:

1. The association of a GF with its unique cell membrane receptor

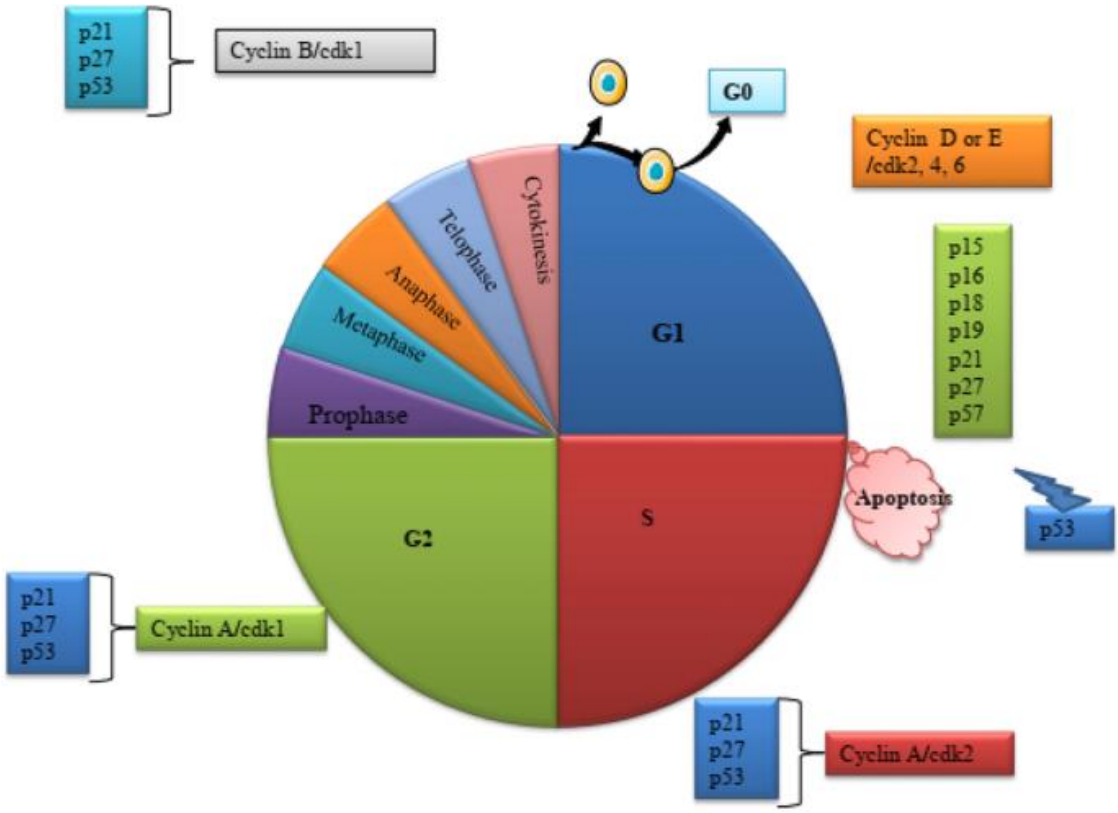

Fig. 2. 1: Regulation of the cell cycle and the cyclin-cyclin dependent kinase complexes (Karidio and Sanlier, 2021).

Keys

P- Tumour surpressor protein

G^0- Resting state or gap phase

cdk- Cyclin dependant kinase

G1 and G2- Growth phase 1 and 2

S- DNA synthesis phase

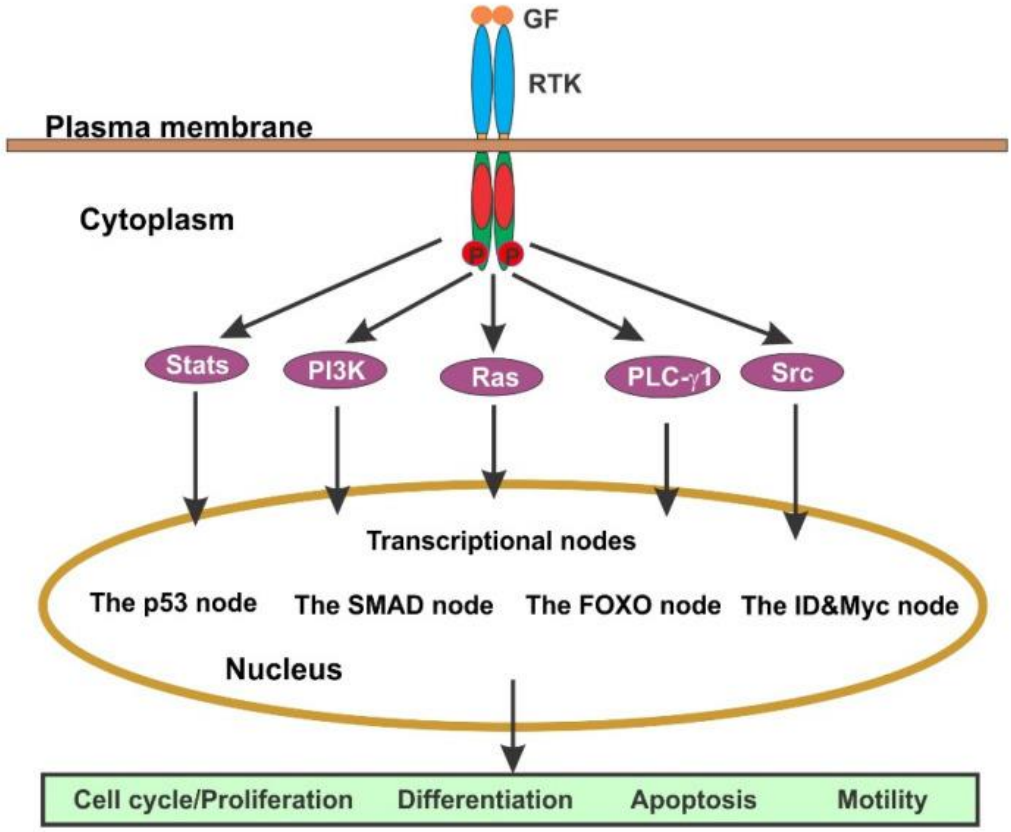

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Fig. 2.2: Growth factor signalling pathways through Receptor tyrosine kinases (RTKs)(Wang, 2021).

Keys

GF- Growth Factor

RTK- Receptor Tyrosine Kinase

PI3K- Phosphatidylinositol 3-kinase

PLCγ- phospholipase Cγ

Src- non-receptor protein tyrosine kinase

STATs- Signal Transducers and Activators of Transcription

2. A brief and transitory activation of the GFR that causes many signal-transducing proteins, including a Ras, to become active on the plasma membrane's inner leaflet.

3. Signal transduction molecules deliver the signal to cytosolic or nuclear locations, where it causes specific genes of cell cycle to start to be transcribed.

4. The cell's entry into the cell cycle, resulting in cell division (Wang, 2021).

This mechanism, which is frequently blocked in cancer, enables rogue cells to produce their own internal signals that promote proliferative differentiation and environmental independence. When GFR gene alterations take place, which facilitate activation in the absence of GFs or when GF overproduction resulting in an autocrine signaling loop, cancer cells can produce their own growth stimulatory signals.

Other elements of cell signaling

Constitutive prompt of internal signaling components is an alternative method for making tumour cells GF independent. When a GFR is triggered, for instance, the Ras protein can be activated, altering it from a quiescent state to an active, signal-producing one. Ras, for instance, is normally switched off in normal cells and does not signal unless a GFR is engaged. After that, the Ras protein are able to release additional downstream signals that can stimulate division. This signaling pathway is dysregulated in tumour cells as a result of the ability of structurally altered Ras proteins to constantly transmit growth-enhancing signals into the interior of the cell deficient of GFs (Wang, 2021).

2.1.2 Tumour microenvironment in cancer development

A growing tumour microenvironment (TME) is a complex, dynamic phenomenon. Although the tumour microenvironment may vary depending on the form of tumour, immune cells, extracellular matrix, stromal cells and blood vessels are common elements. Anderson and Simon (2020), regarded the TME as an important inducer of cancer progression. Immune surveillance, which takes place during the elimination phase (cell death) to inhibit the formation of malignancies, depends on innate and adaptive immunity (Bates *et al.,* 2018). If immune control over the changed cells is weakened during the elimination phase, tumour development will result. As the tumour stroma and tumour cells grow, the immunosuppressive processes become more

pronounced. Despite the immune system's capability to detect and abolish cancer cells, the tumour continues to grow until it escapes detection. Immune cells like T-helper cells, natural killer cells, CD8+ T cells, dendritic cells and proinflammatory macrophages (M1) influence the immune responses against tumour growth (Bates *et al.*, 2018). Tumour cells elude the immune system through down-regulation or loss of tumour antigens, production of immunosuppressive substances like exosomes, IL-10 and transforming growth factor, loss of adhesion molecules like ICAMI. They can also develop resistance to apoptosis through up-regulation of BCL-2 and other anti-apoptosis molecules (Labani-Motlagh *et al.,* 2020).

Chemicals produced by tumour cells contributes to the TME which abates effective immune responses against tumour (Baghban *et al.,* 2020; Anderson and Simon, 2020). The lytic enzymes secreted by M1 macrophages, however, promotes antibody-dependent cell-mediated cytotoxicity (ADCC) that destroys tumours. Basophils release chemokines like CCL3 and CCL4 to promote CD8+ T cell recruitment into the inflammatory tumour tissue (Anderson and Simon, 2020). Eosinophils are drawn to solid tumors by the substances that the tumour cells release (Anderson and Simon, 2020). By secreting granzyme A and TNF-α via degranulation and by binding to NKG2D ligands (NKG2DLs) to cancer cells, eosinophils regulate T-cell activation in addition to inducing anti-tumor responses (Labani-Motlagh *et al.,* 2020).

Lastly, neutrophils of the N1 type promote the stimulation of anti-tumour T cell responses, the stimulation of cancer cell apoptosis by TRAIL and the production of ROS (Anderson and Simon, 2020).

2.1.3 Carcinogens and mutagens

The phenotypic changes a cell experience during the malignant transformation process are a reflection of the progressive genetic mutations that are acquired (Chatterjee and Alfaro-Moreno, 2023). This multi-step process, which may take twenty years or longer, is not an instantaneous change from normal to malignant growth (Chatterjee and Alfaro-Moreno, 2023). Critical genes, comprising oncogenes, tumour suppressor genes, and DNA repair genes, can become mutated, causing genetic instability and a progressive loss of differentiation. Cancer cells are able to control cell division through the development of their own vascular system, which causes tumours to grow larger (angiogenesis)(Zuazo-Gaztelu and Casanovas, 2018). The altered cells are able to

communicate with one another, proliferate out of control, infiltrate nearby tissues, and eventually move through the lymphatic or blood systems to distant organs (Fares *et al.*, 2020).

Cancer can develop as a consequence of both inherited genetic genes and environmental factors (such as radiation, viruses, and chemical carcinogens). The initial stage of carcinogenesis, which results in the accumulation of mutations, is triggered by DNA damage brought on by mutagens (Kumari *et al.*, 2021). Mutational events are assumed to be the main cause of genetic and epigenetic changes like DNA breaks, DNA formation, changes to microRNA, DNA crosslinks, nucleotide mismatches, modification of histones, and DNA methylation. Yet, DNA repair processes are focused on maintaining the DNA to ensure that genetic stability is achieved; any deviations from these meticulously regulated systems might lead to the emergence of cancer (Fig. 2.3). Physical, biological, and chemical mutagens can all result in this varied damage to DNA (Temko *et al.*, 2018). Physical substances that are strong carcinogens, include lead, arsenic, cadmium, and mercury. These mutagenic substances act together with DNA-binding proteins to adhere to DNA and change its base sequence, or they interfere with DNA repair mechanisms, growing the rate of alterations and resulting in carcinomas with various origins (Kumari *et al.*, 2021). Additionally, these mutagens can increase the amount of ROS accumulation that binds to nucleic acids, speeds up apoptosis, and raises oxidative stress, which can result in the growth of tumors and unclear abnormal cellular checkpoints. Melphalan, benzidine, and diethylstilbestrol are a few examples of chemical mutagens that mediate base-analog and induce pyrimidines to shift and cause mutagenesis in healthy cells. Other mutagens include DNA intercalating agents, deaminating agents, and alkylating agents. Studies have also identified a number of microbial and viral infectious mutagens that compromise the biological integrity of cells. A few examples of mutagens that are known to increase cellular damage and have been linked to carcinogenesis are the human papillomavirus, hepatitis B and C, and Epstein-Barr viruses (Kumari *et al.*, 2021).

Another prevalent mutagen is Ultraviolet (UV) light, which inhibits apoptosis and causes healthy cells to become malignant (particularly skin tumours) (Laikova *et al.*, 2019). The DNA molecule is excited by these UV rays, which lead to crosslinking and single-strand breaks (SSBs), which then result in the creation of pyrimidine dimers

(Kumari *et al.,* 2021). In general, cytosine (C) and thymidine (T) are more photosensitive and form dimers when exposed to UV radiation. These dimers weaken H-bonds, alter the DNA's helical shape, and prevent the replication fork from moving forward. Furthermore, numerous animal models have demonstrated that UV radiation has the capacity to both initiate and advance malignant melanoma. Additionally, it modifies cytokines like TNF-α and interleukins (ILs), which indirectly impacts the manufacturing of matrix metalloproteinases (MMPs) and encourages melanoma invasion and metastasis (Kumari *et al.,* 2021) (Fig. 2.3).

2.2 History, Epidemiology and Biology of breast cancer

Cancer of the breast, which develops in and around breast tissues, is the most occurring malignancy in women. The history of breast cancer is as long as human history. It was first discovered as early as 3,000 – 2500 BC and was documented in an old Egyptian text Edwin Smith Papyrus named after Edwin Smith (Lakhtakia and Chinoy, 2014). The literature stated that breast cancer was untreatable when it feels cool to touch, protruding, and spread around breast (Lakhtakia and Chinoy, 2014).

Over time, striking increase in the possibility of having breast cancer has been observed. In 2020, the incidence was 2.3 million globally, out of which 685, 000 deaths were recorded (Arnold *et al.,* 2022). Breast cancer has the highest frequency in the world, with 7.8 million cases reported during a five-year period (Arnold *et al.,* 2022). In the early nineties, breast cancer incidence in Nigeria was a few hundred (Pearson, 1963; Ihekwaba, 1992), to thousands per year in recent times (Todua *et al.,* 2015; Arnold *et al.,* 2022) . One of the lowest breast cancer survival rates globally occurs in Nigeria. Nigeria recorded 50% of all incidents of fatality in 2012 (Bray *et al.,* 2012) and as of 2020, Nigeria continues to be one of the countries with a high rate of 20.1% per 100,000 people breast cancer fatality (Sung *et al*., 2021) (Fig. 2.4). This low survival rate in Nigeria has been attributed to the nature of the different breast cancer subtypes, low awareness, inadequate healthcare infrastructure, limited access to care, and a lack of government interest in non-communicable diseases.

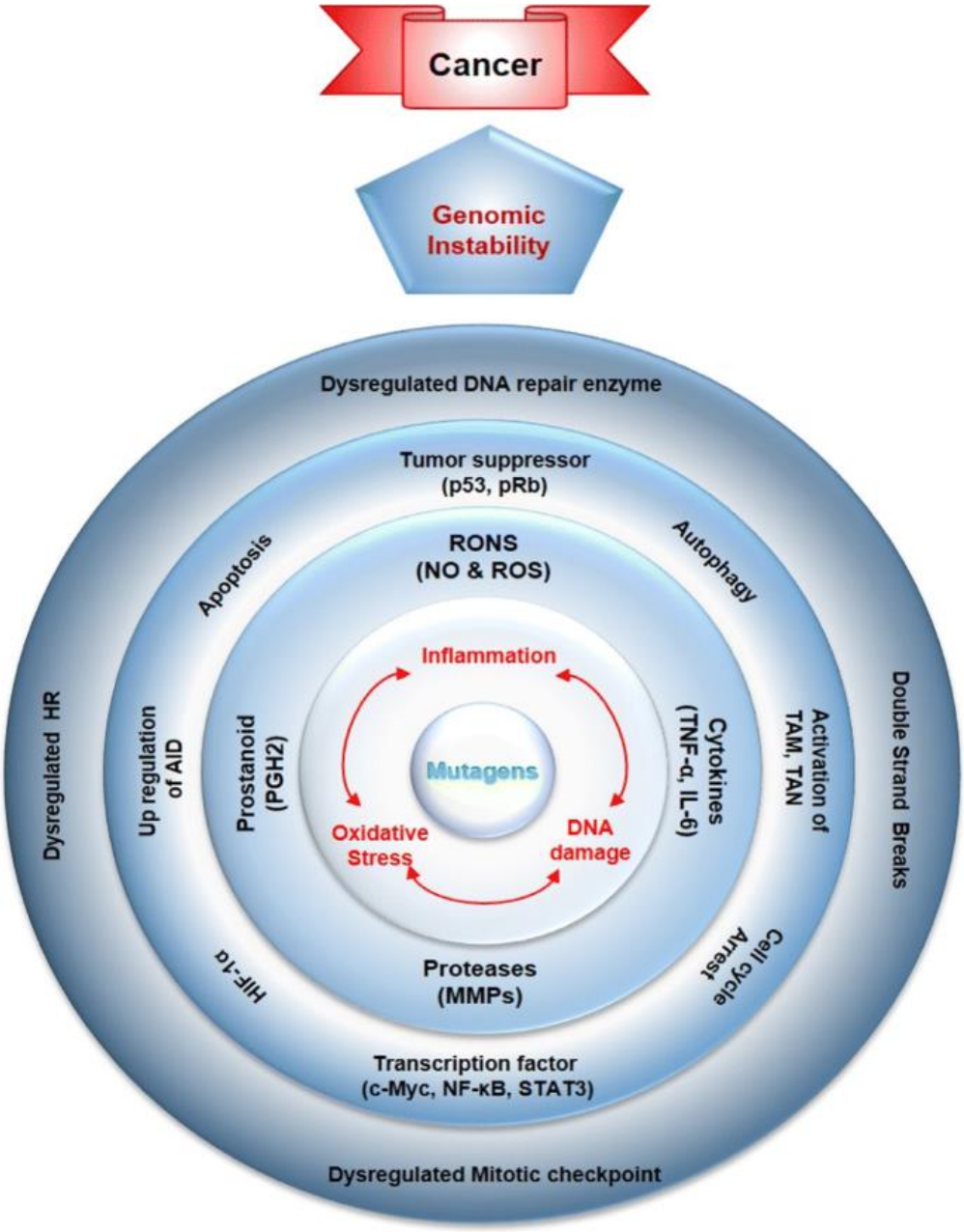

Fig. 2.3: The systematic basis of inflammatory responses and oxidative damage initiated by mutagens leading to genomic instability and consequently tumorigenesis (Kumari *et al.*, 2021).

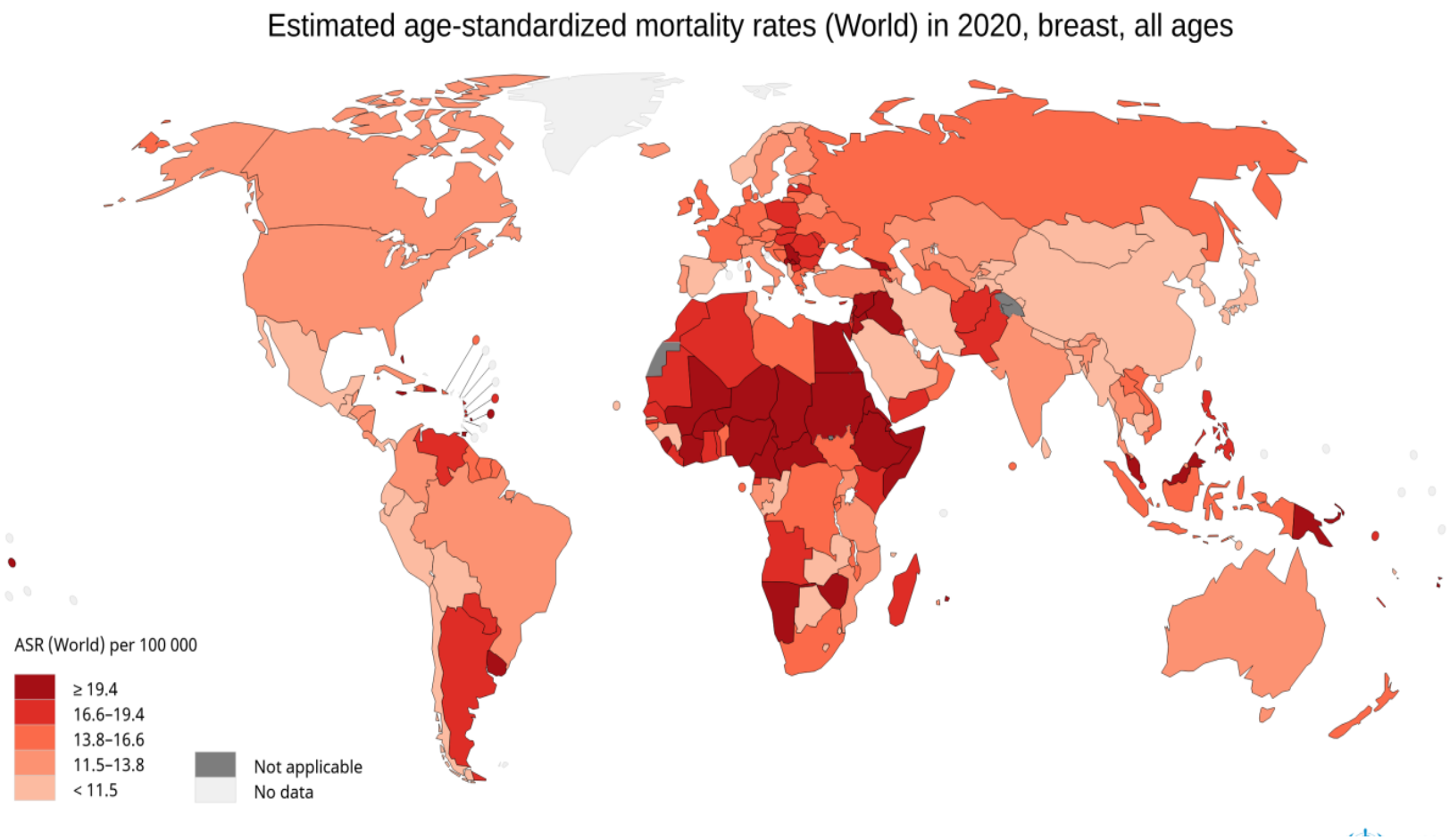

Fig. 2.4: Mortality rate of breast cancer across the world (Sung *et al.*, 2021)

2.2.1 Categories of Breast Cancer (Plichta *et al.*, 2018)
The different breast cancer types includes.

1. **Ductal Carcinoma *in Situ* (DCIS):** It falls within the non-invasive or pre-invasive categories. Other names for it include intra-ductal carcinoma and stage 0 breast cancer. The duct's lining cells develop into malignant cells during this condition, but they do not move to the neighboring breast tissue through the duct's walls. Most women who have breast cancer at this stage can be treated because it is in its early stages.
2. **Invasive Breast Cancer (IDC/ILC):** Breast malignancy that has extend to the tissues around the breasts are invasive. Although, invasive ductal carcinoma and invasive lobular carcinoma, which both begin in the milk duct lining and the lobule, respectively, are the two most frequent kind of breast cancer, the majority of cases fall into this category.
3. **Triple-negative Breast cancer:** The tumour cells produce little HER2 protein and neither estrogen nor progesterone receptors. It makes up between 10% and 15% of all cases of breast cancer and develops and spreads more quickly than other invasive types.
4. **Inflammatory Breast Cancer (IBC)**: IBC develops when cancer cells block the lymphatic capillaries in the skin, giving the breast an irritated appearance. Only 1% to 5% of all breast cancers are this uncommon subtype, which is unusual.
5. **Breast Paget disease:** Nipples and areola of the breast are usually impacted. DCIS or IDC are frequently present.
6. **Breast angiosarcoma:** The cells lining the lymphatic and blood arteries are where this uncommon type of breast cancer grows. It frequently develops following problems from prior radiation therapy for the breast, and it has a predisposition to grow and spread quickly.
7. **Phyllodes Tumour:** It appears in the breast as a connective tissue tumour. Most often affecting women in their 40s, it is a rare kind of breast cancer. Majority of phyllodes are benign tumours, although 25% of them are malignant.

2.2.2 Breast cancer symptoms, diagnosis, and treatment

Breast cancer may cause several signs and symptoms which include:

1. Lump in breast and under arm after menstruation with painless sensation
2. Change in size, contour, temperature of breast
3. Itching, burning sensation and nipple retraction
4. A reddish surface like orange skin in advance case

Breast cancer treatment and survival depend on early diagnosis. Breast cancer is usually detected using a variety of tools which includes magnetic resonance imaging (MRI), ultrasound, mammography, genetic tests, clinical and self-evaluation (Black and Richmond, 2019). A hormone receptor test is carried out once the presence of cancer cells in the breast has been confirmed in order to ascertain how receptive the cancer cells are to hormones like progesterone, oestrogen, and human epidermal growth factor and to select the most appropriate course of treatment.

The recommended treatment for breast cancer is neo-adjuvant chemotherapy, while additional effective options include surgery, radiation, and immunotherapy. Usually, the therapies are combined to have the most effective treatment (Korde *et al.,* 2021). Chemotherapy can be combined with radiotherapy, surgery, or immunotherapy, but the responsiveness of a patient is different from one patient to another (Selli and Sims, 2019). This has led to the advocacy of personalized medicine, where the genome of a patient are used to determine which treatment option will best suit the patient (Adeniji *et al.,* 2021).

2.2.3 Stages and grades of breast cancer (Plichta *et al.,* 2018)

According to how far the cancer cells have spread, there are five distinct stages of breast cancer (Fig. 2.5).

Stage 0: The non-invasive stage, commonly known as the precancerous stage. This stage is easily treatable. Ductal carcinoma in situ is the most prevalent kind (DCIS).

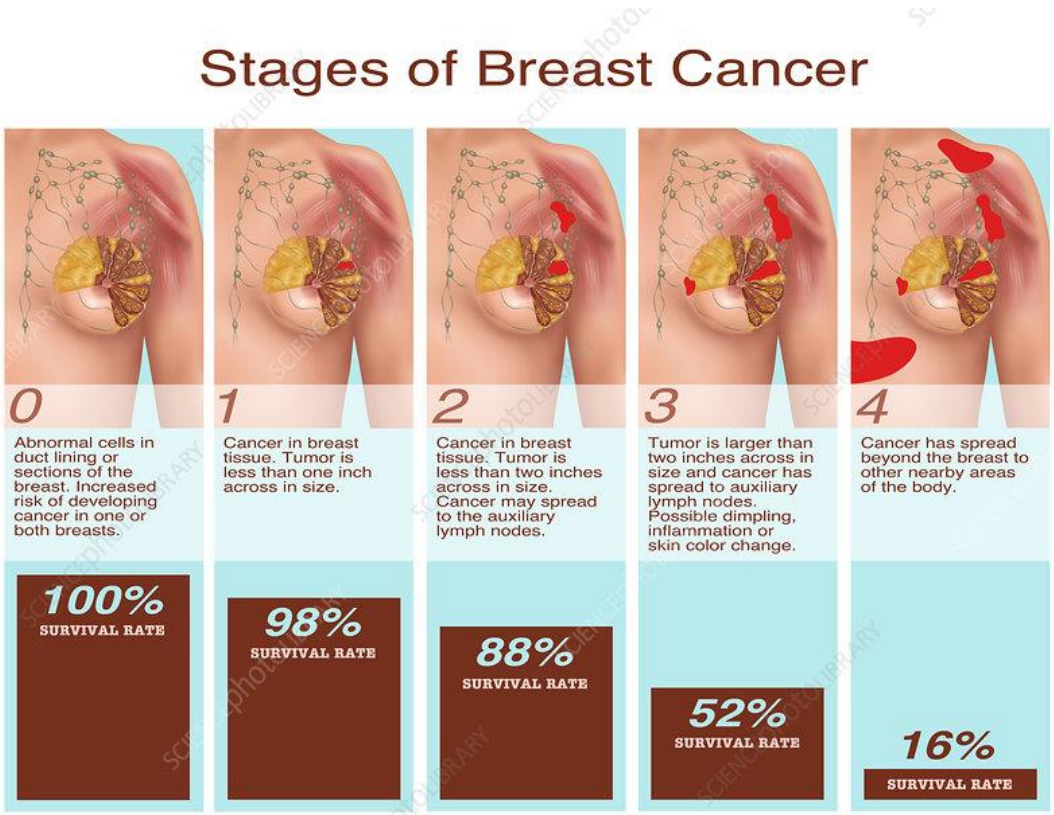

Fig.2.5: Stage of breast cancer and survival rate at detection.

Source: ("Breast Cancer Stages, Illustration Photograph by Gwen Shockey - Pixels," Last Accessed: April 5, 2023).

Stage I: A very early stage of invasive carcinoma, the tumour cells have moved to nearby healthy breast tissue but are still contained within a small area. Stage I is further subdivided into the following two categories:

1. Lymph nodes are cancer-free, and the tumor measures up to 20millimeters.

2. Breast cancer with a tumour size of 20 millimeters or less and tiny clusters of cancer cells in the lymph nodes.

Stage II: This no longer just affects a tiny portion of the breast. It indicates how many lymph nodes might contain malignant cells. There are two subcategories within this stage as well.

One of the following is used to determine Stage IIA:

1. The lymph nodes under the arm have developed up to 20 millimeter-sized breast cancer.

2. There is a 20–50 millimeter breast tumour present but yet to spread to the lymph nodes. The following standard determines Stage IIB:

1.A 20 to 50millimeter breast tumour is visible, and the tumour has progressed to one–three surrounding lymph nodes.

Stage III: This has spread beyond a small area of the breast. The criteria for Stage IIIA are one of the following:

1. Breast tumor present or missing, but four to nine surrounding lymph nodes are cancerous.

2. One to three nearby lymph nodes are cancerous, and the breast tumour is larger than 50 millimeters.

In stage IIIB, the tumour has extended to the chest area beneath the breast;

1. The skin may be swollen or inflamed because the cancer may have spread there.

2. It may have pierced the skin, leaving a wound or ulcerated region.

3. Up to nine axillary nodes (lymph nodes under the arms) or nodes adjacent the breastbone may have been affected by its spread.

In stage IIIC, there may be any size tumour in the breast or none at all.

The malignancy, however, might have migrated to one of the following locations:

1. Ten or more axillary (underarm) lymph nodes

2. Lymph nodes close to the collarbone,

3. A few lymph nodes under the arms and those near the breastbone

Stage IV: The tumour has metastasised from the breast to adjacent lymph nodes and other parts of the body at this stage, which is the most advanced. This suggests that additional organs could be affected, including the liver, brain, lungs, and bones. Breast cancer may already be in stage IV when it is initially identified or be a widespread recurrence of an earlier instance.

2.2.4 Grades of Tumour in Breast Cancer

Cancer cells are graded based on how much they look like normal cells. The grade is important in the prediction of prognosis and identification of the best treatment that can be used for a patient. There are three categories of tumour grades:

1. **Grade 1:** The lower grade, grade 1, shows that the cancer is less likely to spread and is growing more slowly. This implies a score of 3-5 for invasive carcinoma, although in DCIS, the cells typically exhibit ER-negative and PR-negative estrogen and progesterone receptors.
2. **Grade 2:** Grade 2 is the intermediate grade, where the cancer is developing more swiftly than grade 1 cancers but more slowly than grade 3 cancers. This is based on a 6,7 score for invasive breast cancer.
3. **Grade 3:** is a high grade that denotes a cancer that is spreading more quickly. In DCIS, these cells frequently lack ER- and PR-positive progesterone and estrogen receptors. With an 8.9% likelihood of developing into invasive breast cancer, high grade DCIS is more likely to progress (Fig. 2.6).

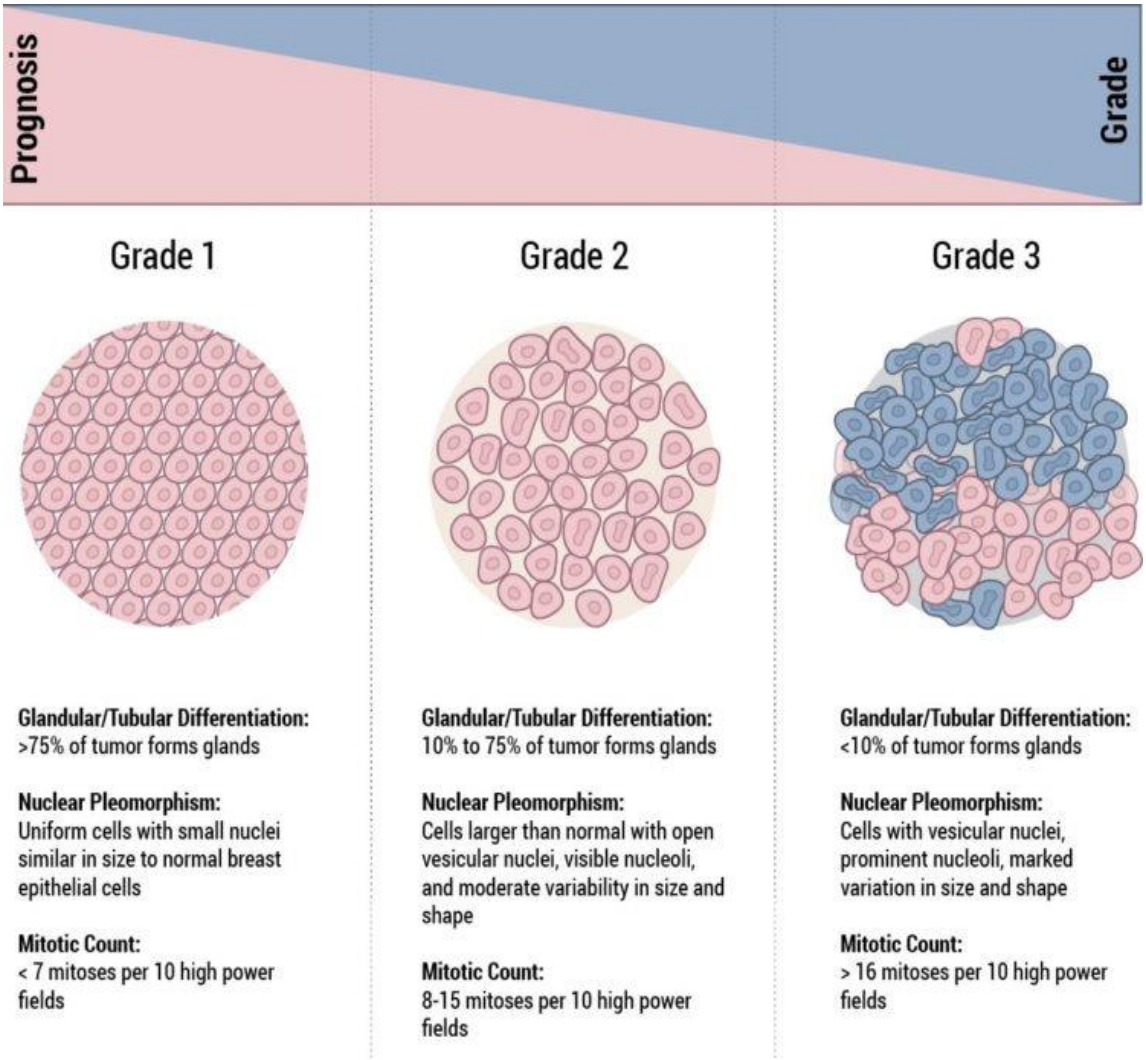

Fig. 2.6: Different categories of breast cancer tumour grades. Source: ("Staging & Grade - Breast Pathology | Johns Hopkins Pathology,") last access: April 5, 2023

Necrosis, or dead or dying cells, is a sign that the tumour is expanding swiftly. The presence of dead or dying cells in the breast duct is known as "*Comedo necrosis*." A high grade of DCIS is frequently associated with *comedo necrosis*, which increases the likelihood that it will progress to IDC.

2.3 Risk factors for breast cancer

Risk factors are things that could make someone more likely to get a certain disease. Breast cancer risk factors can be categorized in two different groups:

1. Adjustable risk factors

These are risk factors that can be altered for example life style which may include alcohol consumption, smoking, physical in-activity.

2. Non-adjustable risk factors

These are factors that cannot be altered or changed but their effect can be controlled through changes in life style e.g. age, hormones, genes.

2.3.1 Modifiable risk factors

Consumption of Alcohol

Adult females who consume alcohol have higher possibility of having breast cancer and the risk increases with increase in alcohol use (Ying *et al.*, 2015). Among breast cancer survivors, the relationship between alcohol consumption and overall survival is debated; while some studies find no decrease in overall survival, others suggest an increased risk of breast cancer-specific mortality, particularly in certain patient subgroups, with higher risk observed in cases of moderate to heavy alcohol consumption (LoConte *et al.*, 2018). Notably, among women with oestrogen receptor-positive breast cancer, those consuming seven or more drinks per week exhibit a 90% heightened risk of asynchronous contralateral breast cancer, exceeding the 30% increased risk found in a multicenter case-control study (LoConte *et al.*, 2018). Additionally, it has been demonstrated that drinking alcohol between a woman's first period and her first pregnancy may raise her possibilities of developing breast cancer (Allen *et al.*, 2009).

Body Mass Index (BMI), Overweight & Obesity

Body Mass Index, a measure of body fat based on height and weight is an essential factor for breast cancer. Poor dietary habit was implicated in poor prognosis of breast cancer (Lin *et al.*, 2019). Research have shown that obesity and higher BMI might cause some epigenetic modifications that affect development of breast cancer and its prognosis(McCullough *et al.,* 2016; Johansson and Flanagan, 2017). Hair *et al*. (2015)found that breast tissue underwent several BMI-related alterations. Also, they claimed that individuals with high body mass indices had higher rates of methylation in the genes involved in metabolism of energy, epithelial-stromal interactions, and inflammatory response (Hair *et al*., 2015).

Other adjustable factors include smoking, pregnancy and physical inactivity.

2.3.2 Non-modifiable risk factors

i. Age: A significant factor that influence breast cancer in women is age. An analysis of the morbidity factors for the Polish population revealed a direct rise in the fraction of women aged 40 to 59, followed by a plateau and a slight decline in the fraction of people aged 70 and older (Kamińska *et al.,* 2015). The ultimate age of diagnosis for breast cancer in Nigeria has been between 39 - 45 years since 1963 (Pearson, 1963; Ihekwaba, 1992; Zheng *et al*., 2018). The age at which neoplastic changes in breast diagnosed and the production of the oestrogen receptor detected in the tissue samples from the tumour under study showed a very fascinating association (Kamińska *et al.,* 2015). Contrary to oestrogen negative (ER-) tumours, which are more frequent up to the age of 50 and then reach a plateau beyond that, neoplasms exhibiting estrogen receptor overexpression oestrogen positive (ER+) are characterized by an increase in frequency with age (Kamińska *et al.*, 2015)

ii. Hormonal contribution to breast cancer development

Hormones are assumed to be the key cause of breast cancer. The triggering of mitosis by oestrogen and progesterone to increase division epithelial cells of the breast comes with likelihood of mutation leading to tumour formation (Pike *et al.*, 1993; Trabert *et al*., 2020). Based on this, cancer cells in breast tissues are said to either respond to hormones if they have hormone receptors or not respond to hormones if they do not

have hormone receptor. This characteristic of breast cancer tissues has been used to molecularly define its subtypes and has been used to proffer management options for breast cancer patients (Ades *et al*., 2014; Łukasiewicz *et al*., 2021). The molecular subtype classifications are:

1. Progesterone Receptor (PR) Positive or Negative
2. Oestrogen Receptor (ER) Positive or Negative
3. Human Epidermal Growth Factor Receptor 2 (HER-2) Positive or Negative.

Survival rate of breast cancer sufferers have also been attributed to their hormone receptor status. Patients that are ER/PR positive tends to have better prognosis compared to patients that are ER/PR negative (Dunnwald *et al.*, 2007; Lamb *et al.*, 2019). Additionally, clinical trials have demonstrated that adjuvant hormonal and/or chemotherapeutic regimens improve the survival benefit for females with hormone receptor-positive cancers (Dunnwald *et al.*, 2007; Lamb *et al.*, 2019).

iii. Family History of Breast Cancer

Family history is another crucial factor; 5–10% of cases are connected to a family history (Liu *et al*., 2021). Also, according to Liu *et al*., (2021) patients' breast tumours are typically less aggressive if they don't have a family history.

iv. Genetics of breast cancer

Genetics with relation to breast cancer has evolved into a key component of breast cancer care. It affects guidelines for screening, follow-up, prophylaxis, and treatment for women carrying germ line breast cancer susceptibility genes. It is crucial to identify minor groups of patients that have a different prognosis or a different response to treatment. Pathogenic mutations in the breast cancer predisposition genes (BRCA 1 & 2) are the most noteworthy inheritable risk factor for disease development, particularly early-onset breast cancer (EOBC)(Criscitiello and Corti, 2022). About 10% to 20% of EOBC cases are thought to be hereditary (Siddig *et al.*, 2021). Additionally, research has revealed that the therapeutic importance of the germline BRCA1/2 status in determining a patient's treatment type for breast cancer (Jiang *et al.*, 2021). The BRCA genes predict vulnerability to platinum-based chemotherapy, indicating the ability of the treatment to disrupt DNA repair paths (Siddig *et al*., 2021).

In addition to the BRCA 1 and 2 genes, other high- to moderate-risk genes with pathogenic mutations include the tumour protein p53 (TP53), the associate and localizer of BRCA2, PALB2, the checkpoint kinase 2 (CHEK2), and mutant ataxia-telangiectasia (ATM) (Abdel-Razeq *et al*., 2021). The bulk of hereditary breast cancer occurrences are brought on by alterations in the *BRCA1 or 2* genes (Abdel-Razeq *et al*., 2021). Hereditary mutations in the TP53, BRCA1, or PALB2 genes account for one in eight cases of invasive breast cancer in Nigerian women (Adedokun *et al*., 2020). The lifetime risk of breast cancer is increased by up to 80% due to several highly penetrant genes (BRCA1, BRCA2, PTEN, TP53, CDH1, and STK11), which also is responsible for up to 25% of hereditary cases (Abdel-Razeq *et al*., 2021). An additional 2%–3% of instances are caused by rare but moderate-penetrance gene mutations (such as those in the CHEK2, BRIP1, ATM, and PALB2 genes), which each carry a two times increase in risk (Abdel-Razeq *et al*., 2021). Other moderate risk genes include *BARD1*, *BRIP1*, *MRE11A*, *NBN*, *RAD50*, *RAD51C*, *XRCC2*, *RAD51D*, *ABRAXAS*. Prediction models indicate that it is unlikely that there will be any further high-penetrance genes that have not yet been discovered (Shiovitz and Korde, 2015). However, there has been continuous discovery of intriguing alleles of low-penetrance single-nucleotide polymorphisms (SNPs) that add to the risk in a polygenic manner (Shiovitz and Korde, 2015). Unlike inherited or germline mutations, breast cancer can also be caused by sporadic or spontaneous mutation usually known as sporadic breast cancer.

Sporadic breast cancer arises from the episodic increase of acquired, uncorrected alterations in somatic genes without the involvement of germline genes. An early event in spontaneous tumour is the activation of oncogenes, frequently in conjunction with inactivation of tumour suppressor genes. This is likely followed by subsequent, independent mutations in at least four or five additional genes, the order of which is probably less important.

It has been determined that cancer causing genes such as MYC, Cyclin D1, and HER2/neu have an early effect on sporadic breast cancer (Sirisena *et al*., 2018). Other genes that are linked to sporadic breast cancer include *XRCC2*, *PHB, CDH1* and *ATM* (Sirisena *et al*., 2018). BRCA1/2 mutational inactivation in sporadic breast cancer is

uncommon because inactivation demands that both gene copies be altered or completely eliminated (Sirisena *et al*., 2018).

2.4 Epidemiological barriers to the early diagnosis of breast cancer

Late diagnosis is the hallmark of breast cancer in most developing countries which has led to an increase in the mortality of patients due to poor prognosis. Studies have identified some obstacles to early diagnosis and early presentation of breast cancer in different countries. Early detection of breast cancer in the South Indian population has been hindered by low rates of Breast Self-examination (BSE) practices, infrequent routine clinical examinations of breast, inadequate mammography services, high screening costs, and challenging travel conditions for mammography (D'Almeida and Latha, 2021). Inadequate awareness of the disease, information about it, preventative measures, and early detection programs are part of the obstacles to early identification of breast cancer in Ethiopia (Getachew *et al*., 2020). In Indonesia, two barriers were discovered. These included patients seeking alternative therapies and inadequate knowledge on breast cancer's early warning symptoms (Pratiwi and Hamidiyanti, 2020). According to Akuoko *et al*. (2017), socio-cultural elements including tradition, belief, and fear play a role in how women approach getting medical care for cancer. Women of sub-Saharan Africa also lack awareness on timely detection tests for breast cancer. All of these papers demonstrated that, depending on the population, various groups have different barriers to early diagnosis of breast cancer.

Nigeria is one of the nations in the world with a very high rate of breast cancer mortality among women (Unger-Saldaña, 2014). To reduce this growing mortality rate, there is a need to enhance diagnosis and treatment facilities in Nigeria. Breast cancer diagnosis in Nigeria begins with a physical examination by a physician followed by mammography, chest x-ray and tissue biopsy check (Jedy-Agba *et al*., 2017).

Breast tumours are characterized into several categories during diagnosis based on how aggressive and distinct the breast cells are from tumour cells. Grade 1 is a benign, slow-growing breast cell; grade 2 is a rapidly differentiating breast cell that develops into a tumour cell; and grade 3 is an aggressive, rapidly growing aberrant cell (Fig.2.6). Grade 3 was the most prevalent grade of diagnosis among Nigerian women with breast cancer out of all of these grades (Adisa *et al*., 2012; Usman *et al*., 2019).

Barriers to breast cancer timely detection in Nigeria have many faces, starting from patients' delay in getting diagnosed to structural barriers in the process of diagnosis. Patient delay may be due to disease ignorance (lack of awareness), poor health seeking behavior, denial and dismissal of symptoms, social and cultural beliefs, and seeking alternative medicines (Pruitt *et al*., 2015).While structural barriers such as a lack of infrastructure, access to transportation, and inadequate health policies may exist (Pruitt *et al*., 2015; Ogunkorode *et al*., 2017).

2.5 Breast cancer in Nigeria

Cancer is a key concern to global health, it has impaired the life expectancy in many countries. Nigeria is one of the countries experiencing highly reduced life expectancy (mortality due to cancer at age < 50) (Sung *et al*., 2021) (Fig 2.7). The most frequent malignancy among women and generally in Nigeria is breast cancer 38.7% & 22.7% respectively according to data form GLOBOCAN published in 2021 (Fig. 2.8a&b). The biology of breast cancer in Nigerian women is characterised with a highly aggressive phenotype, resulting in poor prognosis (Banjo *et al*., 2008; Adisa *et al*., 2012). High frequencies of Triple Negative Breast Cancer (TNBC) have been observed among Nigerian women. The features of TNBC include high invasiveness, high metastatic potential, tendency for relapse, and poor prognosis with a variety of genetic variations compared to European or American populations (Wright *et al*., 2018). Standardized TNBC treatment protocols are still lacking because TNBC tumours do not express ER, PR, or HER2. Additionally, TNBC tumours are not responsive to endocrine therapy or HER2 treatment (Yin *et al*., 2020). This aggressive phenotype commonly experienced by Nigerian women is not the only limiting factor for good prognosis of the disease. There are other structural and social barriers deterring patients from accessing good quality health care. Breast cancer risk factors for in Nigerian women include age, menarche age, parity, length of nursing, history of the disease in family, height, BMI, presence of benign tumour in breast, and alcohol usage. These elements were combined in a risk prediction model that Wang *et al*. (2018) proposed for Nigerian women.

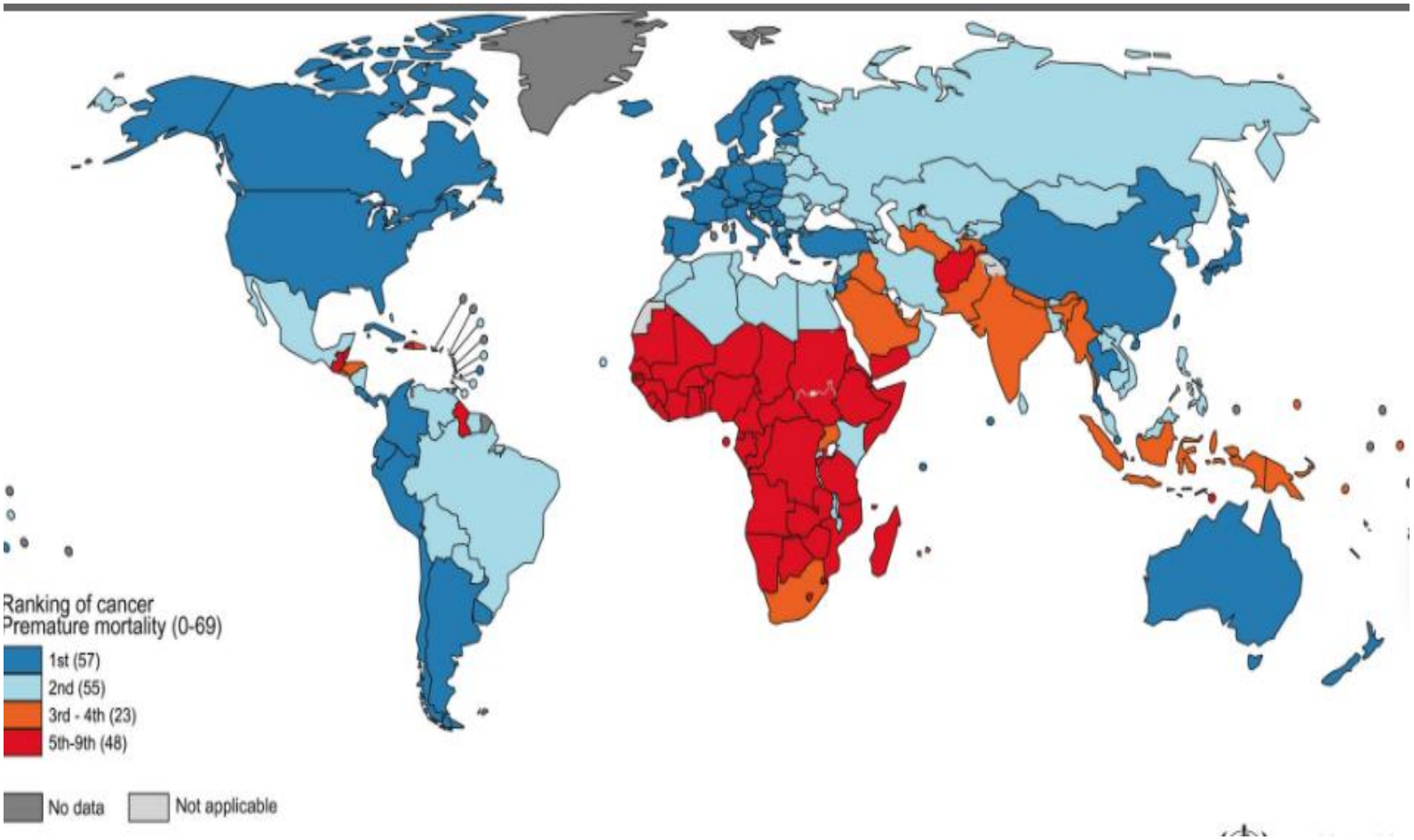

Fig. 2.7: Global life expectancy for breast cancer (Sung *et al*., 2021).

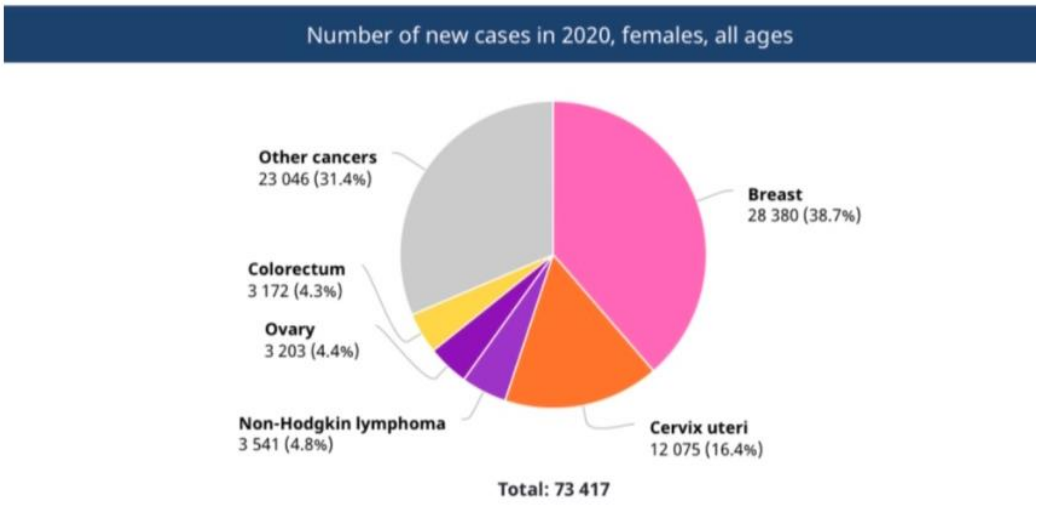

Fig.2.8a: Incidence of breast cancer among females in Nigeria (Sung *et al.*, 2021).

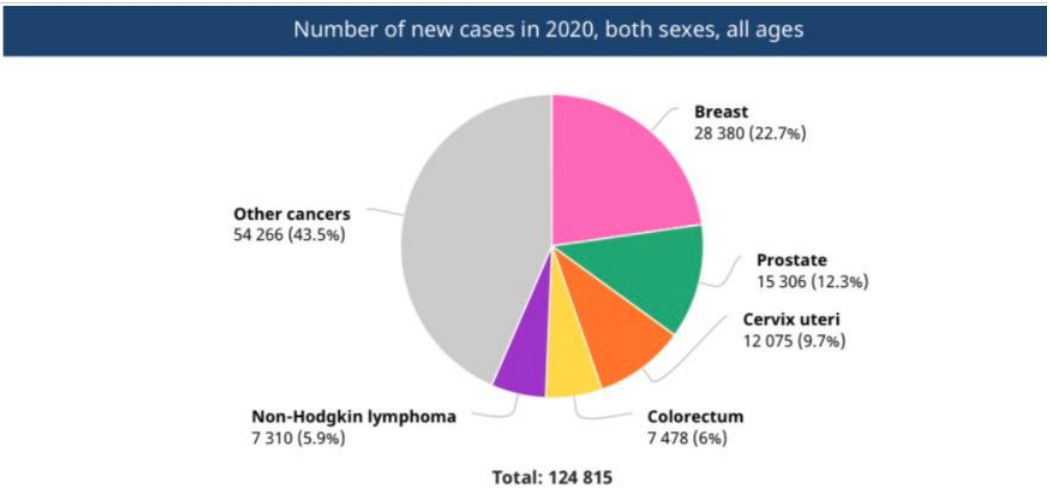

Fig. 2.8b: Incidence of breast cancer in Nigeria (Sung *et al.*, 2021).

How these risk factors are modulating the genes in the development of breast cancer among women in Nigeria is not yet known (Adeniji *et al.,* 2020). Being a female is an additional risk factor as females have 100 times increased rate of developing breast cancer compared to men (Łukasiewicz *et al.,* 2021).

2.6 Discovery of effective genetic markers for risk assessment of breast cancer

Development of cancer of the breast has a genetic basis which could be familial (inherited) or sporadic (spontaneous). There are several candidate genes that have been identified and linked to the occurrence of inherited breast cancer in the world that can be potentially used as genetic markers for early diagnosis of breast cancer. These candidate genes have also been checked among the Nigerian population, with a few of them linked to breast cancer. According to Zheng *et al*. (2018), mutations in BRCA 1 & 2, PLAB2 and p53 were linked to the risk of having cancer of the breast in Nigeria. Nevertheless, other populations have linked some other genes and their SNPs to the development cancer of the breast. In Asian populations, *TNF-alpha, Enos, RAD50, CCND1, NBS1* and *SULT1A1* are said to have a possible association with breast cancer (Abbad *et al*., 2018; Zheng *et al*., 2018). Many of these genes are population-specific due to racial and geographical differences (Abbad *et al*., 2018; Zheng *et al*., 2018).

The importance of the above-mentioned genes is evident in their protein function. Mutations in genes can be missense or nonsense. Loss of function mutations of genes were linked to breast cancer, especially *BRCA1 and 2, and Tp53* genes (Ergul and Sazci, 2001; Wendt and Margolin, 2019). BRCA1 and 2 are phosphoproteins that function as tumour suppressors and are important in the DNA repair pathway and cell apoptosis (Heijink *et al*., 2019). When there is exposure to a DNA-damaging agent, the expression of BRCA1 is down regulated in the presence of p53, which consequently leads to either growth arrest, senescence, or apoptosis of the cell (Arizti *et al*., 2000; Alvarado-Ortiz *et al*., 2020). p53 has the ability to inhibit inflammation by acting as an antagonist to nuclear factor kappa B (NF-kB), known to promote cancer progression (Weisz *et al*., 2007; Alvarado-Ortiz *et al*., 2020). In cancer cells, mutation in p53 gene can lead to over production of p53, resulting in cancer-induced gain of function of p53 that leads to activation of NF-kB pathway in response to cytokines involved in the pathway (Weisz *et al*., 2007; Alvarado-Ortiz *et al*., 2020) (Fig. 2.9). However, loss of function

of p53 have been identified among women who have breast cancer in Nigeria (Zheng *et al*., 2018).

A process by which breast cancer develops has been hypothesized to be an inflammatory process involving several cells attracted by chemokines and cytokines that mediate inflammatory responses in the body (Zhao *et al*., 2021). Influx of immune cells and activated fibroblasts cause inflammation in the tumor microenvironment, and the tumour reacts to these substances by secreting cytokines, chemokines, and growth factors (Zhao *et al*., 2021). Depending on the chemical mechanism that is engaged, some of these chemokines and cytokines either stimulate tumor growth or result in cell death. The NF-kB pathway is activated by the cytokines like Interleukins (IL) 6, 8, 11, and 10 and TNF-α, which support carcinogenesis (Castro *et al*., 2014). Some of these cytokines (IL-6 and TNF-α) have been connected to the development of ductal cancer (Castro *et al*., 2014; Masjedi *et al*., 2018). Cytokines have also been implicated in systemic inflammation that is associated with some factors attributed to breast cancer, such as obesity, age, menopause, etc. Serum levels of these cytokines were observed to be higher in patients against healthy controls and correlated with tumour stage and poor patient survival (Felix *et al*., 2018). Numerous SNPs in the IL-10, IL-6, and TNF-α cytokine genes have been linked to various levels of gene transcription, which in turn determine inter-individual variations in their production (Chae *et al*., 2016; Gaiolla *et al*., 2021). Numerous SNPs in the IL-10, IL-6, and TNF-α cytokine genes are connected to various levels of gene transcription, which in turn dictate inter-individual variations in their output (Chae *et al*., 2016). The SNPs are the most common variation known to affect various biological functions, including those that lead to cancer (Zou *et al*., 2020). The SNPs of TNF-α have been implicated in breast cancer and other cancer types. Two *TNF-α* SNPs (*488A and 859T*) were significantly linked to the possibility of having bladder cancer among the Caucasian population (Marsh *et al*., 2003; Gautam and Srivastava, 2018). *TNF-308* was associated with triple-negative breast cancer among Indian and Northeast Chinese Han women (Xu *et al*., 2014; Ahmad *et al*., 2020), while *TNF-308* was associated with triple-negative breast cancer among the Asian population (Li *et al*., 2015; Ahmad *et al*., 2020) as well as with breast cancer among Iraqi women (Abed and Dhabaan, 2020).

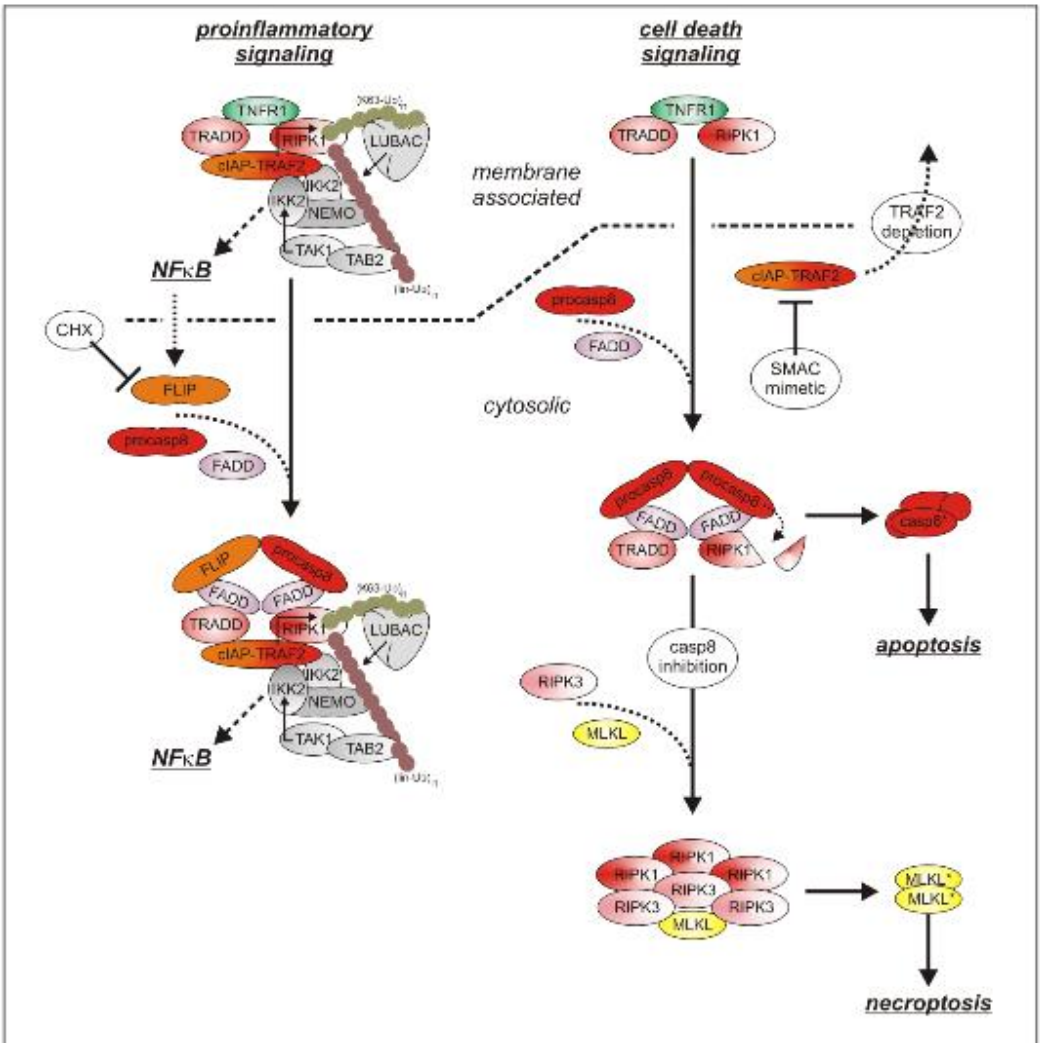

Fig.2.9: Signaling pathway of TNF-α with TNFR1 and 2

(Weisz *et al*., 2007; Wajant and Siegmund, 2019; Alvarado-Ortiz *et al*., 2020).

2.6.1 Inflammation and breast cancer development

The immune system and cancer have been linked since leukocytes were first discovered in the stroma of malignant tissue in 1863 (Quail and Joyce, 2013). It was discovered that a population of B&T-cells present in the interductal stroma or adjacent to ducts were the primary cause of inflammation. A less obvious trend is the diffusion of stromal infiltration, which is predominantly composed of macrophages and T cells. Peripheral monocytes that move to the site of a developing tumour (like ductal carcinoma in situ) and get specialized into macrophages due to the hormones released by the tumours are the main source of tumour associated macrophages (TAMs). The abundant cytokines and growth factors in the microenvironment have a significant impact on how plastic macrophages are. The frequent cytokines and growth factors in the environment substantially influence the plastic phenotype of macrophages, which can alter in response to changes in the microenvironment (Grohmann *et al*., 2019). Macrophages are grouped into two categories: M1 (typically activated & proinflammatory) or M2 (alternatively activated, regulatory and homeostatic) (Brown *et al*., 2012). Typically-activated macrophages (M1) chiefly produce cytokines such as IL-12, TNF-α, and IL-10 while TGF-β is produced by M2, which also have immunosuppressive properties. Numerous M2 subpopulations in TAMs express growth factors that support angiogenesis, matrix remodeling, immune evasion, and tumour-promoting cytokines like VEGF and MMP2 and MMP9 (Quail and Joyce, 2013). According to Allen and Jones, (2015), it was discovered that colon cancer cell lines pushed macrophages to an M1, tumour promoting phenotype, while breast cancer cell lines pushed them to an M2 (antitumour phenotype).

2.6.2 Tumour Necrosis Factor alpha (TNF-α)

TNF-α, which has a molecular weight of 26 kD and is membrane-bound, is converted by the TNF-converting enzyme (TACE) into soluble TNF, which has 76 amino acid terminal residues and a molecular weight of 17 kD (Jang *et al*., 2021)(Fig. 2.10). Under natural conditions, TNF-α exists in equilibrium as a monomer, dimer, and trimer, bound and soluble, with the trimer being the physiologically active form. TNF-α is a part of the TNF superfamily, which also consists of the ligands TRAIL/Apo-2 ligand, that induces apoptosis, and LIGHT, which is involved in T cell activation, as well as

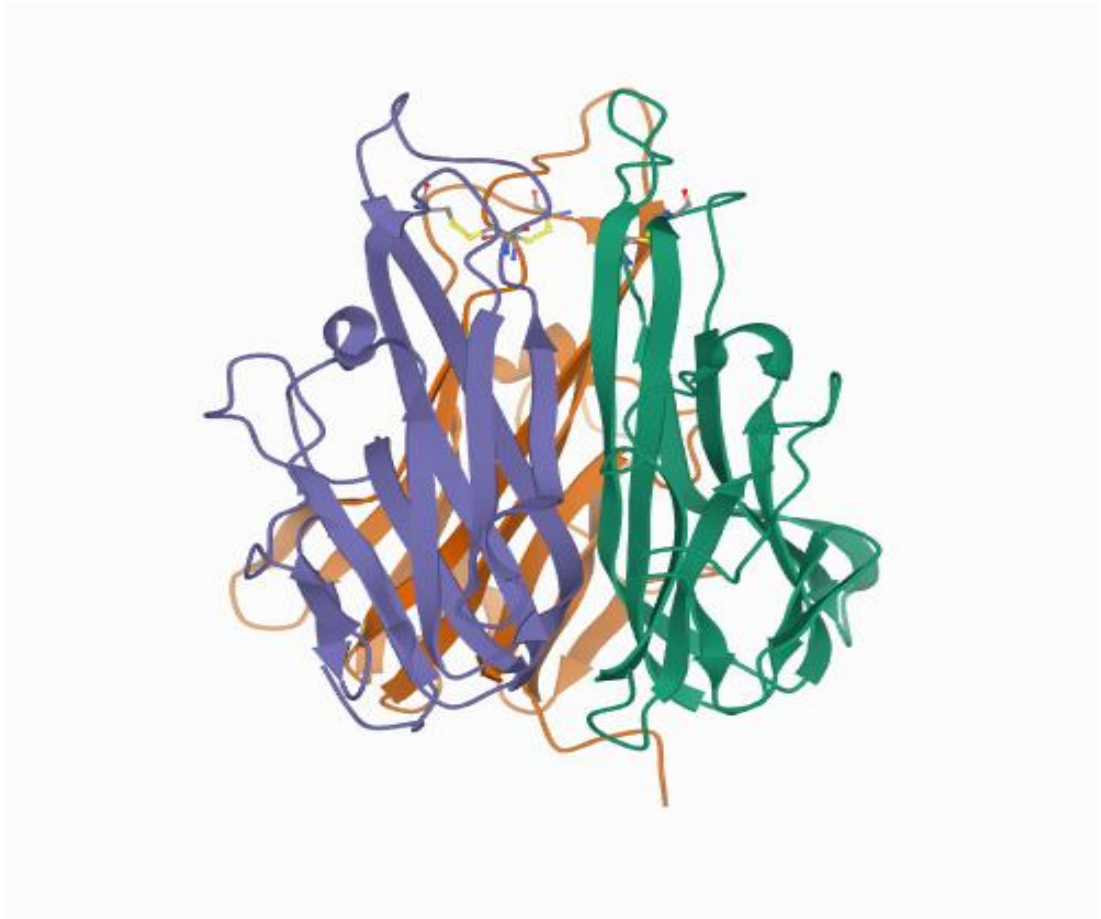

Fig. 2.10: The structure of human TNF-α at 26 Ångstroms resolution. TNF-α is a homotrimer that consists of wedge-shaped monomers (*colured in green, brown and purple*) that are associated in an antiparallel sandwich structure. (Fitzgerald *et al*., 2001).

Lymphotoxin a and b, Fas ligand, and CD40 ligand (Mauri *et al*., 1998; Jang *et al*., 2021). TNF-α- is a pleiotropic cytokine that operates on several cells and affects each cell differently. It is important in the activation of neutrophils, macrophages, and monocytes. TNF-α may cause apoptosis in cell lines, although this impact is typically reliant on oestrogen therapy and the production of specific epidermal growth factors. TNF-α was found in several tumour forms, including breast and ovarian tissue, as well as hemorrhagic malignancies (Azmy *et al*., 2004; Mercogliano *et al*., 2020) . Expression of TNF-α in breast cancers may promote tumour progression, according to some studies, due to its ability to increase the transcription and stability of the epidermal growth factor (EGF) receptor, which can lead to cell division and carcinogenesis (Zuo *et al*., 2011; Mercogliano *et al*., 2020) . Its expression in a variety of cells was found to relate with increasing tumour grade and node involvement in inflammatory breast cancer, and patients with progressive phenotypes had significantly higher levels of TNF-α (Hong *et al*., 2018).

The *TNF-α* locus contain several SNPs that have been linked to both communicable and non-communicable disorders (Tables 2.1 and 2.2). The promoter region of TTNF-α single nucleotide polymorphisms were investigated in prostate cancer. Prostate cancer incidence was 17-fold higher at the 488 gene, and polymorphisms at the -308 locus were linked to increased tumor stage (Bandil *et al*., 2017). Other investigations have demonstrated that the expression of *TNF-238A* allele, known to suppress TNF-α transcription, may protect against stomach cancer, but the risk of gastric cancer was not increased by the expression of the *TNF-308A* allele, which is known to up-regulate TNF-α expression (Uthansingh *et al*., 2022). The *TNF-α* SNPs have been implicated in several communicable diseases such as *TNF-308* was linked Urinary tract infection among Indian population (Sharma *et al*., 2021). Likewise, *TNF-308A* was linked to malaria susceptibility (Surjyapratap *et al.*, 2023). *TNF-308A* has also been implicated in SARS CoV-2 infection in Iraqi Kurdish population (Ali, Niranji, and Al-, 2022).

2.6.2.1. TNF-α protein and genetic variants in Nigerian Population

The association of TNF-α protein and gene variants with various diseases has been studied in Nigeria (Table 2.3). Especially in malaria studies, TNF-α SNPs have been extensively researched for their association with disease severity, susceptibility, and outcomes.

Table 2.1: Association of TNFα and receptor Single nucleotide polymorphisms (SNPs) with different cancer types

S/N	*TNFα SNPs*	Association	Reference
1.	*TNFα SNPs (488A and 859T)*	The risk of bladder cancer among Caucasian population	(Marsh *et al.,* 2003)
2.	*TNFα receptor 1A (TNFRSF1A)*	Sporadic breast cancer among Northeast Chinese Han women	(Xu *et al.,* 2014)
3.	*TNFα 308*	Triple negative breast cancer among an Asian population	(Li *et al.,* 2015)
4.	*TNFα 308*	Breast cancer among Indian women	(Ahmad *et al*., 2020)
5.	*TNF α 238 AG*	Associated with prognostic parameters of Classical Hodgkin lymphoma	(Gaiolla *et al*., 2021)
6.	*TNFα -863C/A, − 857 C/T, − 308 G/A, and − 238 G/A*	Connected with hepatocellular carcinoma risk	(Wungu *et al*., 2020)
7	*TNFR1 rs767455AG/GG and rs234649AA*	cervical precancerous lesions progression	(Da Rocha *et al*., 2019)

Table 2.2: Association of TNF α and receptor SNPs with infectious diseases

S/N	*TNFα SNPs*	Association	Reference
1.	*TNF α 238 A*	Linked to severe clinical outcome of falciparum malaria in Southwest Nigeria	(Olaniyan *et al*., 2016)
2.	*TNF-α-308 G/A*	No association with urogenital schistosomiasis	(Marume *et al*., 2021)
3.	*TNF 308/238*	Associated with reproductive tract infections among Indian women	(Sharma *et al*., 2021)
4.	*TNF 489*	GG genotype linked with higher TNF-α levels in septic shock.	(Georgescu *et al*., 2020)
5	*TNF 308*	*TNF-308 G/A* genotype protective against SARS in infants and Adults	(Paim *et al*, 2021)
6	sTNFR1	Higher serum level associated with severity of COVID 19	(Mortaz *et al*., 2021)

Table 2.3: TNF-α protein and genetic variants in Nigerian Population

S/N	*TNF-α/ SNPs*	Association	Reference
1	*TNF-α 308*	Severe malaria susceptibility	Ojurongbe *et al.*, 2018
2	*TNF-α 308/244*	No association with malaria outcome	Oyedeji *et al.*, 2017
3	TNF-α level	Influences malaria outcome	Ajibaye *et al.*, 2014
4	Higher mean level of TNF-α	Gestational diabetes mellitus	Mohammed and Aliyu, 2018
5	TNF-α	Associated with breast cancer metastasis	Felix *et al.*, 2018

TNF-α 238 has been found to be associated with the clinical outcome of malaria, while TNF-α 308 has shown no such association (Olaniyan *et al.*, 2016; Oyedeji *et al.*, 2017). However, an association between TNF-α 308 and severe malaria has been documented (Ojurongbe *et al.*, 2018). TNF-α has also been hypothesized to play a key role in the coexistence of malaria and type 2 diabetes in endemic areas (Ademola *et al.*, 2023). Higher serum levels of TNF-α have been associated with gestational diabetes (Mohammed and Aliyu, 2018).

2.6. 3 TNF-α Receptor (TNFR1 and 2)

Tumour Necrosis Factor alpha (TNF-α) begins its function by binding to two different receptors, TNFR1 (p55, p60, CD120a or TNFRSF1A) and TNFR2 (p75, p80, CD120b or TNFRSF1B) (Li *et al*., 2014) (Fig. 2.11).Their interactions induce the activation of other cytokines and promote tumorigenesis through initiation of NF-KappaB signaling pathway (Liu *et al*., 2017).The blockage of the two receptors in breast cancer cell lines showed truncation of tumour growth according to Geerts *et al*., (2020). The *TNFR1* gene, on chromosome 12p13 codes for a 60 kDa protein, and the TNFR2 gene, which is on chromosome 1p36.2 and produces an 80 kDa protein, are transmembrane glycoproteins that are a part of the superfamily of TNF receptors, which has 29 members in humans (Martínez-Reza *et al*., 2017). They both have similar extracellular domains with cysteine-rich repeated units of 40 amino acids, but they have different intracellular domains. TNFR1 is either activated by soluble or membrane-bound TNF-α whileTNFR2 is only activated by membrane bound TNF-α with the majority of TNF-α biological effects occurring via TNFR1 activation (Martínez-Reza *et al*., 2017). When ligands bind to TNFR1, it activates the IKK complex, which then triggers the NF-KappaB, inflammatory cytokines and growth factors. It is imperative to stress that the nonselective CylinD1, BCL2, and superoxide dismutase are anti-apoptotic factors that are significantly affected by NF-B activation brought on by TNF-α. Consequently, when NF-KappaB activation is insufficient, the late response to TNF-α activation is apoptosis. TNF/TNFR1 activation attracts a protein known as TNFR associated death domain (TRADD), which is activated in two separate ways. The Fas-associated protein with death domain (FADD) activates the caspase-8 and caspase-3 pathways, recruited by TRADD. Additionally, TRADD can stimulate the mitochondria to produce ROS and activate the caspase-9 and caspase-3 pathways. Apoptosis can also be influenced by the

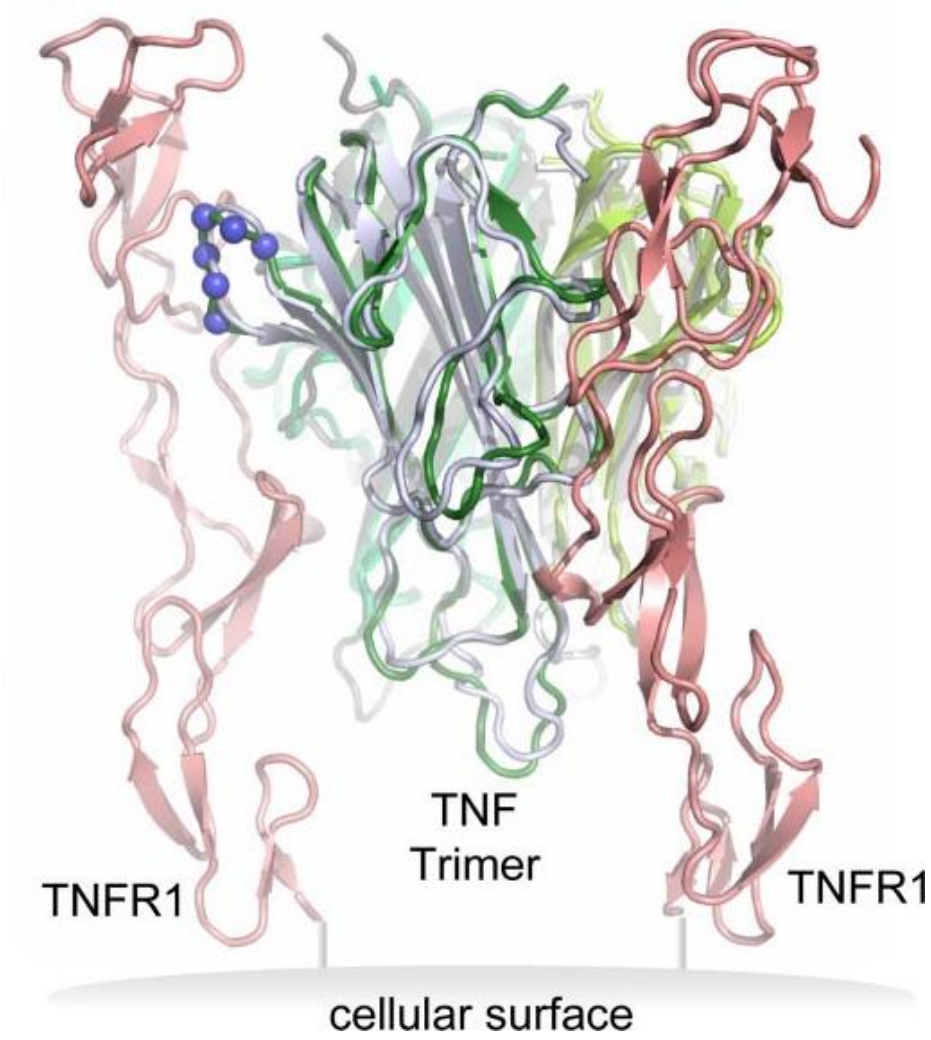

Fig. 2.11: Structure of Model TNF -TNFR1 complex. The TNF-α trimer represented in green, lemon and gray schematics binds to the monomer of TNFR1 in red schematics on the cell surface (Shibata *et al*., 2007).

accumulation of intracellular ROS and continuous activation of Jun amino-terminal kinase (JNK). On the other hand, TNFR2 lacks a death domain, is expressed on hematopoietic cells, and is found in specialized tissues, however the exact mechanism by which it activates cell signaling is unknown. Numerous investigations showed that TNFR2 can bind to TNFR-associated factor 2 (TRAF2) directly, in addition to NF-B and MAPK signaling pathways (Martínez-Reza *et al.,* 2017).

2.7 Methods in SNP identification and qualitative analysis

A quantitative research methodology is required for SNP identification. Allele-specific polymerase chain reaction is a useful technique for locating TNF- SNPs (PCR). Under typical PCR settings, SNPs can be effectively distinguished with the use of allele-specific PCR, a less expensive technique. Two forward allele-specific primers with different tails and a common reverse are used to differentially amplify two alleles of a single SNP. Agarose gel electrophoresis is then used to resolve these products. Destabilizing 3′ primer end with a mismatch is used to increase the PCR specificity. This technique for SNP identifictaion is straightforward and affordable and does not call for PCR tweaking (Lefever *et al.,* 2019). Microarray for SNP is another good method that can be used to identify SNPs of genes but this method is quite expensive and it can be used for a genome wide SNP detection. Since the approach for this study is a candidate gene approach, allele specific PCR is a good and reliable method to use. Some studies have used allele specific PCR to identify SNPs associated with breast and other cancer types in populations (Marsh *et al*., 2003; Lefever *et al*., 2019; He *et al*., 2022). Furthermore, studies on factors contributing to delayed diagnosis of breast cancer are usually done through qualitative approach which allows for the capture of lived experiences of concerned individuals (Getachew *et al.*,2020).

CHAPTER THREE

METHODOLOGY

3.1 OBJECTIVE 1

Factors preventing early diagnosis of breast cancer in Nigeria.

Questions regarding the subject area, "early breast cancer diagnosis in Nigeria" were addressed using a mixed method approach that combined qualitative and quantitative data collection techniques. (Fig.3.1).

3.1.1 Study Design

A qualitative study using a phenomenological approach was used and this research design focused on the lived experiences of participants (Neubauer *et al.,* 2019).

3.1.2 Study location

The study was carried out in Ibadan, South-West Nigeria at the University College Hospital and the Breast Cancer Association of Nigeria (BRECAN), Ibadan branch.

3.1.3 Study population

The first population studied was composed of women with breast cancer who were visiting University College Hospital, Ibadan. The second population recruited for this study was breast cancer healthcare professionals at University College Hospital, Ibadan, and administrators of the Breast Cancer Association of Nigeria, Ibadan and Ondo chapters.

3.1.4 Sampling design / technique

Non-Probability sampling technique was used for identifying and selection of participants. All participants had equal chances of selection into the study (Showkat and Parveen, 2017).

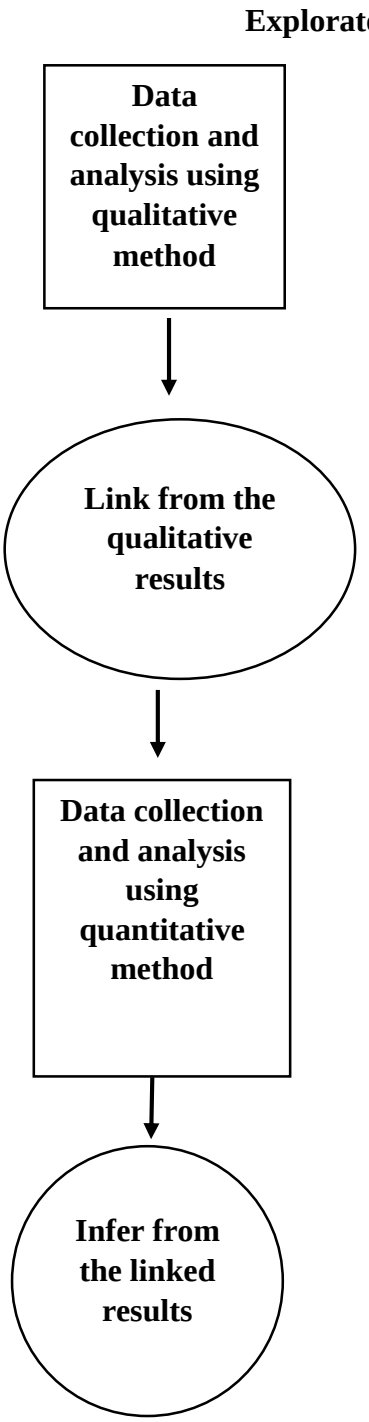

Fig. 3.1: Exploratory mixed method research design (Curry *et al.*, 2013)

3.1.5 Selection of participants

The selection of participants for this study followed the standard for qualitative research to obtain maximum saturation level. Muellmann *et al.,* (2021) stated sample size 10 is good enough for Key Informant Interviews (KII) for community readiness assessment. This was adopted for this study. Likewise, Hennink and Kaiser (2022) stated that saturation can be reached in a qualitative research with 5-20 sample size.

3.1.6 Data collection procedure

Ethical approval of the research proposal was given by the Joint Ethical Committee of the University of Ibadan and University College Hospital (UI/UCH) (UI/EC/20/0271) (See appendix) prior to the start of the study. Recruitment of participants was done after full explanation of the study had been given and written consent obtained.

Semi-structured interviews were conducted with those in the listed groups below:

Group 1: Health care service provider and stake holders,

Group 2: Partner organizations in health care awareness programmes,

Group 3: Diagnosed breast cancer patients.

3.1.7 Protocol for the conducted interviews

Step 1: Key informant interviews were conducted among Health care service providers and stake holders. Each interview lasted 30mins to 1hour. The primary health care service provider included:

i. Medical doctors/Oncologist/Radiologists that are involved in women health care and breast cancer diagnosis
ii. Pathologists who are directly involved with diagnosis of breast cancer patients

Step 2: Key informant interviews were conducted with key management personnel at the partner organization in health care awareness programme for breast cancer in both Oyo States.

Each interview was kept confidential, and every transcript was coded with the identification number of participants. The interviews were documented using audio

devices and notes were taken during the interviews. The documented interviews were transliterated into text for analysis.

3.1.8 Data analysis methods

Data were analysed using a mixture of thematic and narrative analysis methods to capture the lived experiences of patients regarding timely diagnosis of breast cancer as well as that of health workers. Codes were deduced from transcripts and used to identify recurrent concepts and patterns. These were then reorganized into descriptive themes and narration.

3.2 OBJECTIVES 2-4

Identification of key genetic variants of TNF-α cytokine as possible predictor of breast cancer among Nigerian Women using quantitative methods

3.2.1 Study design and location

A case-control design was used for this objective. It included 100 breast cancer and 100 non-cancer patients in the University College Hospital, Ibadan.

3.2.2 Study population

The study population was made up of women at different stages of breast cancer at diagnosis and controls (women without cancer). Only women were considered for this study because breast cancer is known to be more prevalent among women than among men (Afolayan *et al*., 2012). Breast cancer patients (100) and individuals without breast cancer (100) as controls were enrolled for the study as determined by the calculated sample size (Formula 3.1).

3.2.3 Inclusion and Exclusion criteria

Inclusion criterion: Women that have been diagnosed and confirmed to have breast cancer but had not commenced treatment and were between 20-70 years old.

Inclusion criteria for controls: Controls included in the study were individuals without breast cancer or other cancer types, or ailment that could interfere in their TNF production. They were women in the same age range (20-70 years) as the recruited breast cancer patients.

Exclusion criteria for the cases included women who were yet to be confirmed to have breast cancer histologically and who were less than 18 years of age. Exclusion criteria for controls included women with any form of cancer and who were less than 18 years of age.

3.2.4 Sample size Calculation

The sample size was calculated using the incidence rate of breast cancer in Nigeria (26.7%). The formula below was used (Kirkwood, 1988)

$$n = 2\left[\frac{(Z_{\alpha} + Z_{1-\beta})^2\, \pi\, (1-\pi)}{(P_1 - P_2)}\right] \qquad \text{(Formula 3.1)}$$

n= Sample size

Z_{α} = Standard normal value corresponding to 95% confidence interval set at 1.96

$Z_{1-\beta}$ = Standard normal value corresponding to 80% statistical power set at 0.84

P_1 = Prevalence of breast cancer in the population (26.7%) (Sabiu *et al.,* 2017)

P_2 = 50% probability of exposure within the control population (Yanna *et al.,* 2011)

Π = Average prevalence = 0.3835

$$n = 2\left[\frac{7.84\ (0.2356)}{0.054}\right]$$

n= 68.41 adjusting the sample size for 20% non-response

nf= = = 85

n= 85

multiplying the sample size with 1.2 design effect

n= 85 X 1.2= 102 ¬ 100

Therefore, n= 100

3.2.5 Ethical consideration and Sample collection

Ethical approval of the research proposal was given by the Joint Ethical Committee of the University of Ibadan and University College Hospital (UI/UCH) (UI/EC/17/0054) (See appendix) prior to the commencement of the study. Recruitment and enrolment of breast cancer patients registered in the University College Hospital, University of Ibadan, Ibadan as well as individuals without cancer as controls was done after informed consent was obtained and full explanation of the study was given.

Blood samples (5ml) were obtained through venepuncture by qualified physicians. Case notes of consenting patients were accessed for their ages, weight, height, use of birth control, fertility hormone usage, family history of cancer etc.

3.2.6 Plasma and DNA extraction from blood samples

Blood samples (5mls) were collected once from the enrolled participants for Plasma and DNA extraction. The blood samples were collected in Ethylenediaminetetraacetic Acid (EDTA) bottles to prevent coagulation and each bottle was well labelled. Each sample was spun down at 4000rpm for 5mins, plasma was removed into corresponding labelled cryovials and stored in -20℃ for Enzyme Linked Immunosorbent Assay (ELISA) analysis.

DNA was extracted from each sample using Qiagen kit according to manufacturer's instruction. To each 3ml of blood sample, 6ml of Red Blood Cell (RBC) lysis solution was added to lyse all red blood cells in the sample. The mixture was then spun and supernatant was decanted. This step was repeated twice using 3mL of RBC lysis solution until supernatant was clear enough. To the supernatant, 3mL White blood cell lysis solution was added (to break open all the white blood cells releasing the nuclei contents), the mixture was allowed to homogenize at room temperature. RNAse (25µL) was added at 37℃ for 30 minutes to digest all RNA contaminants. Proteinase K (25µL) was added at 55℃ for 1 hour to digest all protein contaminants. Protein precipitation solution of 1.5mL was added to each sample to precipitate out protein contaminant. DNA of each sample was precipitated in iso-propanol (4mL) and washed in 70 % ethanol (3mL). The extracted DNA was quantified using Nanodrop spectrophotometer (NanoDrop™ 2000/2000c manufactured by Thermo Scientific™) and the stability of the DNA was checked on 1%agarose gel. The optical density (OD) ratio of the DNAs used were between1.8-2.0. Extracted DNA was suspended in TE buffer and stored in the -20℃ freezer.

3.2.7 Determination of Levels of Tumour Necrosis Factor alpha (TNF-α) and soluble TNF-α receptor 1(sTNFR1) proteins in plasma samples

Different levels of TNFα and sTNFR1 proteins were determined in breast cancer patients and controls through Enzyme Linked Immunosorbent Assay (ELISA) using a protocol previously described by Wallace and Stacey (Wallace and Stacey, 1998). Plasma samples (2mLs) separated from the blood samples collected from each participant were used for ELISA.

All samples including standards were ran in duplicates. Serial dilution of the standard was performed in ranging concentrations of 10ng, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.25pg/ml, 15.6pg/ml and 7.8pg/ml, Assay Diluent A served as the zero standard (0 pg/ml). 100ul of Standards and Samples were added to the ELISA plate and incubated for 2 hours at 37℃. This was followed by series of washing with wash buffer four times. 100µl of diluted Detection Antibody solution was added to each well and incubated for 1 hour at 37℃. The wash step was repeated and 50ul of Assay Diluent B was added followed by addition of 100ul of diluted Avidin-HRP solution to each well. Incubation was done for 1 hour at 37℃. The last wash step was performed and 100ul of TMB Substrate solution was added and incubated in the dark for 15-30 minutes at 37°C. To stop the reaction, 100ul of Stop Solution was added to each well. Absorbance was read at 450 nm within 30 minutes of stopping the reaction.

3.2.8 Polymerase Chain Reaction (PCR) analysis for detection of *TNF-α* and *TNFR1A* SNPs

The PCR was carried out using allele specific primers (Table 3.1). The primers were obtained for the following SNPs: *TNF-α* (rs1800629 *308G>A*, rs361525 *488G>A*, rs1799964 *380G>A*, rs1800610 *1032C>T*, rs1800750 *238G>A*, and rs9282876 *859C>T*) and TNFR1A (rs1800693). The final volume for the PCR cocktail was 20 µL; 2µL of genomic DNA template, 0.5µL of each primer, 10µL of master-mix (comprising of $MgCl_2$ dNTPs, Taq polymerase and PCR buffer) and 7µL of molecular grade water. The PCR amplification programme was optimized to 94℃ for 2mins followed by 9cycles of 94℃ for 30 secs, 65°C for 30 secs, 72℃ for 1min and 35 cycles of 94°C for 30 secs, 55°C for 30 secs, 72°C for 1 min and final extension of 72°C for 5mins using Applied Biosystems GeneAmp PCR System 9700.

Polymerase chain reaction products were electrophoresed in 1.5% agarose gels and visualized with UV illumination.

3.2.9 Sequencing and analysis of SNPs in *TNF-α* gene

Randomly selected samples were sequenced using Nanopore technology (Minion). The procedure included preparation of DNA library by mixing 400ng of DNA with 2.5µL of fragment mix reagent (FRA). This was then incubated at 30°C and 80°C for 1min each. The prepared DNA library was cooled for 5mins. Then, 34µL of sequencing buffer was added to the DNA library as well as 25.5µL of loading beads and 4.5µL of nuclease free water to make a reaction volume of 75µL.

The sample mix was then loaded on the flow cell of the sequencer after priming. The sequencing process was initiated, and bases were called.

3.3 STATISTICAL ANALYSIS

Data generated from genotyping TNFα and receptor SNPs were scored and entered on Microsoft-excel with other metadata collected. Statistical analysis was performed using statistical software (GraphPad Prism 6). Descriptive statistics, unpaired t-Test, ANOVA and Fisher's exact test were used to analyse results with odd ratios at $\alpha_{0.05}$. Fisher's exact test was performed to determine the association of *TNF-α and TNFR1* gene variants with breast cancer. Unpaired *t Tes*t and ANOVA were used to determine the differences in the mean concentration of TNF-α level in both cases and control. Population association analyses was done using Haploview genetic software 4.2 to determine deviations of *TNF-α SNP* genotypes from Hardy-Weinberg equilibrium in the population. The sequence data were sorted and aligned to human reference genome using NCBI BLAST tool.

Table 3.1: Primer sets used for the allele-specific PCR

	Sense primer	Anti-sense primer
TNF+488G/ 238G/ 308G	5'-GCATCCCCGTCTTTCTCCAC	5'-ATAGGTTTTGAGGGGCATGG
TNF+488G/ 238G/ 308A	5'-GCAT CCCCGTCTTTCTCCAC	5'-AT AGGTTTTGAGGGGCATGA
TNF+488G/ 238A/308G	5'-GCATCCCCGTCTTTCTCCAC	5'-GAAGACCCCCCTCGGAATCA
TNF+488A/ 238G/308G	5'-GCATCCCCGTCTTTCTCCAT	5'-GAAGACCCCCCTCGGAATCG
TNF 1032T	5' -CCGGGAATTCACAGACCCC	5'-CAAAGGAGAAGCTGAGAAGAT
TNF 1032C	5'-CCGGGAATTCACAGACCCC	5' -CAAAGGAGAAGCTGAGAAGAC
TNF 859C	5'-CTACATGGCCCTGTCTTCG	5' -AAGGATAAGGGCTCAGAGAG
TNF 859T	5'-TCTACATGGCCCTGTCTTCA	5'-AAGGATAAGGGCTCAGAGAG
TNF 380G	5'-GGCTGGGTGTGCCAACAAC	5'-CCTGCATCCTGTCTGGAAG
TNF 380	5'-GGCTGGGTGTGCCAACAAC	5'-TCCTGCATCCTGTCTGGAAA
TNF 238G/A	5'- AAACAGACCACAGACCTGGTC-3'	5'-CTCACACTCCCCATCCTCCCGGATC
TNF 308G/A	5'-GAGGCAATAGGTTTTGAGGGCCAT-3'	5'-GGGACACACAAGCATCAAG
TNFRA1+1V56+10 A	5'-CACTGAGGACTCAGGTGAGGAGTA	5'ATTAAACCAATGAAGAGGAGG
TNFRA1+1V56+10 A	5'-CACTGAGGACTCAGGTGAGGAGTG	5'ATTAAACCAATGAAGAGGAGG

CHAPTER FOUR

RESULTS

4.1 Socio-demographic information of interviewed participants

Socio-demographic information of participants are documented and summarized in the Table 4.1 and 4.2 below. Majority of the participants are well educated with 56% having tertiary education (Table 4.1). Most of the breast cancer patients (92%) were Christians. Majority of the health workers interviewed had more than 10 years of working experience. The group of the health professionals examined include gynaecologist, clinical oncologist, radiotherapist, pathologist, and representative from non-governmental organization focused on breast cancer awareness (Table 4.2).

4.2 Factors contributing to delayed diagnosis in Southwest Nigeria.

The participant interviews, several themes and subthemes describing the causes of a delayed diagnosis in breast cancer patients in South-West Nigeria emerged. Three fundamental steps were found to make up the breast cancer diagnosis process: detection, hospital visit, and diagnosis (Fig 4.1). Analysis of the interview responses identified structural delays at each stage of a breast cancer diagnosis.

4.2.1 **Method of detection of breast cancer by patients (Self report)**

Breast self-examination (BSE) was the primary early detection approach most frequently used. Eighty-eight percent of the women surveyed claimed that they self-detected breast lumps before going to the hospital. Only a few other people also experienced breast pain. This shows that BSE knowledge is rising in Nigeria and that more people are practicing it. Health professionals, however, criticized the procedure as a late post-symptomatic strategy that shouldn't be depended upon given the aggressive nature of breast cancer seen in this area.

Table 4.1: Socio-demographics of breast cancer patients interviewed (n=25)

Socio-demographics	Frequency	Percentage (%)
Age in years		
≤40	7	28
41-50	5	20
51-60	10	40
≥61	3	12
Education		
No formal education	2	8
Primary	4	16
Secondary	5	20
Tertiary	14	56
Occupation		
Skilled Labour	11	44
Unskilled Labour	13	52
Unemployed	1	4
Religion		
Muslim	1	4
Christian	23	92
None	1	4

Table 4.2: Socio-demographics of Health workers interviewed (n=10)

Socio-demographics	Frequency	Percentage (%)
Years of Service		
5-10	3	30
≥ 11	7	70
Education		
Primary	0	0
Secondary	0	0
Tertiary	10	100
Occupation		
Gynecologist	1	10
Non-governmental organization	2	20
Radio Oncologist	5	50
Clinical Oncologist	1	10
Pathologist	1	10

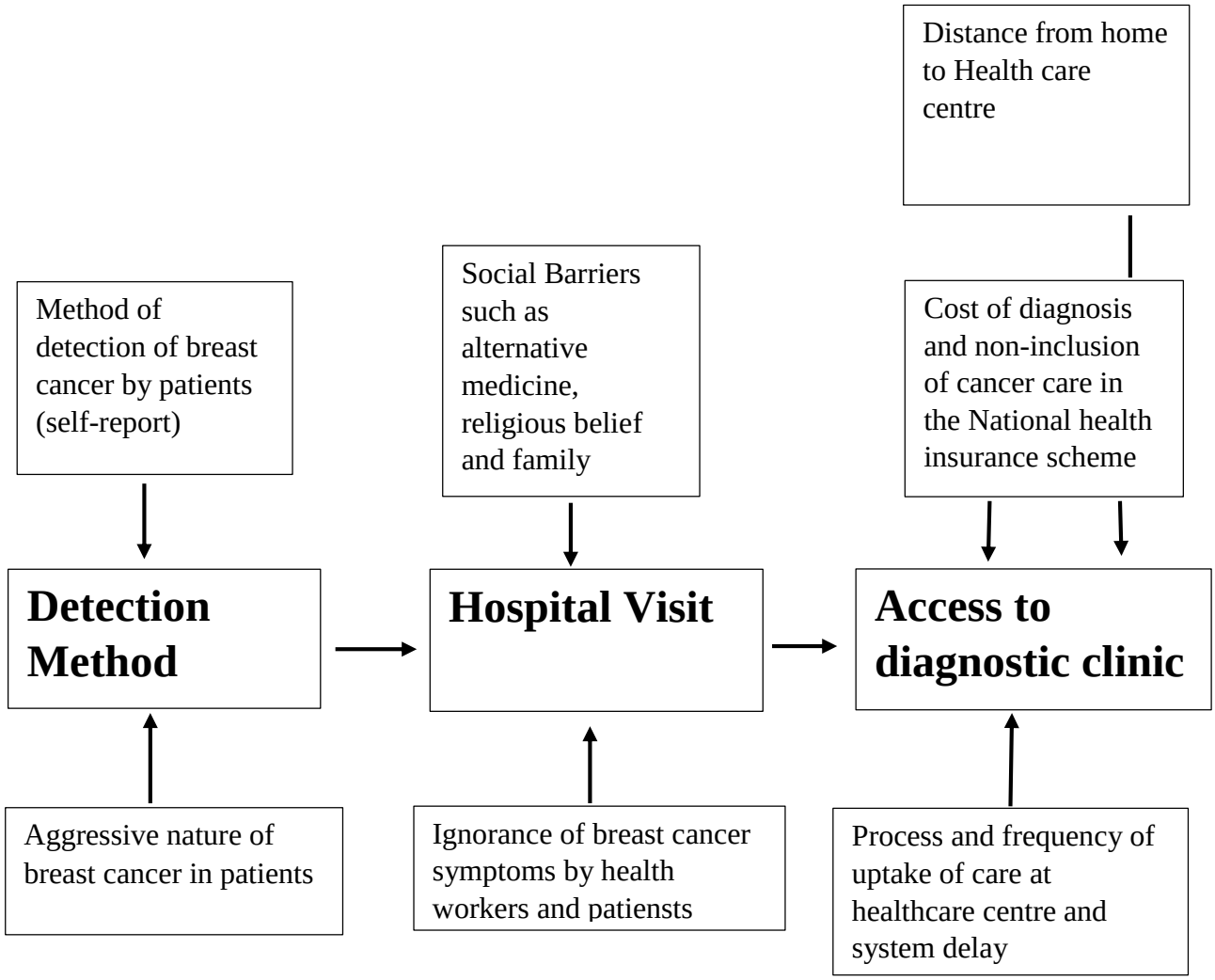

Fig. 4.1. Emerging themes (*in bold*) and sub-themes from the In-depth and Key informant interviews. These themes and subthemes highlight the factors contributing to delayed diagnosis of breast cancer in Southwest Nigeria.

Codes for respondents

Case (1-25): Breast cancer patients

Key informants

Key (1-8): Health workers**; Key (9 and 10):** Service providers at breast cancer association of Nigeria

Narratives includes:

Case 14: *"I notice one lump when I was checking myself because I am the type that normally checks."*- (Patient 38 years old, in-depth interview).

Case 15: *"I notice small seed, the first time when I notice am na last year 2020"*- (Patient, 40 years old, in-depth interview)

Case 17: *"I notice when I dey rub my body, I see small seed"*- (Patient, 49 years old, in-depth interview)

> Key 3:*"We still have a lot of women with late stage breast cancer because by the time a lump is felt or symptoms are felt and a woman presents to know if it's cancer, that time when you are feeling the lump it's already sort of an advanced disease. Most of the disease picked up in the developed world are* (from) *non symptomatic screening so it makes you know* (the) *diagnosis quite early and then treatment can then lead to cure"*
>
> (Radiologist, years of experience: 11yrs, key informant interview)
>
> Key 4: *Let me just cite an example for you. If a patient notices a lump, the day a patient notices a lump, it has been there for more than one year and this is the problem. And it's painless, once it's painless, they cannot even seek help. But still there are some breast cancers that don't present with lump, it can only be discovered maybe by accident -You go for something else then you now discover that"*
>
> (Clinical Oncologist, years of experience: 10yrs, key informant interview)

4.2.2 Hospital Visit

Many patients said they went to the hospital right away after finding a lump in their breasts. Some patients who informed a family member or a religious leader were also advised to go to the hospital right away. The experience of a key source from a non-governmental organization, however, revealed that some health professionals who are more in touch with grassroots communities, like the Primary Health Care Centres, were unaware of the breast cancer signs. Although some patients choose to go directly to a tertiary hospital rather than using these centres, those that attend PHC were thought to be at danger of being misinformed.

Narrative includes:

> Key 9: "*The primary health centre should have little knowledge about breast cancer because (in) one of the cases we handled last year, she would have better survived if she presented early but the truth was that she presented early but she presented to the wrong people so she met at the primary health care centre. She was complaining of pains and the truth is that before even you start feeling pains you know that is cancer related it's no longer stage one. The PHC gave her pain killers till it became something else.*."
>
> (Health care volunteer 1, Key informant interview)

4.2.3 Access to Diagnostic clinic

Access to diagnosis has many delay facets emerging as sub-themes, these include the following:

i. Distance from home to health care centre

In Nigeria, tertiary institutions provide the majority of the healthcare services for cancer patients. Patients must travel a great distance to get these services, and referrals from both public and private institutions are frequent. Additionally, it was reported that the majority of the facilities were in a state or despair, which led to an increase in the number of individuals using the few operational facilities.

Narratives include:

Case 2: "*I had to travel a distance of 1hour 30minutes to get diagnosed*."

(Patient, 24 years old, in-depth interview)

Case 3: "*It's far now, like I come from Warri to Ibadan (about 7hours travel)*."

(Patient, 55 years old, in-depth interview)

Key 10: "*We don't have a lot of radiotherapy centres ……., the system in Abuja has broken down, that in Kaduna has broken down there are none, so we don't have enough facilities*,"

(Healthcare volunteer 2, key informant interview)

ii. Inadequate knowledge on advanced technology by health care workers for breast cancer diagnosis.

It was stated by the health care workers interviewed that the some medical practitioners sometimes have trouble correctly identifying early neoplastic alterations in cells. This makes it necessary for medical personnel to receive training in order to advance their knowledge of breast cancer early diagnosis.

Narrative include:

Key 3: "*A lot of people are not trained because breast is……. though, there are radiologists out there but you need additional training to be able to consistently interpret the mammograms*"

(Radiologist, years of experience: 11years, key informant interview)

Key 4: "*Training of more expertise, we need more hands in this field we need more hands, in developed world(s) they separate radiation oncologist from clinical oncologist right"*

(Clinical Oncologist, years of experience: over 10 years, key informant interview)

iii. Cost of diagnosis and non-inclusion of cancer care in the National health insurance scheme

In Nigeria, there is currently no health insurance program for cancer, hence, individual's expenditure for diagnosis and treatment of breast cancer are significant. This affects the number of patients who visit medical facilities and delays some people from receiving the necessary medical care.

Narratives include:

Case1: "*I have spent now, I have spent plenty that's why we did not come last year. No money*." (Patient, 55 years old, in-depth interview)

Key 4: "……..*I think financially, like I have a patient I asked to do mammogram and when she cost it, it was around 10,000 and now I have not seen the patient in 2 months."*

(Clinical Oncologist, years of experience: over 10 years, key informant interview)

> Key 9: "*Government should make Breast cancer a health priority and include breast cancer in national health insurance scheme so if people are poor and they are sure that they are going to get some subsidized treatment, then they will go to the hospital*"

(Healthcare volunteer 1, key informant interview)

> Key 3: "*What this health scheme offers is not up to what the patients actually need, as most of these schemes do not cover terminal illnesses like cancer, but for(covers) normal diseases that patients can pay out of their own purse for*"

(Radiologist, years of experience: 11yrs, key informant interview)

4.2.4. Process and frequency of uptake of care and system delay

Due to the scheduling of patients by medical staff, a patient's time receiving care at a health care facility may be prolonged.

Narrative include:

Key 1: "*not only the cost, even the scheduling of the patient(s) when they go…… they say a woman has a breast lump, just to schedule the person, they will say come in two months' time, come in six weeks' time*",

(Gynaecologist, years of experience: 10years, key informant interview)

4.2.5 Different dimensions of social barriers to early diagnosis of breast cancer in Nigeria emerged from the interviews as well as.

Some of the factors that emerged from the interviews as social barriers include:

i. Family support and care

Most of the patients claimed to have family support and care, although this negates the claims of officials from non-government organization (NGO). The NGO perceived that patients do not have enough family support which is one of the causes of late presentation.

Case 1: "*I tell them, all of them know and none of their attitude change. Na them even say make I come UCH*."

(Patient, 55 years old, in-depth interview)

Key 10: "*It's not the cancer itself that kills but you know depression……., a lot of people come with family problems, (for example) that their husbands would have abandoned them thinking that it's a death sentence*"

(Healthcare volunteer 2, key informant interview)

Key 9: "….*fear of what people will say, people will begin to castigate them. We've had a wife that was sent away by her husband in fact her husband's family sent her away, they even took the children from her I think she is late now*…"

(Healthcare volunteer 1, key informant interview)

ii. Emotion, psychology, self-denial

Majority of breast cancer patients suffer from emotional break-down, self-denial and thought out perception of their community. After diagnosis, some patients are still in denial of being diagnosed with breast cancer. Some do not like some treatment options such as surgical removal of the breast because they think they may not be seen as a woman again by their husbands.

Key 1: "….*I will lose my breast so……., it also affects their gender perception to say would I still be a woman if I don't have one breast? They are also afraid of acceptability in the community……. that it will not lead to marital crisis*"

(Gynaecologist, more than 10 year experience, key informant interview)

Case 4: "*I have not experienced breast cancer."* A patient after confirmed breast cancer diagnosis

(Patient, 28 years old, in-depth interview)

Key3: "….*some people when you tell them they have cancer they say "God forbid or i reject it in Jesus name*".

(Radiologist, years of experience: 11yrs, key informant interview)

iii. Alternative medicine / religious belief

Due to cost and some other factors such as religious belief, breast cancer patients often time try alternative medicine for diagnosis and cure. This perception often leads to delay in presentation to appropriate health care centres.

> Key 3: "*A lot of factors might be involved; first of them is ignorance, then secondly religious beliefs and denial, some people when you tell them they have it they say "God forbid or i reject it in Jesus name"……. "They want to try alternative medicine and when they come back when it's already late".*

(Radiologist, years of experience: 11yrs, key informant interview)

Key 4: "*Information deficit and religious belief as some people believe that it is spiritual"*

(Clinical Oncologist, years of experience: over 10 years, key informant interview)

4.2.6 Knowledge barrier as factors preventing early diagnosis of breast cancer in Nigeria include:

i. Knowledge on breast cancer before diagnosis

Some patients do not have basic knowledge about breast cancer prior to diagnosis.

Case 5: "*Rara o!, (No Oh! Translation), I don't know*"

(Patient, 34 years old, in-depth interview)

Case 6: "*Until I had experienced it I didn't have any knowledge about it*"

(Patient, 53 years old, in-depth interview)

ii. Current awareness status of breast cancer in Nigeria

There is need to create more awareness about breast cancer as lack of awareness is one of the factors affecting early diagnosis of breast cancer.

Key1: "*I also think that the awareness about it is not enough,……. it's not enough in the community because there is till a lot of ……."*

(Gynaecologist, more than 10 year experience, key informant interview)

Key 9: "*it does but the thing is the field is wide and the number of organizations doing this are not much unlike HIV and other you know there is no money in Cancer unlike maternal and child care, HIV, USAID…..*"

(Healthcare volunteer 1, key informant interview)

iii. Level of education

It was perceived that most patients that present late stage breast cancer at hospitals are patients with no formal education and usually attribute the cause to other factors.

Key 2: "……*in our society those who are educated who have at least a secondary school level or first degree level (of) education, they are more responsive to any scientific based evidence but those who are not educated will always attribute any and everything to certain factors spiritual or mysterious*"

(Pathologist, years of experience: over 10 years, key informant interview)

4.3 Socio-demographics factors of breast cancer patients for quantitative analysis

Mean age of breast cancer was 45.81±10.66 and most of the participants (96%) had no family history of breast cancer (Table 4.3). Most of the breast cancer patients had not use birth control (80%) or fertility hormone pills (95%), with 34% of the breast cancer patients having tertiary education while 17 % had no formal education (Table 4.3). The body mass index (BMI) of participants was analyzed and divided into categories of underweight (18.5), normal (18.5-24.9), overweight (25-29.9), and obese (30). Approximately 55.6% of the breast cancer patients had a normal BMI, while 5.6%, 22.2%, and 16.7% were underweight, overweight, and obese, respectively (Fig. 4.2).

Table 4.3: Description of breast cancer patients recruited for this study

Demographics	Breast cancer patients
Age	
Mean	45.81±10.66
Education	
No formal education	17% (17)
Elementary	17% (17)
Secondary school	32% (32)
Higher or tertiary education	34% (34)
Family history of breast cancer	
Yes	4% (4)
No	96% (96)
Use of Birth control	
Yes	20% (20)
No	80% (80)
Use of fertility hormone	
Yes	5% (5)
No	95% (95)

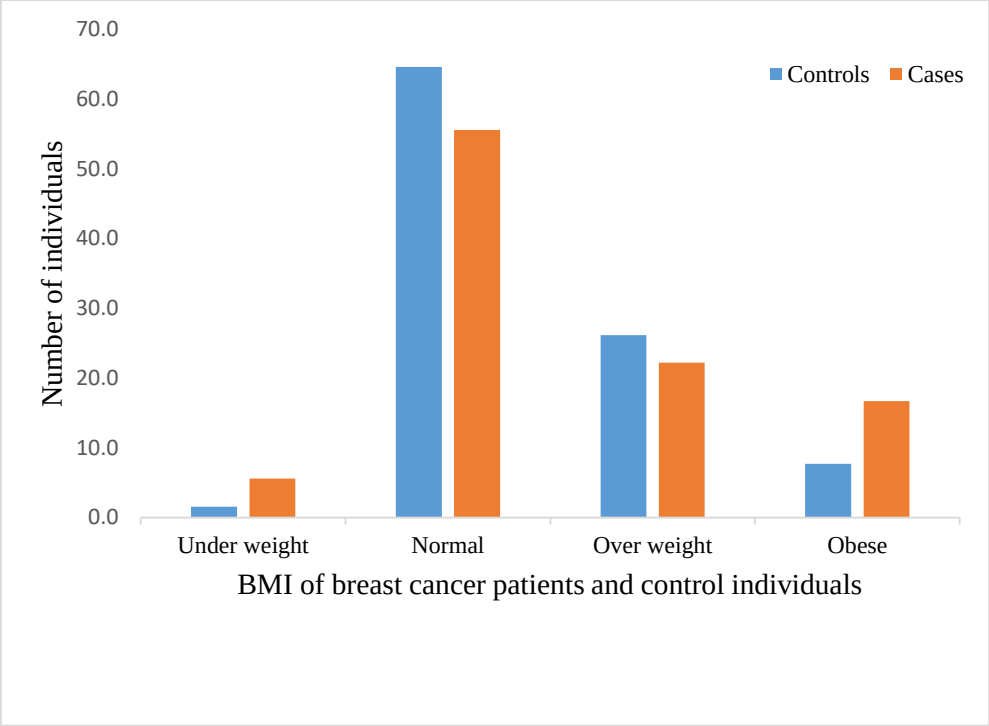

Fig. 4.2: Body Mass Index (BMI) in breast cancer patients and controls individuals.

Breast cancer patients were grouped based on their occupational status, which is a reflection of their socioeconomic status. The patients were grouped into categories of unemployed, unskilled labourers, and skilled professionals. Unskilled labourers had the highest frequency among the breast cancer patients studied (56.5%), while unemployed and skilled professionals had 8.7% and 34.8% frequencies, respectively (Fig. 4.3). Tumour grades at diagnosis of patients were documented, and 58% had a grade 2 tumour at diagnosis, while 19% and 23% presented with grade 1 and grade 3 tumours, respectively (Fig. 4.4).

4.4 Detection of TNF-α by ELISA

Using unpaired t-Test, the results on Fig 4.5 showed that the mean level of TNF-α in plasma samples of breast cancer patients (mean=96.13pg/ml ± 15.01) was significantly lower when compared to control individuals (mean= 174.4pg/ml ± 32.46) ($p=0.02$). Using multiple comparison test, TNF-α levels in plasma of breast cancer patients diagnosed with grade 3 tumour was significantly lower than TNF-α levels in plasma of control individuals with mean difference of 129.7 (CI= 26.69 to 232.8) ($p<0.05$) ($R^2=0.12$) (Table 4.4)

4.5 Detection of soluble-TNF-α receptor 1 by ELISA

The mean level of soluble-TNF-α receptor 1 in women with breast cancer (mean=5.038ng/ml ± 1.203) was lower but not significantly different from the mean level in compared to control individuals (mean =7.446ng/ml ± 1.861) (Fig. 4.7).

4.6 Genotyping of *TNF-α* SNPs in participants

In this study, the genotyping of six *TNF-α* SNPs was performed using allele-specific PCR with ten pairs of primers. This allowed for the differential amplification of each allele of every SNP, which was then visualized through gel electrophoresis as demonstrated in Figure 4.7a & b. Specifically, the SNPs genotyped were *TNF-α308G>A, 488G>A, 380G>A, 1032C>T, 238G>A,* and *859C>T*. Examples of the gel pictures for *TNF-α 859T* is shown in Fig. 4.7 a & b, *TNF-α 859C &1032T* in Fig 4.8a while *TNF-α 238A &308G* in Fig.4.8b, others are in appendix.

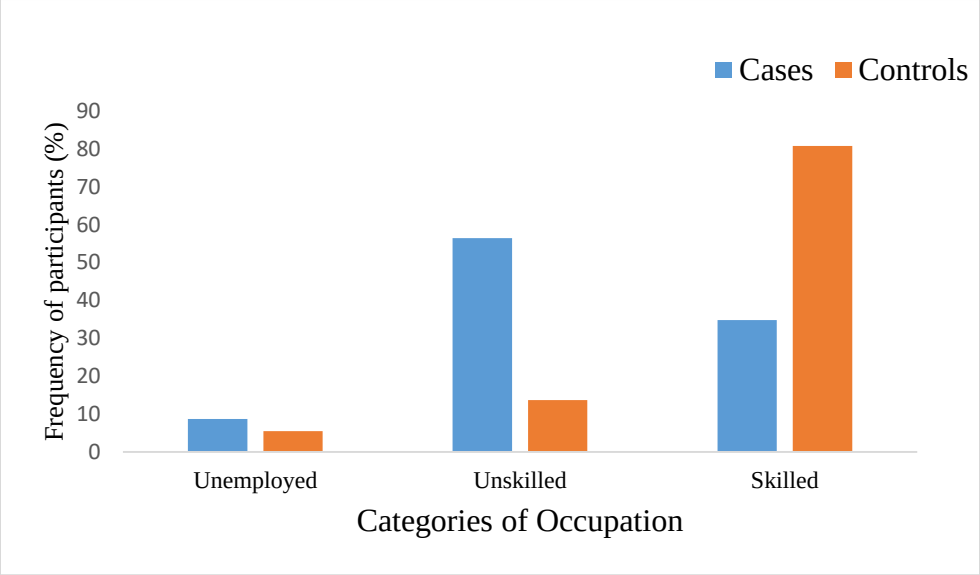

Fig. 4.3: Occupational status of study participants.

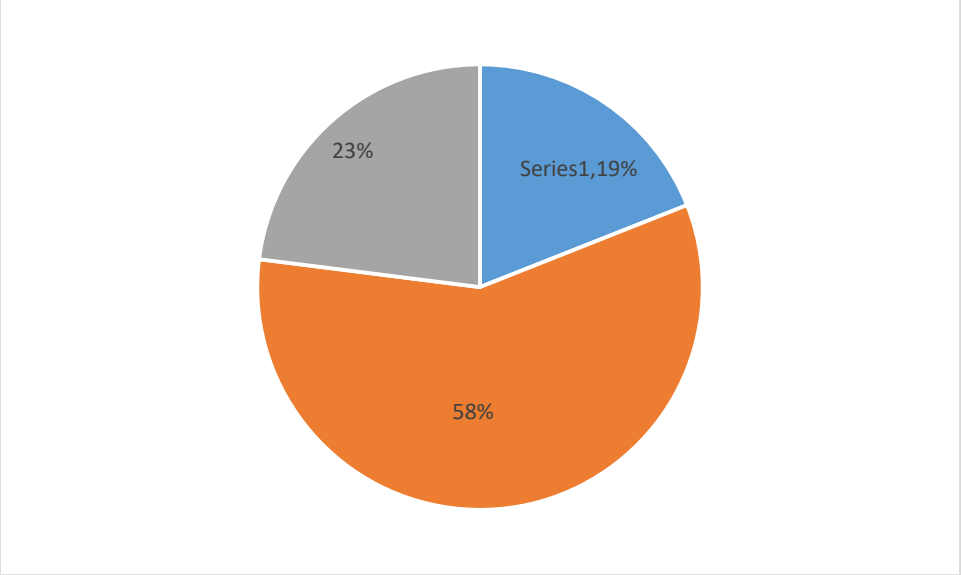

Fig. 4.4: The frequency of tumour grades of breast cancer patients at diagnosis.

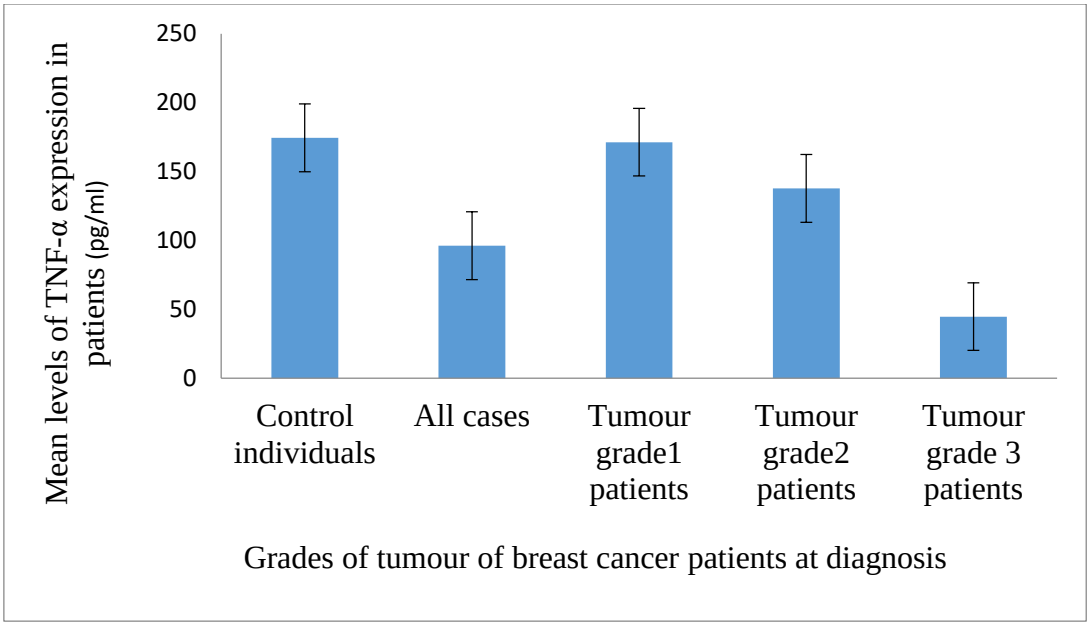

Fig. 4.5: Level of TNF-α expression in participants.

A significant difference was observed in mean levels of *TNF-α* in cases and controls especially between controls and patients with grade 3 tumour at diagnosis ($p<0.05$).

Table 4.4: Differences in mean levels of TNF-α in breast cancer patients compared to control individuals and grades of tumour

Comparison Test	Mean Diff.	95% CI of diff
Controls vs breast cancer patients	78.27	11.37 to 145.2*
Controls vs Grade 1 breast cancer patients	3.160	-92.86 to99.18
Controls vs Grade 2 breast cancer patients	37.40	-33.56 to108.4
Controls vs Grade 3 breast cancer patients	129.7	26.69 to 232.8*
Grade1 breast cancer patients vs Grade 2 breast cancer patients	34.24	-77.82 to 146.3
Grade 1 breast cancer patients vs Grade 3 breast cancer patients	126.6	-53.39 to 306.5
Grade 2 breast cancer patients vs Grade 3 breast cancer patients	92.32	-25.80 to 210.4

*Significant values at $p<0.05$ $R^2=0.12$

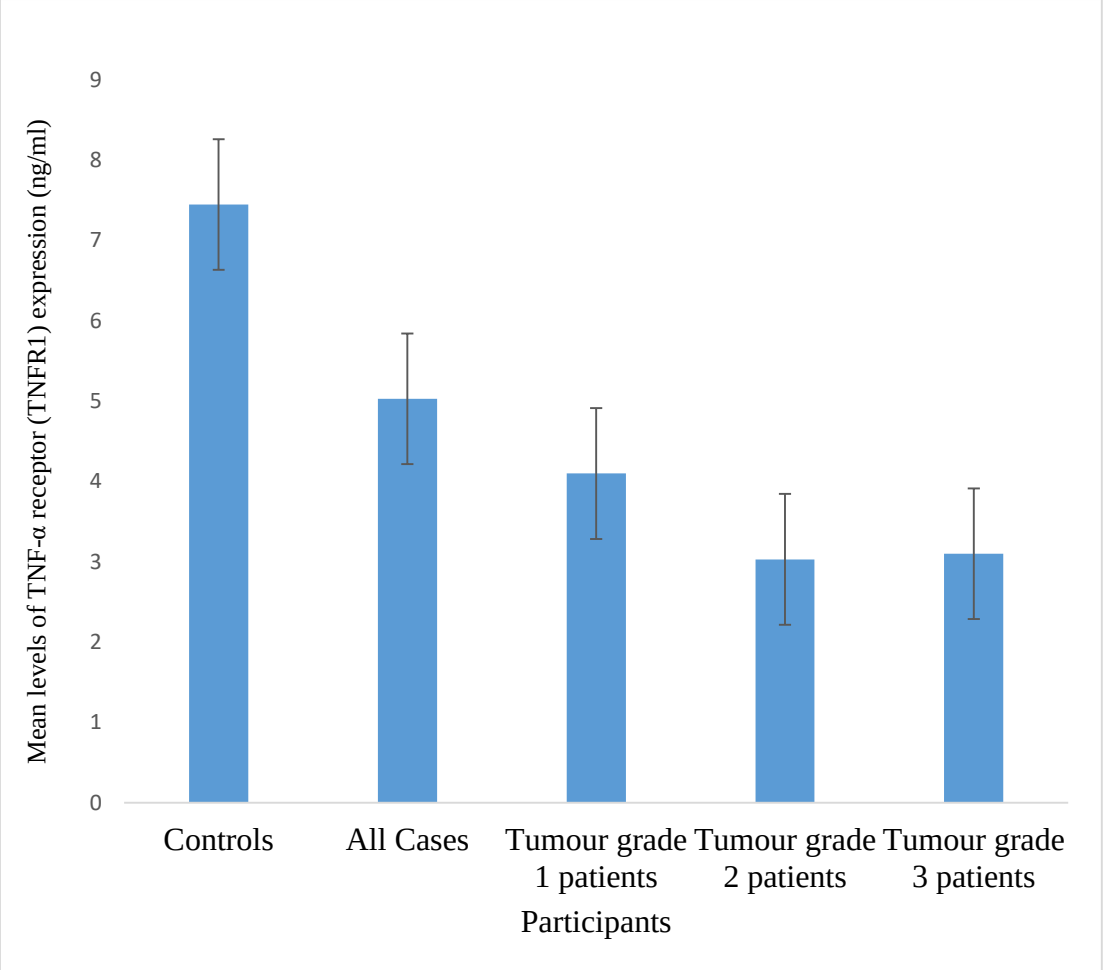

Fig. 4.6: Expression of soluble-TNF-α receptor1 among participants.

The mean level of soluble-TNF-α receptor1 was significant different among participants ($p>0.05$).

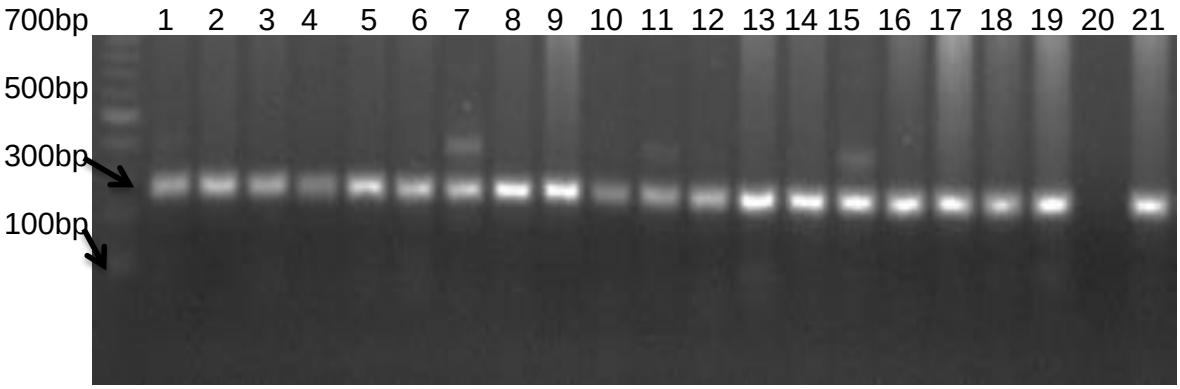

Fig. 4.7a: Amplification of *TNF-α 859T* in control samples

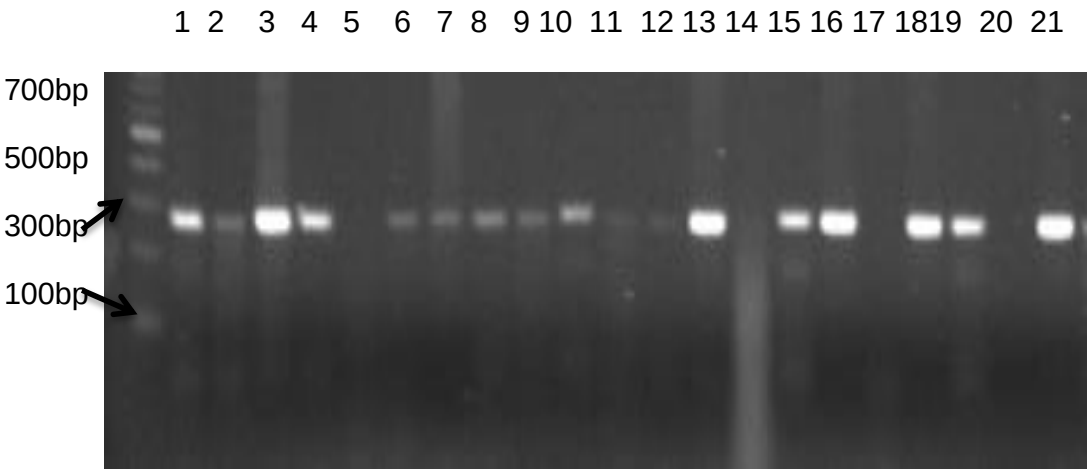

Fig. 4.7b: Amplification of *TNF-α 859T* in breast cancer samples

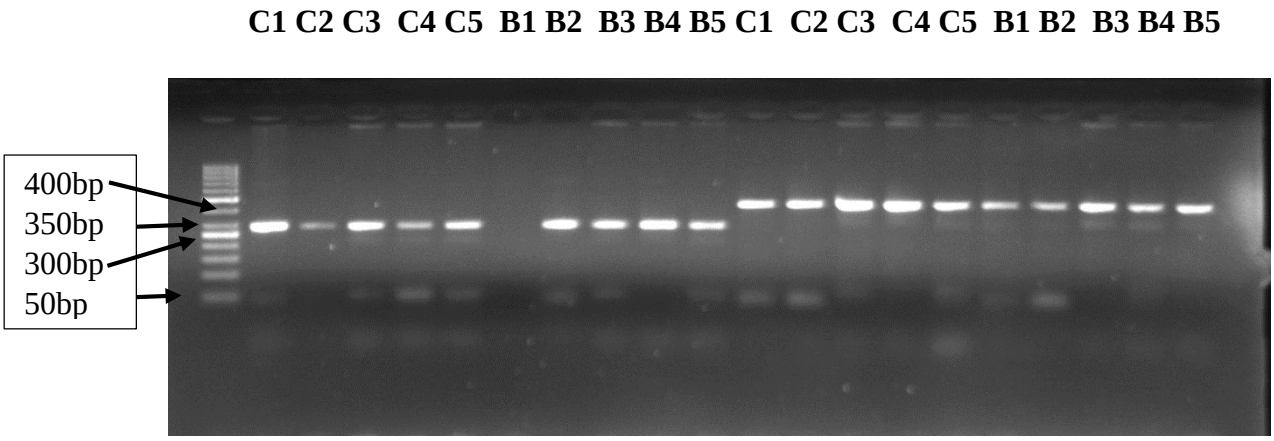

Fig. 4.8: Amplification of *TNF-α 859C* and *TNF-α 1032T* in cases and controls
Keys
B-Cases
C- Controls

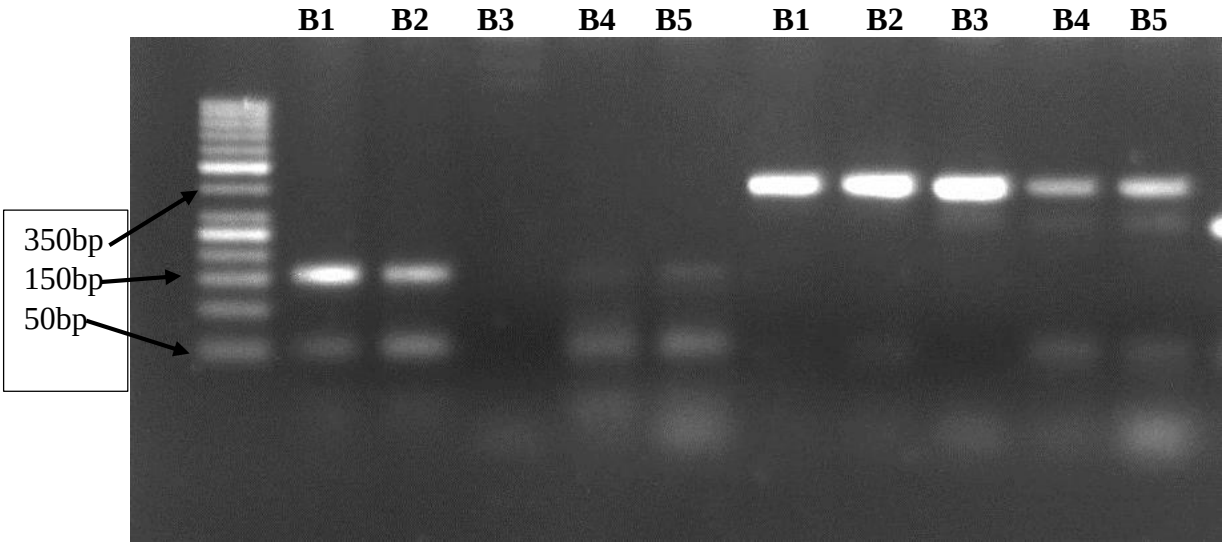

Fig. 4.9: Amplification of *TNF-α 238A* and *TNF-α 308G* in cases

Keys
B-Cases

4.6.1. Distribution of TNF-α SNPs in Breast cancer patients compared with control individuals.

The frequency distribution of the alleles of each SNP varied among participants. Using Fisher's exact test, the frequency distributions of alleles in controls were compared to cases. The frequency of *TNF-α 488G* was significantly lower among cases compared to controls ($p<0.05$), with an odds ratio of 0.24, indicating that the presence of this allele might reduce the risk of breast cancer (Table 4.5). On the other hand, there was no significant difference in the frequency of *TNF-α 488A* among participants (Table 4.6).
The frequency distributions of *TNF-α 238A* (Table 4.7) and *TNF-α 238G* (Table 4.8) were not significantly different between cases and controls ($p>0.05$), with odds ratios less than 1.
Similarly, the frequency distribution of *TNF-α 308A* was also significantly lower among cases compared to controls ($p<0.05$), with an odds ratio of 0.33. This suggests that the presence of this allele reduces the odds of having breast cancer (Table 4.9). Although the frequency of *TNF-α 308G* showed no significant difference in cases and controls, it had an odds ratio less than 1 (Table 4.10).
The frequency distributions of *TNF-α 859C* (Table 4.11) and *TNF-α 859T* (Table 4.12) were not significantly different between cases and controls ($p>0.05$), with odds ratios approximately 1 indicating no association with breast cancer.
Furthermore, the frequency distribution of *TNF-α 380G* was significantly lower among cases compared to controls ($p<0.05$), with an odds ratio of 0.51, indicating that the presence of this allele reduces the risk of breast cancer (Table 4.13). Conversely, there was no significant difference in the frequency of *TNF-α 380A* among participants (Table 4.14).
There was no significant difference in the frequency distribution of *TNF-α 1032T* in cases compared to controls (Table 4.15). However, a significant difference was observed in the distribution of *TNF-α 1032C* among cases and controls, showing an increased odds ratio greater than 1 (2.1). This suggests that the absence of this allele might increase susceptibility to breast cancer (Table 4.16).

Table 4.5: *TNF-α 488G* allele showed a protective association against breast cancer.

	Case	Control	Total
Observation			
Present	85	96	181
Absent	15	4	19
Total	100	100	200
P value			**0.0140***
Strength of association			
Relative Risk			0.5948
95% confidence interval			0.4500 to 0.7864
Odds ratio			0.2361
95% confidence interval			0.07542 to 0.7391

***p<0.05.** *The frequency of TNF-α 488 was significantly lower among cases compared to controls*

Keys

Present- number of individuals with *TNF-α 488G*

Absent- number of individuals without *TNF-α 488G*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.6: *TNF-α 488A* showed no significant association with breast cancer

Observation	Case	Control	Total
Present	86	87	173
Absent	14	13	27
Total	100	100	200
P value			**1.0000**
Strength of association			
Relative Risk			0.9587
95% confidence interval			0.6470 to 1.421
Odds ratio			0.9179
95% confidence interval			0.4076 to 2.067

Keys

Present- number of individuals with *TNF-α 488A*

Absent- number of individuals without *TNF-α 488A*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.7: No significant association was found between *TNF-α 238 A* and breast cancer

Observation	Case	Control	Total
Present	81	87	168
Absent	19	13	32
Total	100	100	200
P value			**0.3350**
Strength of association			
Relative Risk			0.8120
95% confidence interval			0.5857 to 1.126
Odds ratio			0.6370
95% confidence interval			0.2956 to 1.373

Keys

Present- number of individuals with *TNF-α 238A*

Absent- number of individuals without *TNF-α 238A*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.8: No significant association between *TNF-α 238G* and breast cancer

Observation	Case	Control	Total
Present	91	96	187
Absent	9	4	13
Total	100	100	200
P value			**0.2507**
Strength of association			
Relative Risk			0.7029
95% confidence interval			0.4753 to 1.039
Odds ratio			0.4213
95% confidence interval			0.1253 to 1.416

Keys

Present- number of individuals with *TNF-α 238G*

Absent- number of individuals without *TNF-α 238G*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.9: *TNF-α 308A* indicated a protective association against breast cancer.

Observation	Case	Control	Total
Present	79	92	171
Absent	21	8	29
Total	100	100	200
P value			**0.0149***
Strength of association			
Relative Risk			0.6380
95% confidence interval			0.4837 to 0.8415
Odds ratio			0.3271
95% confidence interval			0.1373 to 0.7794

***p<0.05.** *The frequency was significantly lower among cases compared to controls*

Keys

Present- number of individuals with *TNF-α 308A*

Absent- number of individuals without *TNF-α 308A*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.10: *TNF-α 308G* showed no significant association with breast cancer

Observation	Case	Control	Total
Present	85	90	175
Absent	15	10	25
Total	100	100	200
P value			**0.3928**
Strength of association			
Relative Risk			0.8095
95% confidence interval			0.5678 to 1.154
Odds ratio			0.6296
95% confidence interval			0.2682 to 1.478

Keys

Present- number of individuals with *TNF-α 308G*

Absent- number of individuals without *TNF-α 308G*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.11: *TNF-α 859C* showed no significant association to breast cancer

Observation	Case	Control	Total
Present	86	85	171
Absent	14	15	29
Total	100	100	200
P value			**1.0000**
Strength of association			
Relative Risk			1.042
95% confidence interval			0.6947 to 1.562
Odds ratio			1.084
95% confidence interval			0.4931 to 2.383

Keys

Present- number of individuals with *TNF-α 859C*

Absent- number of individuals without *TNF-α 859C*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.12: *TNF-α 859T* also showed no significant association with breast cancer

Observation	Case	Control	Total
Present	81	81	162
Absent	19	19	38
Total	100	100	200
P value			**1.0000**
Strength of association			
Relative Risk			1.000
95% confidence interval			0.7023 to 1.424
Odds ratio			1.000
95% confidence interval			0.4933 to 2.027

Keys

Present- number of individuals with *TNF-α 859T*

Absent- number of individuals without *TNF-α 859T*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.13: The frequency of *TNF-α 380G* was significantly lower among cases compared to controls.

Observation	Case	Control	Total
Present	58	73	131
Absent	42	27	69
Total	100	100	200
P value			**0.0369***
Strength of association			
Relative Risk			0.7274
95% confidence interval			0.5554 to 0.9525
Odds ratio			0.5108
95% confidence interval			0.2820 to 0.9251

***P<0.05.** *The presence of TNF-α 380G indicated a protective association against breast cancer.*

Keys

Present- number of individuals with *TNF-α 380G*

Absent- number of individuals without *TNF-α 380G*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.14: *TNF-α 380A* indicated no significant association with breast cancer

Observation	Case	Control	Total
Present	80	84	164
Absent	20	16	36
Total	100	100	200
P value			**0.5813**
Strength of association			
Relative Risk			0.8780
95% confidence interval			0.6302 to 1.223
Odds ratio			0.7619
95% confidence interval			0.3689 to 1.574

Keys

Present- number of individuals with *TNF-α 380A*

Absent- number of individuals without *TNF-α 380A*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.15: *TNF-α 1032T* showed no significant association with breast cancer

Observation	Case	Control	Total
Absent	27	29	56
Present	73	71	144
Total	100	100	200
Strength of association			
Relative Risk			0.9511
95% confidence interval			0.6936 to 1.304
Odds ratio			0.9055
95% confidence interval			0.4882 to 1.680

Keys

Present- number of individuals with *TNF-α 1032T*

Absent- number of individuals without *TNF-α 1032T*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.16: Absence of *TNF- α 1032C* is associated with increased risk for susceptibility to breast cancer.

Observation	Case	Control	Total
Absent	56	38	94
Present	44	62	106
Total	100	100	200
P value			**0.0158***
Strength of association			
Relative Risk			1.435
95% confidence interval			1.084 to 1.900
Odds ratio			2.077
95% confidence interval			1.180 to 3.653

***P<0.05.** *The frequency of TNF-α 1032C was significantly lower among cases compared to controls*.

Keys

Present- number of individuals with *TNF-α 1032C*

Absent- number of individuals without *TNF-α 1032C*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

4.6.2. Genotype distribution of *TNF-α* among participants

The genotype distribution of each SNP was analysed and presented in graphs. Notably, the genotypes of *TNF-α* among participants exhibited high heterozygosity for all SNPs, as evidenced in Figures 4.10 to 4.15. The frequency of genotypes GA, GG, and AA for *TNF-α 488, 238,* and *380* (Figures 4.10, 4.11, and 4.14, respectively) did not show any significant differences between cases and controls. Similarly, the frequencies of *TNFα 859* CC, CT, and TT genotypes did not exhibit any significant differences between the case and control groups, as illustrated in Figure 4.13. In contrast, the frequencies of genotypes GA, AA, and GG of *TNF-α 308*, and genotypes CC, CT, and TT of *TNF-α 1032*, revealed significant differences between the cases and controls, as demonstrated in Figures 4.12 and 4.15, respectively.

Furthermore, the high heterozygosity observed in the genotypes of *TNF-α* SNPs suggested a deviation from the Hardy-Weinberg equilibrium. This is presented in Table 4.17. Overall, these findings provide insight into the potential genetic factors associated with breast cancer and highlight the importance of further research in the association of *TNF-α* SNPs with breast cancer.

4.7 Analysis of *TNF-α* sequence data

Alignment of *TNF-α* promoter region in participants was done against human reference genome in the National Centre for Biotechnology Information (NCBI) nucleotide data base. This analysis was done as a confirmatory test to show that the amplified region is present in study participants as seen on agarose gel as in Fig. 4.14

4.8 Relationship between variance in *TNF-α* SNP frequencies and TNF-α plasma levels

Regression model, heteroskedasticity test, was used to test the association between variances in frequencies of *TNF-α* SNP and TNF-α plasma levels of participants. TNF-α 308A, 488G, 1032C in breast cancer patients showed significant association with plasma levels of TNF-α in breast cancer patients $p<0.05$ (Table 4.18). In the controls, TNF-α 238A, 488G and 1032T showed significant association with plasma levels of TNF-α $p<0.05$ (Table 4.19).

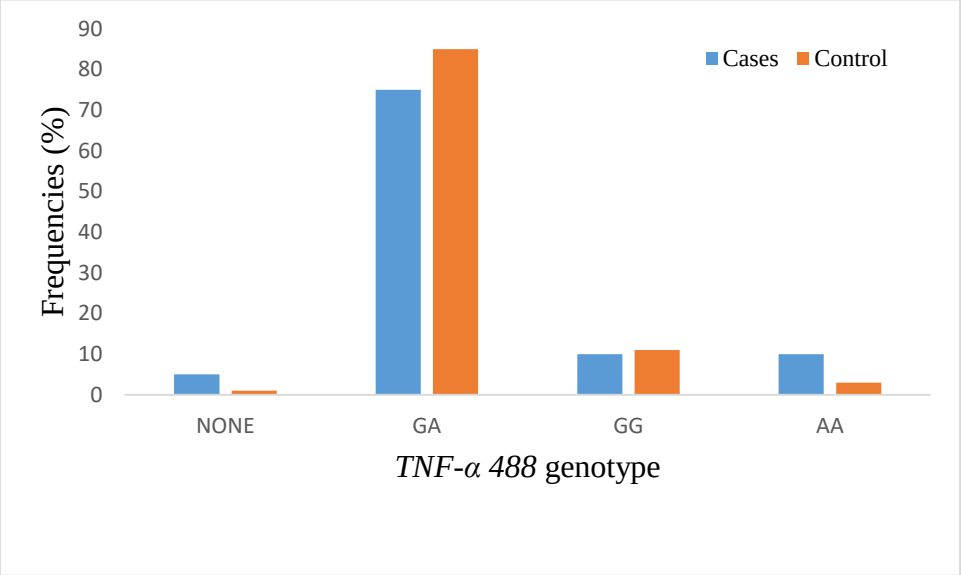

Fig. 4.10: *TNF-α 488* genotype among study participants.

Significant difference was observed in genotype frequencies of *TNF-α 488* (p=0.2841) among controls and breast cancer patients.

Keys

None - none carriers of TNF-α 488;

GA - heterozygous genotype carriers of TNF-α 488;

GG - homozygous wild type carriers of TNF-α 488;

*AA - homozygous mutant type carriers of TNF-α 488***.**

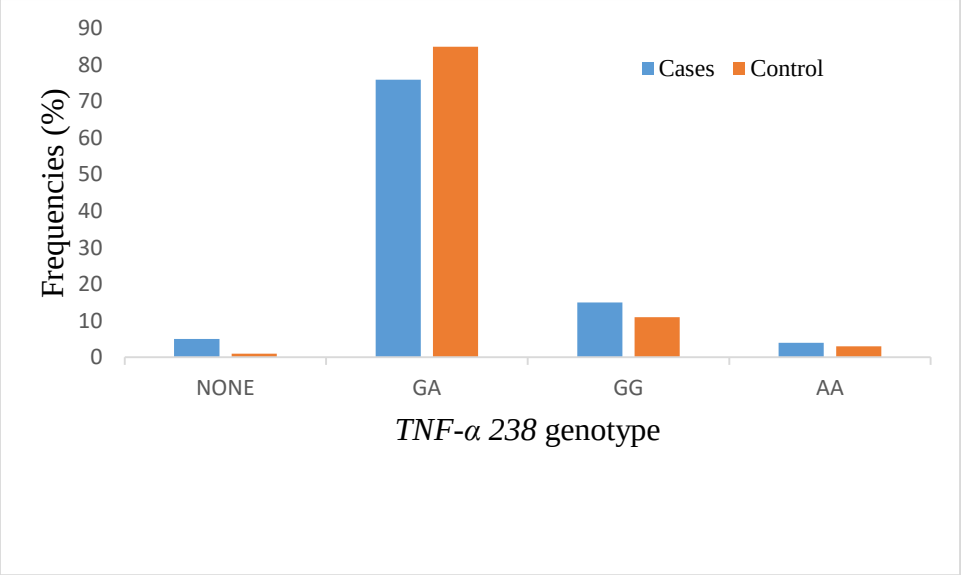

Fig. 4.11: *TNF-α 238* genotype among study participants.

Significant difference was observed **in genotype frequencies of *TNF-α 238* (p=0.7859) among controls and breast cancer patients.**

Keys

None - none carriers of TNF-α 238;

GA - heterozygous genotype carriers of TNF-α 238;

GG - homozygous wild type carriers of TNF-α 238;

AA - homozygous mutant type carriers of TNF-α 238.

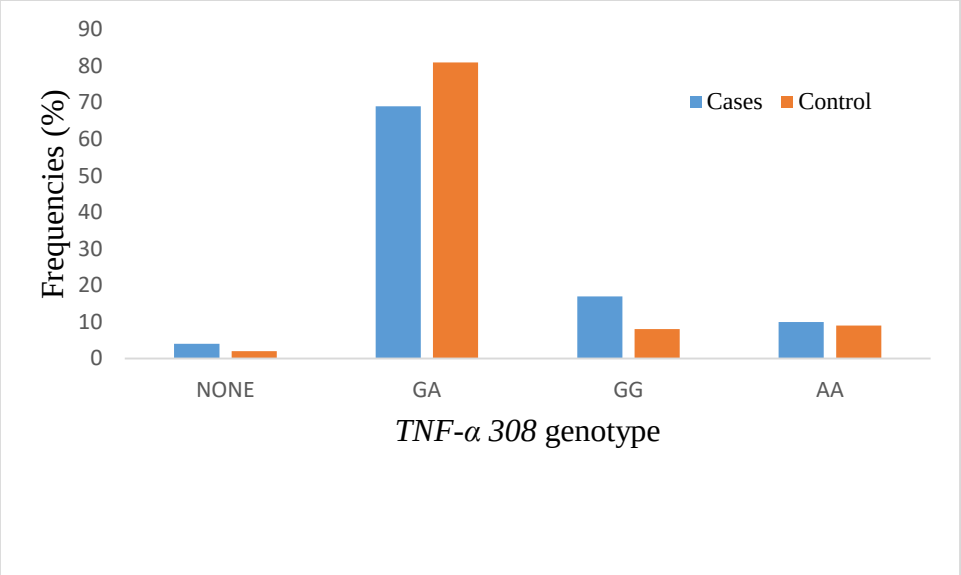

Fig. 4.12: *TNF-α 308* genotype among study participants.

Significant difference was observed in genotype frequencies of *TNF-α 308* (P=0.0213) among controls and breast cancer patients

Keys

None - none carriers of TNF-α 308;

GA - heterozygous genotype carriers of TNF-α 308;

GG - homozygous wild type carriers of TNF-α 308;

AA - homozygous mutant type carriers of TNF-α 308.

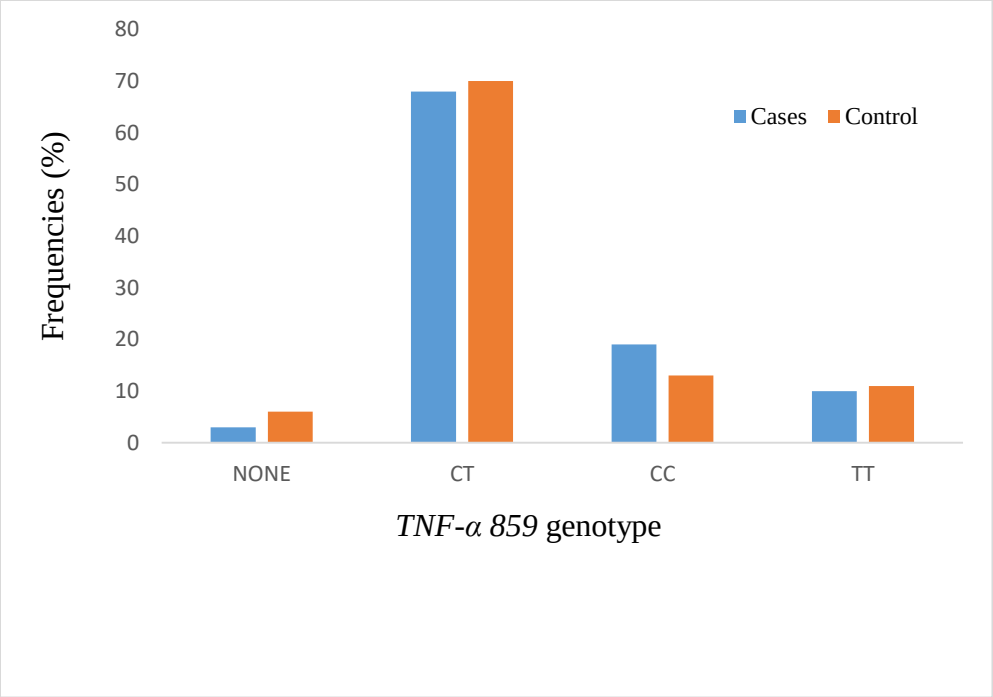

Fig. 4.13: *TNF-α 859* genotype among study participants.

Significant difference was observed in genotype frequencies of *TNF-α 859* (P=0.4922) among controls and breast cancer patients.

Keys

None - none carriers of TNF-α 859;
CT - heterozygous genotype carriers of TNF-α 859;
CC - homozygous wild type carriers of TNF-α 859;
TT - homozygous mutant type carriers of TNF-α 859.

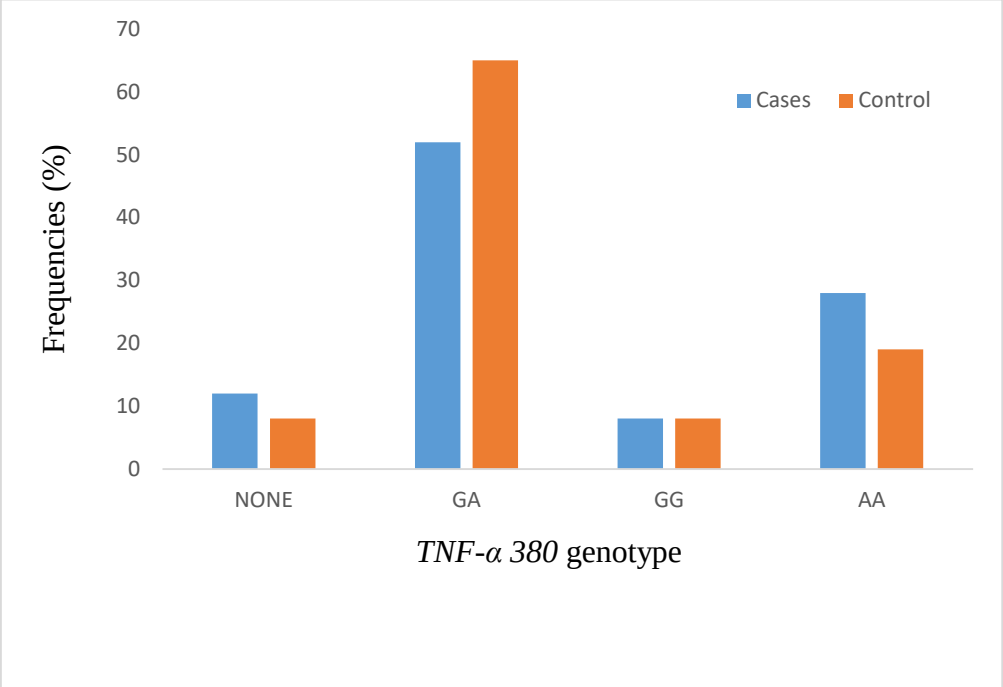

Fig. 4.14: *TNF-α 380* genotype among study participants.

Significant difference was observed in genotype frequencies of *TNF-α 380* (p=0.3014) among controls and breast cancer patients.

Keys

None - none carriers of TNF-α 380;
GA - heterozygous genotype carriers of TNF-α 380;
GG - homozygous wild type carriers of TNF-α 380;
AA - homozygous mutant type carriers of TNF-α 380.

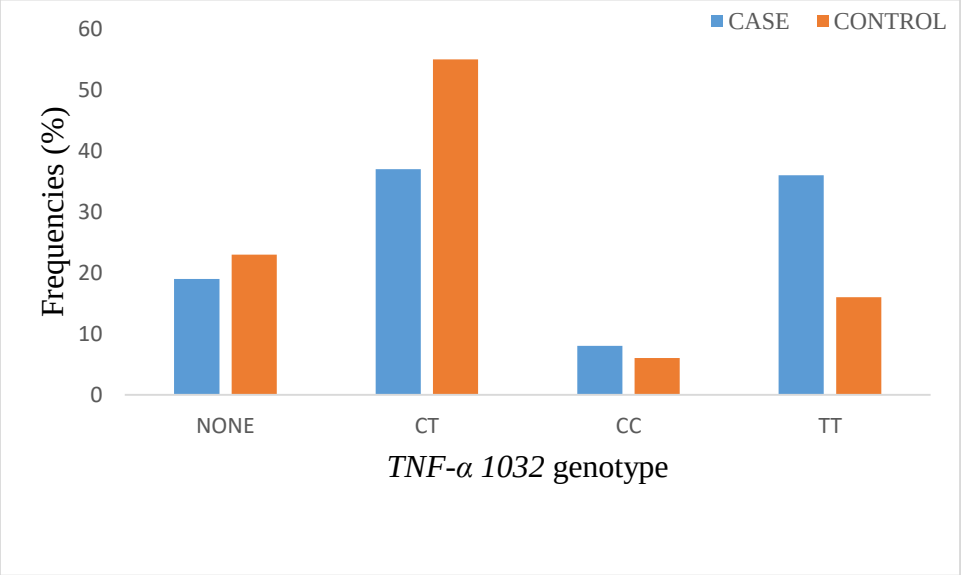

Fig. 4.15: *TNF-α 1032* genotype among study participants.

Significant difference was observed in genotype frequencies of *TNF-α 1032* (p=0.0078) among controls and breast cancer patients.

Keys

None - none carriers of TNF-α 1032;
CT - heterozygous genotype carriers of TNF-α 1032;
CC - homozygous wild type carriers of TNF-α 1032;
TT - homozygous mutant type carriers of TNF-α 1032.

Table 4.17: High heterozygosity in *TNF-α* and TNFR1 genes: a deviation from Hardy- Weinberg equilibrium

S/N	Name	Position	Observed HET	Predicted HET	HWpal	MAF	Alleles
1	rs1800610 (*TNF-α 488*)	31543827	0.743	0.493	4.0786E-13	0.441	G:A
2	rs9282876 (*TNF-α 859*)	31574585	0.693	0.499	5.4113E-8	0.475	C:T
3	rs1800750 (*TNF-α 380*)	31575186	0.644	0.493	2.4926E-5	0.441	A:G
4	rs1800629 (*TNF-α 308*)	31575254	0.787	0.5	1.8255E-16	0.498	A:G
5	rs 1799964 (*TNF-α 1032*)	31575324	0.807	0.494	9.7796E-21	0.443	G:A
6	rs361525 (*TNF-α 238*)	31575254	0.58	0.468	0.0093	0.383	T:C
7	rs 1800693 (*TNFR1A+ IV56+10*)	6330843	0.313	0.473	7.0E-4	0.374	G:A

Alignment: C:\Users\CARTA\Desktop\unimed 2023\PhD New\NC_000006.12[31574685..31574724]multiplealignment.fas

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
Primer308A      ---------- ---------- ---------- ---------- ----------
ref|NC 000      ---------- ---------- ---------- ---------- ----------
Human TNF       ---------- ---------- ---------- ---------- ----------
Sample 1        ---------- ---------- ---------- ---------- ----------
Sample 2        TTCAACAAAG AAATGGTGAA TTTCAGATAA TTTAGAAGAC GAAGGAGAAG
Sample 3        ---------- ---------- ---------- ---------- ----------
Sample 4        ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Primer308A      ---------- ---------- ---------- ---------- ----------
ref|NC 000      ---------- ---------- ---------- ---------- ----------
Human TNF       ---------- ---------- ---------- TTCGCCATGT TGGCCAAACT
Sample 1        ---------- ---------- ---------- -----CCATC TTTAGTACTA
Sample 2        TACTTATGTC CAGGCTGCAA TGTTGGGGAA AGCAAACATA GATAAAGGGT
Sample 3        ---------- ---------- ---------- ---------- ----------
Sample 4        ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
Primer308A      ---------- ---------- ---------- ---------- ----------
ref|NC 000      ---------- -----GTATG GGGACCCCCC CTTAACGAAG -ACAGGGCCA
Human TNF       GGTCTCGAAC TCCTGACCTC AAGTGATCTG CCCACCTGGG CCTCACAGGC
Sample 1        AGGTGTAAGC GGCCYTTTAG ACGCTCCCGT CAGGCCGCAG GCATCCGCCC
Sample 2        GCTTTAGTAA AGCTTCTGTT TTTATAGAAT TTCTCCAGCA TCACCAGGCC
Sample 3        ---------- ---CCCTCCC TAGCCCCCCC CTCCCTCGTA GCGCCTCCTC
Sample 4        ---------- ----GGTTCC GCGACGCCAC ATCGGCGAGC TGCAGAGSGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
Primer308A      -ATAGGTTTT GAGGGGCATG A--------- ---------- ----------
ref|NC 000      TGTAGA---- ---------- ---------- ---------- ----------
Human TNF       CATAGACTTT GTGTAACTTG TGTTTCTTTC TCCTAGATGC ATCTTCAACC
Sample 1        TAGGGGCSCT AAGAGCATAC CCGCTGACAC ACCACTGTTA ACGTCCCGTG
Sample 2        CATAAGAG-T GAGCTCTTTC TGTACTTTGT TCTCCCAATA GTGTAAACCA
Sample 3        GATAGCCCCC CCCCC----- ---------- ---------- ----------
Sample 4        TCTCCCCCCT CCCGAAATTT TATTCGATAT CCTACCATCC CCTCTTATCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
Primer308A      ---------- ---------- ---------- ---------- ----------
ref|NC 000      ---------- ---------- ---------- ---------- ----------
Human TNF       TTGGCAAAAA TAAACCTGTG AATCAGTGGA GATCTGCTCG GTCACTTTTT
Sample 1        GGAAGGGCTG GCTGCGGGGG GACACAAGGT TGAAAGCCGC GACCTCACGA
Sample 2        AAAGTGGACC TGTTGTGATC TCCACTGGAT TCACAGGTTT ATTTTTGCCA
Sample 3        ---------- ---------- ---------- ---------- ----------
Sample 4        ATAATA---- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
```

```
Primer308A   ---------- ---------- ---------- ---------- ----------
ref|NC 000   ---------- ---------- ---------- ---------- ----------
Human TNF    GGTTTACACT ATTGGGGAGA ACAAAAGAAG TACAGAAAAG AGCTTTCACT
Sample 1     AGTTCAGAAC AGCAACCAGC TTAGTAAAGT GATGGGTAAG GCTAGTCGCA
Sample 2     AGGTTGAAGA TGCATCTAGA GAAAGAAACA CAAGTTAAAA GTCTATGTAT
Sample 3     ---------- ---------- ---------- ---------- ----------
Sample 4     ---------- ---------- ---------- ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     310        320        330        340        350
Primer308A   ---------- ---------- ---------- ---------- ----------
ref|NC 000   ---------- ---------- ---------- ---------- ----------
Human TNF    CTTATGGGCC TGGTGATGCA CCAGAAGATT CTATAAAGCA AGCTTTACTA
Sample 1     TTATGTTATA CGCAGAGAGC CCCCAAAGGG CGATGAGTGG GGCAGTACCA
Sample 2     TCGTGAGGCC CAGGTAAGGA TCACCAGGTC AGGAGTTCGA GACCAGTTTG
Sample 3     ---------- ---------- ---------- ---------- ----------
Sample 4     ---------- ---------- ---------- ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     360        370        380        390        400
Primer308A   ---------- ---------- ---------- ---------- ----------
ref|NC 000   ---------- ---------- ---------- ---------- ----------
Human TNF    ACTACCCTTT ATCTATGTTT GCTTTCCCCA ACATTGCCGC CCCTGGACAC
Sample 1     GCACAGCTGC TAGATAGACT GCTACTAA-- ---------- ----------
Sample 2     GCCAACATGG CGAATGAAGT GGCGCACGAG AACGCGATGC TGCTCACGAT
Sample 3     ---------- ---------- ---------- ---------- ----------
Sample 4     ---------- ---------- ---------- ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     410        420        430        440        450
Primer308A   ---------- ---------- ---------- ---------- ----------
ref|NC 000   ---------- ---------- ---------- ---------- ----------
Human TNF    TCAAAGTACT TCTCCTTCGT CTTCTAAATT ATCTAAAAAT TCACTGTTCT
Sample 1     ---------- ---------- ---------- ---------- ----------
Sample 2     AAATGCGAAA ACGGTTAAAC ACCCAATCTG AAAACGAAGT ACAAC-----
Sample 3     ---------- ---------- ---------- ---------- ----------
Sample 4     ---------- ---------- ---------- ---------- ----------

             ....|...

Primer308A   --------
ref|NC 000   --------
Human TNF    TTGTTGAA
Sample 1     --------
Sample 2     --------
Sample 3     --------
Sample 4     --------
```

Fig. 4.16: Sequence alignment of *TNF-α* SNPs in breast cancer patients (samples) with reference human genome from clone RP1-34B20 on chromosome 6p21.31-22.2 (Human), Primer 308A and Ref seq *TNF-α 308*.

Table 4.18: Relationship between variance in *TNF-α* SNP frequencies and TNF-α plasma levels in breast cancer patients

S/N	SNP	CHI square value	P-value
1	**859C**	0.06	0.81
2	**859T**	0.95	0.33
3	**488G**	4.94	0.026*
4	**488A**	2.16	0.1413
5	**380G**	0.70	0.40
6	**380A**	0.50	0.47
7	**308A**	5.01	0.025*
8	**308G**	2.16	0.1413
9	**238A**	2.74	0.0976
10	**238G**	1.03	0.3109
11	**1032C**	5.55	0.0185*
12	**1032T**	0.49	0.4820

***P<0.05**

Table 4.19: Relationship between variance in *TNF-α* SNP frequencies and TNF-α plasma levels in controls

S/N	SNP	CHI square value	P-value
1	**859C**	0.42	0.52
2	**859T**	3.77	0.052
3	**488G**	6.46	0.011*
4	**488A**	2.63	0.1046
5	**380G**	0.25	0.62
6	**380A**	0.35	0.55
7	**308A**	1.18	0.277
8	**308G**	1.02	0.3118
9	**238A**	4.73	0.0297*
10	**238G**	2.9	0.0886
11	**1032C**	0.27	0.6006
12	**1032T**	7.94	0.00048*

*P<0.05

4.9 Genotyping of TNF-α receptor SNP- *TNFR1A+IV56+10*

The TNF-α receptor SNP genotyped was *TNFR1A+IV56+10 G>A.* The frequency of allele G of *TNFR1A+IV56+10* was significantly lower in breast cancer patients compared to controls with odd ratio of 0.3535 (Table 4.20). The frequency of allele A of *TNFR1A+IV56+10* was not significantly different in breast cancer patients compared to the controls (Table 4.21).

Receptor SNP genotypes in the population showed very high heterozygosity which was not significantly different between controls and breast cancer patients (Fig. 4.15). The observed high heterozygosity was a deviation from Hardy-Weinberg equilibrium (Table 4.17).

Table 4.20: *TNFR1+1V56+10A* indicated no significant association with breast cancer

Observation	Case	Control	Total
Present	28	34	62
Absent	52	46	98
Total	80	80	160
P value			**0.4173**
Strength of association			
Relative Risk			0.8511
95% confidence interval			0.6109 to 1.186
Odds ratio			0.7285
95% confidence interval			0.3847 to 1.380

Keys

Present- number of individuals with *TNFR1+1V56+10A*

Absent- number of individuals without *TNFR1+1V56+10A*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

Table 4.21: *TNFR1+1V56+10 G* also indicated a protective association with breast cancer.

Observation	Case	Control	Total
Present	35	55	90
Absent	45	25	70
Total	80	80	160
P value			**0.0024***
Strength of association			
Relative Risk			0.6049
95% confidence interval			0.4426 to 0.8268
Odds ratio			0.3535
95% confidence interval			0.1851 to 0.6753

***P<0.05.** *The frequencies of TNFR1A+IV56+10 was significantly lower among cases compared to control.*

Keys

Present- number of individuals with *TNFR1+1V56+10G*

Absent- number of individuals without *TNFR1+1V56+10G*

P-value- set at $\alpha = 0.05$ to test differences in frequency distribution of SNP

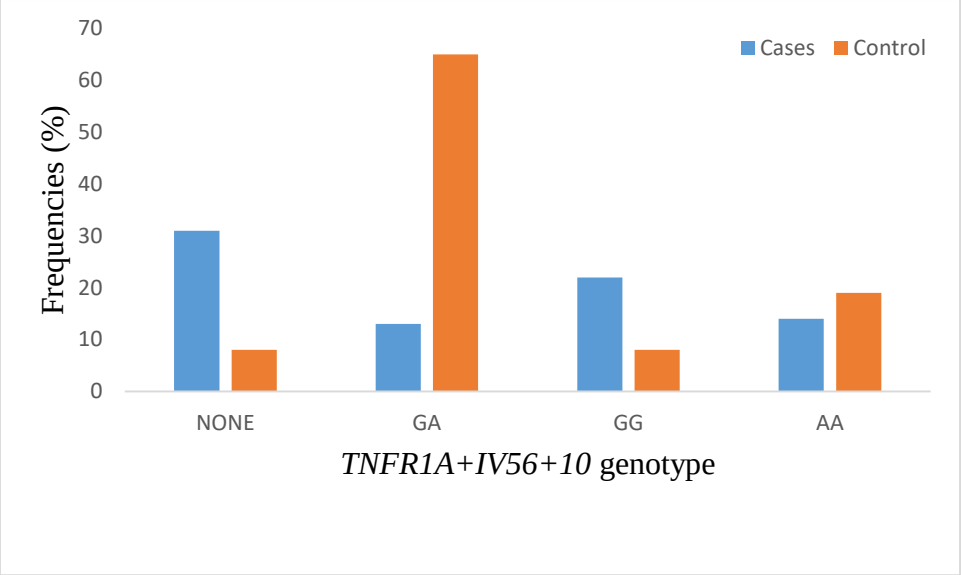

Fig. 4.17: *TNFR1A+IV56+10* genotype among study participants.

Significant difference was observed in genotype frequencies of TNFR1 SNP (*TNFR1A+IV56+10*) (p=0.1147) among controls and breast cancer patients.

Keys

None - none carriers of TNFR1A+IV56+10;
GA - heterozygous genotype carriers of TNFR1A+IV56+10;
GG - homozygous wild type carriers of TNFR1A+IV56+10;
AA - homozygous mutant type carriers of TNFR1A+IV56+10.

CHAPTER FIVE

DISCUSSION

Cancer of the breast is a complex disease that largely affects adult females and a much lower percentage of men (Petrucelli *et al.,* 2022). The incidence of this disease is on the rise in Nigeria and around the world, thus making it a serious public health issue (Azubuike *et al*., 2018; Sung *et al*., 2021; Ntekim *et al*., 2022). In Nigeria, the high rate of mortality due to untimely diagnosis of the disease is persistent. With the multifactorial nature of breast cancer, both genetics and the environment have been implicated in its aetiology, like other cancer types. For breast cancer patients, early detection is essential for treatment and a better prognosis. Increasingly, genetic testing is being used frequently to diagnose diseases early and for prevention although, this is of limited use or non-existent in Nigeria (Wright *et al.,* 2018). Deleterious *BRCA 1 & 2* gene mutations are the most frequently utilised genes for genetic testing for cancer of the breast due to their high penetrance in the disease development. There is limited use of these high-risk genes for risk assessment in Nigeria because only a fraction of breast cancer patients tested are carriers of these gene mutations in the country (Rotimi *et al.,* 2021). Therefore, it is important to identify better risk assessment tools that would help more women determine their breast cancer risk. Consequently, the present study was aimed at identifying important genetic variations of the TNF-α cytokine gene that can be used as potential predictors for breast cancer in Nigeria and to evaluate epidemiological factors affecting early identification of breast cancer in Nigeria.

5.1 Barriers to early diagnosis of breast cancer in Nigeria

To identify the factors that contribute to delayed diagnosis of the breast cancer, in this study, we examined the experiences of patients and healthcare providers in the process of disease detection and confirmatory diagnosis. We found that in Southwest Nigeria, the diagnosis of breast cancer often involved three basic processes: Self-detection, hospital visit and definitive diagnosis. Factors causing a delayed diagnosis were found

at each stage of the process. The method of detection, shown to be primarily breast self-examination, is the first and most significant component that may be related to a delayed diagnosis. It was found that compared to post-symptomatic screening, asymptomatic screening for breast cancer has a lower uptake. Since BSE has been the subject of most awareness information, women in Nigeria are becoming more knowledgeable about breast self-examination (BSE) (Johnson, 2019). In this study 88% of the women surveyed discovered a breast lump with BSE, making BSE the most prevalent detection technique among women. However, BSE was viewed by health professionals from this study as a post-symptomatic screening method, which is ineffective in stopping late breast cancer diagnoses. Due to the aggressive form of breast cancer that is encountered in Nigeria, BSE may not be the most effective early diagnosis tool (Pitt *et al*., 2018). Additionally, health care providers' interviews showed that mostly educated women attend frequent mammogram check-ups at the hospitals. Mammography is a crucial test used for both asymptomatic and post-symptomatic breast cancer diagnosis. The United States' cancer mortality rates have significantly decreased because of early asymptomatic detection (Golemis *et al*., 2018). More advocacy for women to take to asymptomatic screening methods such as clinical breast examination and mammography is required.

On the other hand, other contributing factors were found to influence delayed diagnosis. The cost of diagnosis was also found to be an important factor given that breast cancer like any other cancer in Nigeria was not captured by the National Health Insurance Scheme (NHIS). This makes the cost of diagnosis expensive for women, especially low-income earners. Ayanore *et al.* (2020) also found that the cost of diagnosis at healthcare centres limits the screening for breast and cervical cancer among Ghanaian women. In developed countries such as China, there is an ongoing improvement and inclusion of diagnostic and treatment options for breast cancer patients in the health insurance system and this has been shown to be beneficial to most categories of breast cancer patients (Diao *et al*., 2021). Furthermore, the utilization of the Medicaid program and the Breast and Cervical Cancer Prevention and Treatment Act in the U.S Government policy has enhanced early detection of breast and cervical cancers among low-income earners (DeGroff *et al.*, 2021). To enhance and promote early diagnosis

among women in Nigeria, a policy that subsidizes the cost of breast cancer diagnosis for Nigerian women should be implemented.

From the interviews conducted in this study, all the cancer facilities for both diagnosis and treatment in different regions in Nigeria are facing systemic infrastructural challenges which has resulted in their sub-optimal functioning. These situations have increased the congestion experienced in the facilities, thereby delaying diagnosis and treatment of patients. In line with the above observations, Unger-Saldaña, (2014) reported that systemic delays in the health facilities, such as their unavailability, delays in the consultation process, and inadequacies in the quality of service, are significant barriers to the timely diagnosis of breast cancer in developing nations.

As highlighted by the health workers interviewed in this study, the limitation of trained personnel on advance technological skills involved in the use of equipment such as mammography in the detection of very early changes in breast tissue, is another important factor affecting early diagnosis of breast cancer in Nigeria. According to the American Cancer Society, mammography is the screening method that should be most frequently used. It is therefore important that routine mammography should be part of the health policy and encouraged among women in Southwest Nigeria and Nigeria at large.

5.1.1 Other social barriers preventing an early diagnosis of breast cancer in Nigeria include:

i. Family support

Interviews from key informants in non-governmental organizations showed that most women lack family support, which is why they hide their symptoms till it is too late. This perception negates the claims of the patients interviewed, as many of the patients agreed that they had family support, and the families were the first to know about their condition. Alexander *et al*. (2019) concluded that the majority of patients with breast cancer in India rely on family support rather than social support, consistent with the patient's perceptions of family support in this study. This suggests that family is not a significant factor preventing breast cancer patients from accessing an early diagnostic services and family support is crucial for cancer patients seeking treatment at medical facilities.

ii. Level of Education

According to medical professionals surveyed, education has a significant impact on how breast cancer is diagnosed in Nigeria. They perceived that most people who are diagnosed with advanced-stage breast cancer are uneducated women who are mostly ignorant of the disease and its symptoms. The practice of BSE among women in Turkey was substantially correlated with educational level, according to Gürdal *et al*. (2012). In north-western Ethiopia, Tesfaw *et al.* (2020) discovered a connection between illiteracy and a delay in the identification of breast cancer. The sole sign and symptom of breast cancer that some of the women interviewed for this study were aware of was a lump in the breast, but the majority had only a basic understanding of the disease. This demonstrates the necessity of raising awareness of cancer of the breast among Nigerian women.

iii. Alternative Medicine and Religious Belief

Due to the high expense of diagnosis or their religious beliefs, some of the breast cancer patients interviewed had previously sought alternative medicine treatment. This treatment includes the use of herbs, diet control, exercises and prayers. This has a significant impact on how quickly breast cancer is diagnosed in Nigeria. Some of the women surveyed for this study, are women who delayed their diagnosis and treatment in a hospital for years because they were using alternative medicine, thus allowing progression of the cancer before visiting the hospital. This element was also noted to prevent women in Indonesia from receiving an early diagnosis of breast cancer (Pratiwi and Hamidiyanti, 2020).

iv. Emotion, Psychology and Self-denial

The increased level of awareness of breast cancer has helped some patients in understanding and correcting the previous notion that the diagnosis of cancer is a death sentence. Most patients who were surveyed concurred that better disease prognosis can be achieved with early identification. However, some patients still delay presentation for diagnostic examination because of the fear of being diagnosed with breast cancer or being asked to undergo surgical removal of the breast. This has in turn delayed their

treatment and proper management. A similar scenario was also reported in an Indian population, where the fear of being diagnosed with breast cancer prevented people from getting screened (Dey *et al.*, 2016). Some breast cancer patients find it difficult to accept a breast cancer confirmatory diagnosis and remain in a state of self-denial. Self-denial might be a cogent emotional factor preventing patients from seeking medical attention early after experiencing symptoms (da Silva *et al.*, 2018).

It is critical to address these hurdles comprehensively if we want to enhance patient diagnosis and care of breast cancer. This will entail integrating simple access to screening and diagnostic services, as well as education and awareness.

5.2 Socio-demographics of breast cancer patients

Aging plays a substantial role in cancer occurrence because as we age, our tissues accumulate mutations, some of which are associated with cancer. Cancer risk rises because of these mutations over time. As a result, older people are more likely to get cancer than younger people, which highlights the significance of raising public knowledge about cancer and the advantages of early identification and prevention (Gaiolla *et al*., 2021). The average age of patients in this study was approximately 46 years, similar to the observations of Wang *et al*. (2018). These findings are in accordance with the fact that most patients with cancer of the breast in Nigeria are pre-menopausal, as earlier reported by Olaogun *et al*. (2020).

Cancer of the breast and BMI were not significantly associated in this study. This might be because most patients in this study were premenopausal women. Breast cancer has previously been linked to adiposity (higher BMI), especially in postmenopausal women (García-Estévez *et al*., 2021; Mohanty and Mohanty, 2021; Smith *et al*., 2021). Tran *et al*. (2021) also found no connection between BMI and breast cancer in premenopausal women among a Korean population. However, it has been shown that greater adiposity seems to lessen the possibility of having cancer of the breast among premenopausal women, while as it elevates the possibility in postmenopausal women (Ma *et al*., 2018). Obesity and oestrogen levels were thought to protect against breast cancer in premenopausal women while being risk factors in postmenopausal women. This is because adipose tissue acts as the body's primary source of oestrogen storage following menopause. The main factors contributing to breast cancer in obese postmenopausal

women are elevated peripheral site oestrogen production and high serum oestrogen levels (Mohanty and Mohanty, 2021).

According to healthcare providers' perceptions in the qualitative aspect of this study, one of the main variables hindering an early diagnosis of breast cancer was low educational attainment. Indeed, we found that in this study, 66% of the patients had either tertiary or elementary education. A higher level of education was perceived to raise the incidence of breast cancer according to the meta-analysis by Dong and Qin, (2020), the reason for this remains unclear. Strong evidence suggests that a patient's socioeconomic status (SES) affects the stage of the disease at diagnosis, which in turn has a significant impact on prognosis. According to Azubuike *et al*., (2022), persons with lower incomes in Nigeria had a worse prognosis overall and were diagnosed with advanced-stage cancer. The extent of timely detection and treatment depends on the patient's perception of the tumour, which is greatly influenced by educational level and occupation, both of which have a high correlation to socioeconomic position. This is true even though the level of education and occupation can individually influence the stage of cancer at diagnosis (Liu *et al*., 2017). Since a little above half of the patients in this study (56.5%) worked in unskilled labour, their socioeconomic situation may have an impact on the timing and stage of diagnosis. Lower socioeconomic status influences delay in seeking diagnosis therefore, patients can't afford high diagnostic test fees.

Ninety-six percent of cases in this study had no family history of the disease, implying that sporadic breast cancer development is more common among the cohort of patients studied. It is known that about 5-10% of cancers in and around the breast are hereditary and might be a result of mutations in BRCA 1&2 genes (Arpino *et al*., 2016). Even though sporadic breast cancers are more common, patients with this type of breast cancer have a poorer prognosis when compared with patients with BRCA mutation breast cancer (Arpino *et al*., 2016).

Eighty to ninety-five percent of patients in this study reported never having used birth control or hormonal pills, suggesting that these factors may not pose as risk factors for breast cancer in this group. To confirm this, data from a larger cohort may be needed. Low levels of oestrogen and synthetic progestin are found in contraceptive products, and these products were linked to a 20% increase in the possibility of having cancer of

the breast (White, 2018). In a meta-analysis by Kanadys *et al*. (2021) using studies from several countries, there was no substantial increase in the risk of breast cancer between never-user and ever-user of oral contraceptives among premenopausal and postmenopausal women. However, the occurrence of breast cancer and the use of oral contraceptives for more than five years or before the first full-term pregnancy were previously linked (Kanadys *et al*., 2021)

Tumour grade was identified as a prognostic marker for breast cancer (Rakha *et al*., 2008; Jaroensri *et al*., 2022). Grade 1 tumour is known to be a marker for good treatment response (Amat *et al*., 2002; van Dooijeweert *et al*., 2022), unfortunately, 19% presented with grade 1 among the sampled population for this study. In this present study, data on the histological grading of breast cancer patients in Nigeria were in line with those of Adeniji *et al*. (2020), who found that the majority of women had grade 2 tumours at the point of diagnosis. Although a grade 2 tumour has a better prognosis compared to a grade 3 tumour, a diagnosis of breast cancer with grade 2 is still considered late-stage diagnosis (van Dooijeweert *et al*., 2022).

5.3 Levels of TNF-α and soluble-TNF receptor 1 (sTNFR1) receptor in Plasma of both breast cancer patients and controls.

Breast cancer-associated inflammation typically contains Tumour Necrosis Factor (TNF-α), along with other proinflammatory cytokines, with substantial impact on disease development. Inflammation is frequently described as a favourable environment for the development of tumours. The TNF-α, however, can activate signals for cell division or death under certain conditions (Cai *et al*., 2017). Therefore, research into this cytokine's variations, receptors, and the existence of polymorphisms in carcinogenesis is important. The TNF- α and sTNFR1 expression in the blood plasma of patients and controls were examined in this study. The average level of TNF-α was significantly reduced in breast cancer patients compared with control individuals, particularly in those with grade 3 tumours, demonstrating a negative association between breast cancer and TNF-α. Therefore, it could be concluded that the development and progress of breast cancer in patients is associated with reduced TNF-α levels. The results from this study are comparable to the studies of Arinola *et al.* (2021), Martnez-Reza *et al*. (2017) and Li *et al*. (2009), although some studies have

suggested that greater levels of TNF-α may increase tumour growth (Cai *et al*., 2017; Liu *et al*., 2020). This may be due to the variable levels of sTNF-α and sTNFR 1 and 2, as well as the existence of TNF-α and its receptor polymorphisms. Although markers for inflammation were not investigated in this study, low-grade inflammation has been linked to breast cancer (Berger *et al*., 2018). Low-grade inflammation is the production of chronic inflammatory responses at a lower rate throughout the body (Berger *et al*., 2018).

Furthermore, the level of the sTNFR1 receptor was not significantly different in cases compared to controls. TNF-α is said to promote the growth of breast cancer through binding with TNFR1 and initiation of the NF-κB signaling pathway involved in cell division (Cai *et al.,* 2017). This finding further suggests that TNF-α might likely be protective against breast cancer progression among the study population.

5.4 Association *TNF-α* and receptor SNPs with breast cancer

In this study, the relationship between the SNPs of the *TNF-α and TNFR1* genes with cancer of the breast in a cohort of women was examined. Out of the 14 alleles of 6 SNPs that were analysed, only 5 alleles (*TNF-308A, 488G, 380G, 1032C, and TNFR1A+IV56+10-G*) showed a significantly reduced risk associated with breast cancer. The most-well studied of these alleles is *TNF-308A* which has been linked to increased expression of the *TNF-α* gene and subsequently high production of TNF-α. In a meta-analysis by Yi *et al*. (2018), *TNF-308A* was associated with overall protection against cancer in 13 populations. According to Włodarczyk *et al.* (2020), the protective effect of the *TNF-308A* allele was most noticeable in non-obese women who were carriers of the allele. It was noted that these women exhibited considerably less endogenous DNA damage and blood C-reactive protein concentrations (inflammatory markers) than their GG homozygote carrier counterparts (Kaptoge *et al*., 2010; Włodarczyk *et al*., 2020). Even though some studies have associated protection against cancer with *TNF-308A*, numerous conflicting results showed no association or increased risk with cancer of the breast and other types (Banday *et al*., 2016; Ahmad *et al*., 2020). Some of the reasons for the widespread controversies with this SNP might be attributed to population disparities.

Likewise, TNF 1032 C>T and TNF 488 G>A have also been associated with hepatocellular carcinoma risk and cancer of the bladder respectively (Wungu *et al.,* 2020; (Marsh *et al.,* 2003). Also, TNFR1A+ IV56+10G was linked to cancer in and around the breast in the Chinese population (Li *et al*., 2014). The alleles investigated in this study are known to increase the expression of TNF-α in the body. In high doses, TNF-α destroys tumour vasculature; in low doses, it acts as a vasodilator and chemo-attractant for cells in inflammatory responses, promoting the development of tumour stroma (Marsh *et al.,* 2003). This further explains the low level of TNF-α in patients with grade 3 tumours in our cohort of patients. TNF-α has also been suggested to improve anticancer therapy if administered as adjuvant therapy due to its protective role against cancer at appropriate doses (Shen *et al.,* 2018).

Interestingly, in this study, we found high heterozygosity in the genotyped *TNF-α* SPNs as opposed to high homozygous frequencies found in Caucasian and Asian populations (Marsh *et al*., 2003; Li *et al*., 2014; Ahmad *et al*., 2020). However, a similar observation was seen in the Ivoirian population in a study by Santovito *et al*. (2012). This genotypic occurrence may likely be a result of evolutionary events resulting in a selection for heterozygote genotypes within the population. Although not yet linked to breast cancer, such genotypes may be important in regulating the *TNF-α* gene expression for survival. This is seen from an earlier study where TNF- 308 GA was linked to moderate TNF-**α** production (Marume *et al*., 2021). TNF-**α** 488G, 1032 T, and 238A genotypes had a positive correlation with the level of TNF-**α** in controls. *TNF-488G* and *238A* have been previously linked with higher levels of TNF-α (Chen *et al,* 2019; Mahto *et al*., 2019). This helps to explain why control subjects have moderately higher TNF-α protein production than breast cancer patients. These observed genotypes in TNF-α SNPs may also be a result of natural selection in human evolution that is protective against malaria in endemic areas like Nigeria (Mahto *et al.,* 2019).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

In this study, it was observed that some SNPs at the promoter region of the TNF-α gene is associated with breast cancer among Nigerian women. These SNPs, which include TNF-488G, 308A, and 1032C, are known to increase TNF-α expression and can be used to assess cancer of the breast risk in the Nigerian population. Also, chronic low-grade inflammatory responses were linked with breast cancer, and this study showed a decrease in levels of TNF-α with increasing tumour grade, supporting the idea that low levels of TNF-α might be associated with breast cancer progression and suggesting its possible use as a prognostic marker. The study also showed that post-symptomatic diagnosis such as breast self-examination (BSE) might not be an effective early diagnostic tool in the prevention of breast cancer and improvement of its prognosis in Nigeria.

In this study, it was also found that system delays and other factors, such as the cost of diagnosis, an inadequate health insurance scheme, alternative medicine, and religious beliefs, are among the barriers preventing the timely detection of breast cancer in Nigeria. The factors contributing to the delayed diagnosis of breast cancer in Nigeria are complex and multifactorial but can be addressed through a systematic approach, including improvements in early detection programmes, the provision of detection facilities, and raising awareness of early detection screening methods, such as mammography tests.

6.2 Conclusion

In conclusion, breast cancer is a significant public health issue in Nigeria, and early diagnosis is critical for effective treatment. The reliance on breast self-examination as a diagnostic method appears to be ineffective, with factors such as inadequate awareness, cost of diagnosis, health insurance scheme, alternative medicine, and

religious beliefs contributing to delayed diagnosis. The study also identified key genetic variants of *TNF-α* and *TNFR1* (*TNF-α* receptor) as potential predictors for breast cancer among Nigerian women. The findings underscore the need for increased awareness campaigns and early screening programs, as well as genetic testing and counselling, to improve breast cancer outcomes in Nigeria.

6.3 Recommendations

1. It is suggested that detailed population genomics studies, utilizing GWAS with a large sample size, be conducted in Nigeria to investigate the potential of these SNPs (TNF-488G, 308A, and 1032C) as predictive and prognostic tools for breast cancer among Nigerian women.
2. TNF-308A genetic testing maybe be incorporated into routine tertiary health care check-ups.
3. A policy on free and mandatory routine mammography testing should be made for women 18 and above years in Nigeria. This will help significantly with the early detection of a possible risk of breast cancer.
4. Regular education of healthcare personnel on the use of cutting-edge breast cancer screening technologies will also be required.

6.4 Contributions to Knowledge

1. For the first time in Nigeria, this study showed that TNF-α 308A, 488G, and 1032 C might be suitable as prognostic and predictive markers for breast cancer among Nigerian women.
2. This study showed that the use of BSE as an early diagnosis is ineffective for the prevention and improvement of breast cancer prognosis.
3. BMI, birth control, and hormonal pills are not significant contributors to breast cancer in the study population.
4. A 96% rate of sporadic breast cancer among patients was found in this study. The drivers might be the absence of some TNF-α SNP alleles (TNF-α 308A, 488G and 1032 C and receptor SNP allele (TNFR1A+ IV56+10G)

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The Possible Use of Tumour Necrosis Factor-Alpha Single Nucleotide Polymorphisms (TNFα-SNPs) as Bio-Markers for Breast Cancer in Nigerian Women. A thesis for M.Sc. Degree in Zoology (Cell Biology and Genetics), University of Ibadan

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APPENDIX I

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IAMRAT)
College of Medicine, University of Ibadan, Ibadan, Nigeria.

Director: **Prof. Catherine O. Falade,** *MBBS (Ib), M.Sc., FMCP, FWACP*
Tel: 0803 326 4593, 0802 360 9151
e-mail: cfalade@comui.edu.ng lillyfunke@yahoo.com

UI/UCH EC Registration Number: NHREC/05/01/2008a

Notice of Renewal of Approval

Re: Tumor Necrosis Factor Alpha (TNFα) and its Receptor as Indicators for Development of Breast Cancer among Nigerian women.

UI/UCH Ethics Committee assigned number: UI/EC/17/0054

Name of Principal Investigator:	**Nanfizat Abiket Alamukii**
Address of Principal Investigator:	Department of Zoology, Faculty of Science, University of Ibadan, Ibadan
Date of receipt of annual report:	08/04/2019
Status:	**2nd Approval**

This is to inform you that the UI/UCH Ethics Committee has received your application for the renewal of approval on the above titled research. The report indicates that a total of 200 participants (100 cases and 100 controls) have been recruited so far. It also states that there still about 50 cases and 50 controls to be enrolled. The report does not indicate any adverse event.

The Committee notes the contents of the report and having found it satisfactory, hereby approves your request for renewal of approval for **one year of study only.**

This renewed approval dates from **16/04/2019 to 15/04/2020**. Note that no participant accrued or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the UI/UCH Ethics Committee assigned number and duration of UI/UCH EC approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC at least four weeks before the expiration of this approval in order to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. No changes are permitted in the research without prior approval by the UI/UCH EC except in circumstances outlined in the Code. The UI/UCH EC reserves the right to conduct compliance visit to your research site without previous notification.

UI/UCH EC
16 APR 2019
APPROVED
COLLEGE OF MEDICINE, U.I.

Professor Catherine O. Falade
Director, IAMRAT
Chairperson, UI/UCH Ethics Committee
E-mail: uiuchec@gmail.com

APPENDIX II

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IAMRAT)
College of Medicine, University of Ibadan, Ibadan, Nigeria.
Director: **Prof. Catherine O. Falade,** *MBBS (Ib), M.Sc., FMCP, FWACP*
Tel: 0803 326 4593, 0802 360 9151
e-mail: cfalade@comui.edu.ng lillyfunke@yahoo.com

UI/UCH EC Registration Number: **NHREC/05/01/2008a**

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Epidemiological Survey on the Status of Current Awareness and Barriers to Early Diagnosis of Breast Cancer in Nigeria

UI/UCH Ethics Committee assigned number: UI/EC/20/0271
Name of Principal Investigator: **Nanfizat A. Alamukii**
Address of Principal Investigator: Department of Zoology
Faculty of Science
University of Ibadan, Ibadan

Date of receipt of valid application: 26/05/2020
Date of meeting when final determination on ethical approval was made: **N/A**

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 UI/UCH Ethics Committee.*

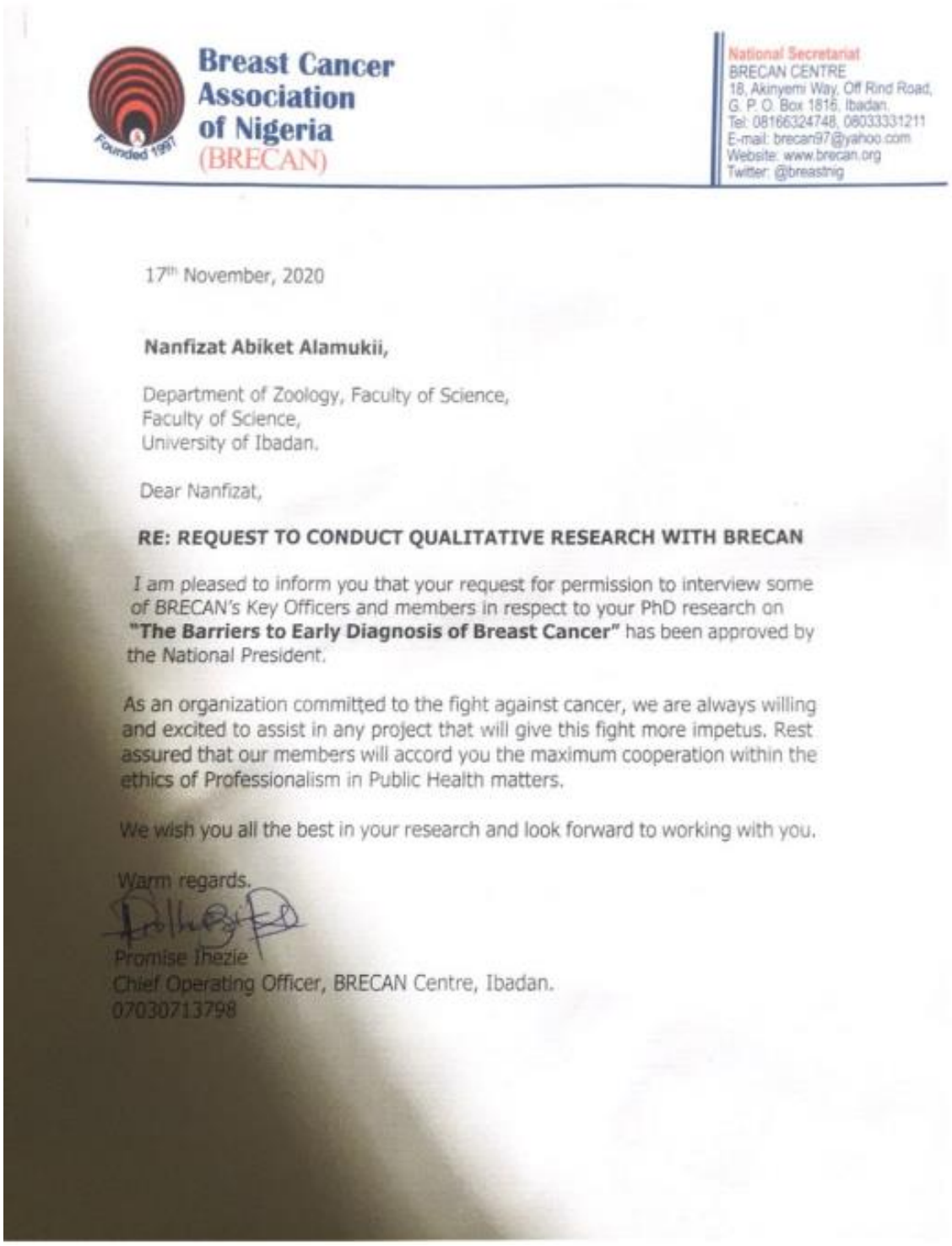
This approval dates from **15/07/2020 to 14/07/2021.** If there is delay in starting the research, please inform the UI/UCH Ethics Committee so that the dates of approval can be adjusted accordingly. Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the* UI/UCH EC *assigned number and duration of* UI/UCH EC *approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC at least four weeks before the expiration of this approval in order to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. *No changes are permitted in the research without prior approval by the* UI/UCH EC *except in circumstances outlined in the Code. The* UI/UCH EC *reserves the right to conduct compliance visit to your research site without previous notification.*

UI/UCH EC APPROVED COLLEGE OF MEDICINE, U.I.

Professor Catherine O. Falade
Director, IAMRAT
Chairperson, UI/UCH Research Ethics Committee
E-mail: uiuchec@gmail.com

APPENDIX III



Breast Cancer Association of Nigeria (BRECAN)

Founded 1997

National Secretariat
BRECAN CENTRE
18, Akinyemi Way, Off Rind Road,
G. P. O. Box 1816, Ibadan.
Tel: 08166324748, 08033331211
E-mail: brecan97@yahoo.com
Website: www.brecan.org
Twitter: @breastnig

17th November, 2020

Nanfizat Abiket Alamukii,

Department of Zoology, Faculty of Science,
Faculty of Science,
University of Ibadan.

Dear Nanfizat,

RE: REQUEST TO CONDUCT QUALITATIVE RESEARCH WITH BRECAN

I am pleased to inform you that your request for permission to interview some of BRECAN's Key Officers and members in respect to your PhD research on **"The Barriers to Early Diagnosis of Breast Cancer"** has been approved by the National President.

As an organization committed to the fight against cancer, we are always willing and excited to assist in any project that will give this fight more impetus. Rest assured that our members will accord you the maximum cooperation within the ethics of Professionalism in Public Health matters.

We wish you all the best in your research and look forward to working with you.

Warm regards.

Promise Ihezie
Chief Operating Officer, BRECAN Centre, Ibadan.
07030713798

APPENDIX IV

UNIVERSITY OF IBADAN, IBADAN, NIGERIA
DEPARTMENT OF ZOOLOGY

A. A. BAKARE, B.Sc., M.Sc., Ph.D. (Ibadan)
Professor and Head of Department
Tel: +234 7032295419; +234 8027389324
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INFORMED CONSENT FORM

IRB Research approval number:

This approval will elapse on:

Research Title: Tumour Necrosis Factor Alpha (TNFα) and its Receptors as genetic determinants for diagnosis of Breast cancer among Nigerian Women

Names of researchers and applicants

The research will be carried out by Miss Nanfizat. A. Alamukii, Department of Zoology, University of Ibadan, under the supervision of Dr. Roseangela. I. Nwuba, Department of Zoology, University of Ibadan and a Co-supervision, Prof Adeyainka G. Falusi, Institute for Advanced Medical Research and Training, University College Hospital, and Prof. Chinedum P. Babalola, F Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ibadan, Ibadan. Ibadan.

Purpose of research

This research seeks to determine role of Tumour Necrosis Factor Alpha (TNFα) and its Receptors Single Nucleotide Polymorphisms (SNPs) in the development of Breast cancer among Nigerian Women and their possible use as biomarkers for the diagnosis and prediction of breast cancer in Nigeria.

Procedure of research, what shall be required of each participant and approximate total number of participants that would be involved in the research

A total number of 260 participants will be enrolled in the research. Each participant is required to donate 5ml of blood samples which will be collected in EDTA bottles by a

qualified physician and samples will be taking to the laboratory for analyses (Polymerase chain reaction and sequence analysis).

Expected duration of the research and participant's involvement.

The study is expected to be over in three (3) years and each participant is expected to per-take in the study only once that is once a participant has donated 5ml of her blood and has answered the questionnaire, she is not expected to per-take again. The participants in this study will be females only.

Risk(s)

Minimal risk will be ensured during collection of blood samples from participants as blood samples will be collected by a qualified physician and undue harm will not be inflicted on patients.

Costs to participants, if any, of joining the research

No cost is required from participants, as participants will be selected from patients who come for regular medical check-ups or registered the Department of Surgery and the Department of Radiotherapy, University College Hospital, Ibadan.

Benefit(s)

The goal of this research is to create knowledge on how TNF$^{\alpha}$ gene is involved in the development of breast cancer. This knowledge will be useful in developing new therapies against breast cancer.

Confidentiality

No direct identification of participants will be made. All data collected during the study will be made confidential as possible.

Voluntariness

Participation in this research is entirely voluntary

Alternative to participation

If you choose not to participate in this study it will not affect your treatment in the hospital in

Consequences of participants' decision to withdraw from research and procedure for orderly termination of participation:

You can also choose to withdraw from the research at any time. Please note that some of the information that has been obtained about you before you choose to withdraw may have been modified or used in reports and publications. These cannot be removed anymore. However, the researchers promise to make effort in good faith to comply with your wishes as much as is practicable.

Modality of providing treatment(s) and action(s) to be taken in case of injury or adverse event(s)

If you suffer any injury in the course of collection of blood sample, you will be treated at the University College Hospital, Ibadan and the researcher will bear the cost of the treatment.

What happens to research participants and communities when the research is over:

The researchers will inform you of the outcome of the research through a news bulletin. During the course of this research, you will be informed about any information that may affect your continued participation or your health.

Any apparent or potential conflict of Interest:

We are not aware of any other information that may cause the researchers not to do their work fear or favour

Statement of person obtaining informed consent

I have fully explained this research to _____and have given sufficient information, including about risks and benefits, to make an informed decision

DATE _____ SIGNATURE _____

NAME _____

Statement of person giving consent

I have read the description of the research or have had it translated into the language I understand. I have also talked it over with the doctor to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have received a copy of this consent form and additional information sheet to keep for myself.

DATE _____ SIGNATURE _____

NAME _____

WITNESS'S SIGNATURE (*if applicable*) _____

WITNESS'S NAME (*if applicable*)

_____

Detailed Contact information

This research has been approved by the Ethics committee of the University of Ibadan and the Chairman of this committee can be contacted at Biode Building, Room T10, 2nd Floor, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, E-mail: uiuchirc@yahoo.com

In addition, if you have any question about your participation in this research, you can contact the principal investigator, Miss N. A. Alamukii, Department of Zoology, University of Ibadan, under the supervision of Dr. Roseangela. I. Nwuba, Department of Zoology, University of Ibadan, and a Co-supervision, Prof. Adeyinka G. Falusi, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, and Prof. Chinedum P. Babalola, F Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ibadan, Ibadan.

Questionnaire section

Summary of research: This research seeks to determine role of Tumour Necrosis Factor Alpha (TNFα) and its Receptors Single Nucleotide Polymorphisms (SNPs) in the development of Breast cancer among Nigerian Women and their possible use as biomarkers for the diagnosis and prediction of breast cancer in Nigeria. Breast cancer is one of the major sources of death among women in Nigeria and early diagnosis is paramount in its treatment. This study requires consented participants to donate 5mls

of blood once which will be collected by qualified physicians. This will be used to check some genetic variations in TNFα and its receptors that can be linked to breast cancer as well as cytokine level of TNFα and its receptors. Participants will also be required to answer some socio-demographic questions which will be used to determine some factors that aid in development of breast cancer.

Participation in this study is entirely voluntary. Non-participation or participation will not affect health care service required in the hospital.

Confidentiality of data

No name or any identifier is required. All data collected during the study will be coded and kept confidential.

Beneficence to participants

The goal of this research is to create knowledge on how TNFα and receptor genes are involved in the development of breast cancer. This knowledge will be useful in the diagnosis and development of new therapies against breast cancer.

Non-maleficence to participants

Risk if any, associated with the collection of blood samples from participants will be kept minimal as much as possible. Participant can choose to withdraw from this study at any point in time without any consequences. No cost or payment is required for participation and in case of any injury suffered as a result of participation in this study, research will bear the cost of treatment in the hospital. In case of any questions, researcher can be contacted through this number-070643565376 or iyabiket@gmail.com

Statement of person giving consent:

I have read and fully understood the research and what is required of me to participate in this research. I have received a copy of the consent form and additional information sheet to keep for myself

DATE:---------- SIGNATURE----------

APPENDIX V

UNIVERSITY OF IBADAN, IBADAN, NIGERIA
DEPARTMENT OF ZOOLOGY

A. A. BAKARE, B.Sc., M.Sc., Ph.D. (Ibadan)
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zoologyibadan@gmail.com

INFORMED CONSENT FORM

IRB Research approval number: ####

This approval will elapse on: dd/mm/yyyy

Title of the research:

Epidemiological survey on the status of current awareness and barriers to early diagnosis of breast cancer in Nigeria

Name(s) and affiliation(s) of researcher(s) of applicant(s):

(For example: This study is being conducted by Nanfizat Abiket Alamukii and Dr. Roseangela I Nwuba, Department of Zoology, University of Ibadan.

Sponsor(s) of research:

Consortium for Advanced Medical Research and Training in Africa (CARTA)

Purpose(s) of research:

To determine the current awareness status of breast cancer in Nigeria and other factors mitigating late stage presentation and early diagnosis of breast cancer in Nigeria.

Procedure of the research, what shall be required of each participant and approximate total number of participants that would be involved in the research:

A total of 150 participants will be interviewed to give valuable information based on their experience with regards to awareness, diagnosis and treatment of breast cancer in Nigeria.

Expected duration of research and of participant(s)' involvement:

It is expected that interviewing participant will take a duration of six months

Risk(s):

Risk if any, associated with interviewing participants will be kept minimal as possible as.

Costs to the participants, if any, of joining the research:

Your participation in this research will not cost you anything.

Benefit(s):

The aim of this research is to determine the current awareness status of breast cancer in Nigeria and other factors mitigating late stage presentation and early diagnosis of breast cancer in Nigeria. Information that will be gathered from this research will in improvement of policies that will positively affect diagnosis and treatment of breast cancer in Nigeria

Confidentiality:

All information collected in this study will be given code numbers and no name will be recorded. This cannot be linked to you in anyway and your name or any identifier will not be used in any publication or reports from this study.

Voluntariness:

Your participation in this research is entirely voluntary.

Alternatives to participation:

If you choose not to participate, this will not affect your treatment in this hospital in any way.

Due inducement(s):

You will be compensated for lost wages; cost of transport to and from the research site but you will not be paid any fees for participating in this research.

Consequences of participants' decision to withdraw from research and procedure for orderly termination of participation:

You can also choose to withdraw from the research at anytime. Please note that some of the information that has been obtained about you before you chose to withdraw may have been modified or used in reports and publications. These cannot be removed anymore. However the researchers promise to make effort in good faith to comply with your wishes as much as is practicable.

Modality of providing treatments and action(s) to be taken in case of injury or adverse event(s):

If you suffer any injury as a result of your participation in this research, you will be treated at the University of Ibadan Teaching Hospital and the research will bear the cost of this treatment.

What happens to research participants and communities when the research is over:

The researchers will inform you of the outcome of the research through a news bulletin. During the course of this research, you will be informed about any information that may affect your continued participation or your health.

Statement about sharing of benefits among researchers and whether this includes or exclude research participants:

If this research leads to commercial products, the University of Ibadan and Cleveland University shall jointly own it. There is no plan to contact any participant now or in future about such commercial benefits.

Any apparent or potential conflict of interest:

No potential conflict of interest

Statement of person obtaining informed consent:

I have fully explained this research to _____ and have given sufficient information, including about risks and benefits, to make an informed decision.

DATE: _____ SIGNATURE: _____

NAME: _____

Detailed contact information including contact address, telephone, fax, e-mail and any other contact information of researcher(s), institutional HREC and head of the institution:

This research has been approved by the Ethics Committee of the University of Ibadan and the Chairman of this Committee can be contacted at Biode Building, Room 210, 2nd Floor, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, E-mail: uiuchirc@yahoo.com and uiuchec@gmail.com

In addition, if you have any question about your participation in this research, you can contact the principal investigator, Name…….....

Department……..... Phone…….. .....

Email…….....

PLEASE KEEP A COPY OF THE SIGNED INFORMED CONSENT.

APPENDIX VI

Interview guides used for each section

Key informant interview guide for health care workers

Age: Gender: Occupation: Number of years in service:

1. What are the breast cancer screening programmes available at this Health Centre and which one is most efficient for early diagnosis?
2. How regular do women visit for routine breast examination?
3. Which option for diagnosis is often used by these women and why?
4. How often do women complain about lumps in their breast after conducting self-breast examination procedure? And what category women do this very often?
5. How regular do you have women presenting with late stage of breast cancer compared to early stage?
6. How long does it take to get routine breast examination and the cost?
7. Do you think the infrastructure in place is efficient enough to provide quality diagnosis at this clinic?
8. What are the policies in place to aid women in early detection of breast cancer?
9. Why do you think women present with late stage of breast cancer at your clinic?
10. Is the clinic offering any services to increase awareness on early diagnosis of breast cancer?
11. What are the constraints in diagnosing women with breast cancer?
12. What do you think about health insurance policies how they affect diagnosis of breast cancer?

In-depth interview guide for managers of partner organizations (Non-governmental organization for Breast cancer in Nigeria)

1. When did this association start?

2. How many awareness programmes have you organized?
3. What are the key messages of these programmes?
4. How many women participate in these programmes?
5. Do you also involve women who have not been diagnosed of breast cancer?
6. How many times do you go / have meetings in a year?
7. Do you stay at your location or visit other environs as well during your awareness programmes?
8. How effective do you think your awareness programme is? How do you test the effectiveness of your programmes?
9. Have you heard or know someone who attended your programmes and later discovered early stage breast cancer due to the knowledge shared at the awareness programme?

In-depth interview guide among breast cancer patients

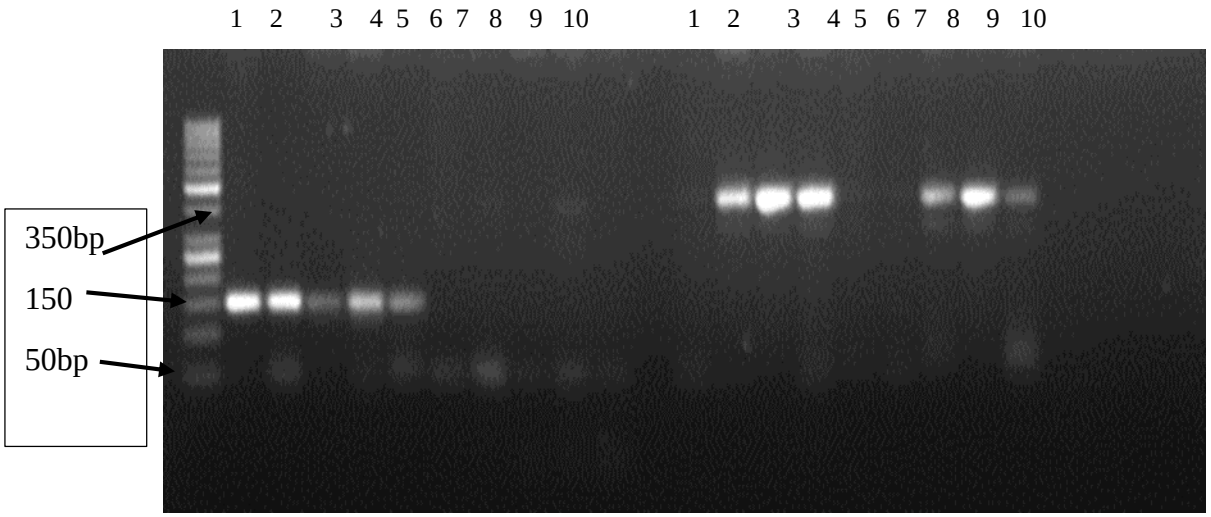
Age: Religion: Education: age at diagnosis: occupation:

1. Can you share
2. How did you detect you had breast cancer?
3. How early did you notice the changes in your breast?
4. Are your relatives aware of your health status? How did they accept it?
5. Do you now know more about breast cancer compared to when you were not yet diagnosed? What additional information do you have about breast cancer?
6. How much did it cost you to get diagnosed, do you think it is expensive? How easy is it to get diagnosed, are the facilities clos to your environment?
7. Were there any delays when you were trying to get tested?
8. Did you encounter any errors in getting diagnosed?
9. Has your community, family and friends attitude changed towards you since you were diagnosed of breast cancer?
10. Have you ever participated or witness awareness campaigns for breast cancer?
11. What do you perceive breast cancer to be?
12. What are your experiences regarding breast cancer diagnosis? What are the constraints?

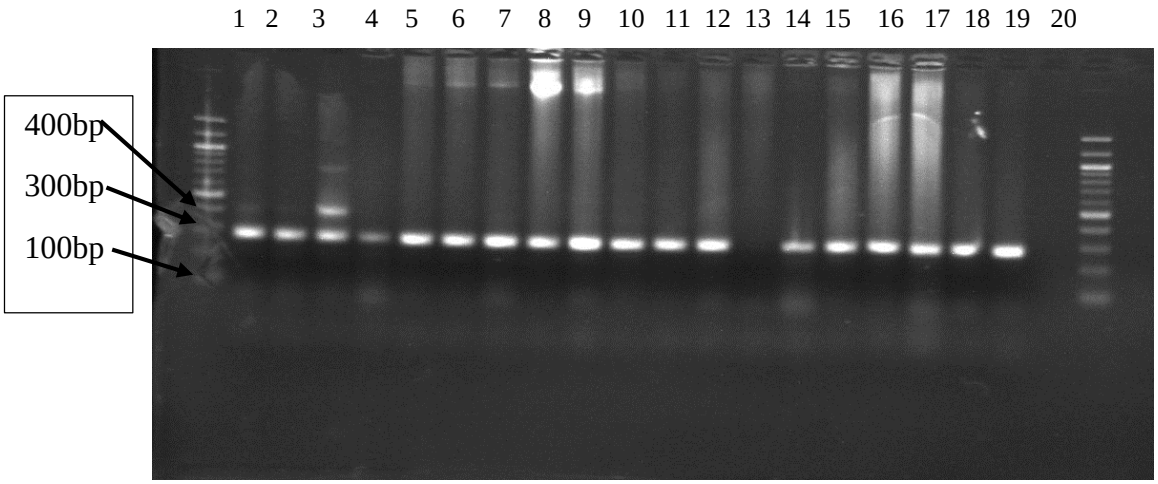
13. What are your suggestions towards improving breast cancer awareness and diagnosis in Nigeria?
14. How far is the health care service center from your home? And how accessible is the road?
15. Do you all have health insurance? How easily did you get how health insurance?
16. What are the advantages of having health insurance?

APPENDIX VII

Gel Pictures



Amplification of *TNF-α 238G* and *488G* in cases and controls



Amplification *TNF-α 380G* among participants