UNIVERSITY OF GHANA

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CIRCULATING CELL-FREE DNA AS A BLOOD BIOMARKER IN MONITORING RESPONSE TO CHEMOTHERAPY IN BREAST CANCER

BY

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DECLARATION

DECLARATION BY CANDIDATE

I hereby declare that except for references to the works of other researchers, which have been referenced accordingly, this thesis is the product of my own research undertaken under supervision in accordance with regulations of the School of Graduate Studies, University of Ghana. This thesis has neither in whole nor in part been presented for another degree elsewhere.

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We hereby declare that the practical work and compilation of this thesis were supervised by us in accordance with the guidelines in supervision of thesis laid down by the University of Ghana.

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DEDICATION

I dedicate this work to my mum, Mrs. Martha Adusei and dad, Mr. Abraham Kweku Adusei as well as my uncle, Professor Partrick Fedinand Ayeh- Kumi for their love and support. A special dedication also goes to my colleagues, friends and loved ones whose words of encouragement kept me going.
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LIST OF ABBREVIATIONS

AIs: Aromatase inhibitors

AJCC: American joint committee on cancer

ALU: Arthrobacter luteus

AMP: Adenosine monophosphate

ATM: Ataxia telangiectasia

BC: Breast cancer

BCS: Breast cancer surgery

BMI: Body mass index

BRCA: Breast cancer associated gene

BS: Bone scintigraphy

CA: Cancer antigen

CAD: Caspase-activated DNase

CEA: Carcinoembryonic antigen

CfDI: Circulating cell-free DNA integrity

CfDNA: Circulating cell-free DNA

CHEK2: Checkpoint kinase 2
CT: Computed tomography

CTCs: Circulating tumour cells

DCIS: Ductal carcinoma in situ

DNA: Deoxyribonucleic acid

EC: Endometrial cancer

EGFR: Epidermal growth factor receptor

ER: Estrogen receptor

Fas: First apoptosis signal receptor

FDG: F-fluorodeoxyglucose

FNA: Fine needle aspiration

GLOBOCAN: Global cancer incidence, mortality and prevalence

GMP: Guanosine monophosphate

HCC: Hepatocellular carcinoma

HER-2: Human epidermal growth factor receptor-2

HRT: Hormone replacement therapy

IDC: Invasive ductal carcinoma

IL: Interleukins

IUDs: Intrauterine devices
MRI: Magnetic resonance imaging

NCCN: National comprehensive cancer network

NETs: Neutrophil extracellular traps

ORC: Origin recognition complex

PCR: Polymerase chain reaction

PR: Progesterone receptor

PSA: Prostate specific antigen

ROS: Reactive oxygen species

satDNA: Satellite DNA

T-DMI: Ado-trastuzumabemtansine

TEs: Transposable elements

TNF: Tumour necrosis factor

TNM: Tumour node metastasis

WHO: World health organization

WHR: Waist to hip ratio
ABSTRACT

Background: Cancer incidence and its related mortality is on the rise and is currently the second leading cause of death globally. Breast cancer has a broad impact in the world, with an estimated 20,996 new diagnoses in 2018 alone. Evidence regarding the accuracy and effectiveness of existing modalities such as magnetic resonance imaging (MRI), computed tomography (CT), bone scintigraphy, chemotherapy and radiation therapy, etc., to evaluate and monitor response to breast cancer treatment is limited. Moreover, such modalities are expensive for patients, and frequently exposes them to radiations which affect their health status. There is, therefore, the need for research into alternative predictive biomarkers that can be used to determine or monitor the effectiveness of breast cancer treatment in resource-limited countries like Ghana without exposing patients to radiation.

Circulating cell-free DNA (cfDNA) is produced at elevated levels in cancer patients than in apparently healthy individuals. Assessing cfDNA as a blood biomarker may be useful in monitoring response to treatment especially chemotherapy in breast cancer since chemotherapy is the primary treatment for breast cancer.

Aim: The study sought to assess circulating cell-free DNA (cfDNA) as a blood biomarker for monitoring response to chemotherapy in breast cancer patients.

Methodology: The study involved 64 females consisting of a test group of 32 breast cancer patients and a control group of 32 apparently healthy controls. Venous blood samples were taken at two time points, one before the commencement of chemotherapy and the other after the 3rd cycle of chemotherapy. Venous blood samples were also taken from the apparently healthy controls as well. ALU species 115 and 247 levels in serum were measured in both the test and control groups. Body Mass Index (BMI) and Waist to Hip Ratio (WHR) of the participants were also assessed.
Results: ALU species 115 and 247 levels in serum were elevated in breast cancer patients than the controls (p-value 0.028 and < 0.001). Circulating cell-free DNA integrity was also higher in the breast cancer patients than the controls. The concentrations of ALU 115 and 247 significantly decreased after the 3rd cycle of chemotherapy in the breast cancer patients unlike the circulating cell-free DNA integrity which increased. The concentrations of both ALU115 and 247 also decreased after the 3rd cycle of chemotherapy among the tumour grades, stages, molecular and histopathological subtypes of the breast cancer patients.

Conclusion: Serum concentration of circulating cell-free DNA was affected by chemotherapy and may be used as a vague biomarker in monitoring response to chemotherapy in breast cancer.
CHAPTER ONE

INTRODUCTION

1.1 Background

Cancer is a major public health concern globally and a leading cause of death. There was an approximated 18.1 million new cases and 9.6 million cancer casualties in 2018 (Ferlay et al., 2019). However, the number of new cases and deaths continues to increase, due to the growing life expectancy, epidemiological and demographic alterations (Ferlay et al., 2019).

In 2018, lung cancer was the most frequently diagnosed form of cancer globally (11.6% of total cases), followed by breast cancer (11.5%) and colorectal cancer (10.2%) (Parkin et al., 2019). Breast cancer has become the most frequently diagnosed cancer in females and a primary cause of cancer deaths in the world (Parkin et al., 2019). Over the past decades, frequency rate of breast cancer has increased in most countries, with some of the fastest increases happening in places such as countries in Africa and Asia (Ferlay et al., 2019).

Breast cancer is becoming a growing public health problem in less developed countries, particularly in Africa, with increasing evidence expected in the coming years. In less developed countries, it is the most common cause of death in females (Brinton et al., 2014). In Africa, it is reported that, several encounters have led to an increased mortality from breast cancer in combination with poor health facilities, absence of population awareness and lack of female education (Yip et al., 2011). In some African countries, it is normal for females to take advantage of outdated care prior to pursuing medication, which can result in diagnostic delay (Pace et al., 2015). This somehow clarifies why more African females are at risk of dieing from the disease than women from developed countries. Breast cancer remains a significant health issue, mainly in Africa, where most cases are detected in the last stages of the disease.
(Girish et al., 2014), despite the progress made in enhancing early diagnosis and treatment. It has been recognized as a unique cancer with a high death rate among women in Ghana (Brinton et al., 2014). This is suspected, due to limited screening, late presentations and advanced stages of diagnosis. Inherited causes comprising of family history of breast cancer and hereditary mutations accounts for 5% to 10% cases of breast cancer in females. Migrant research indicate that, the key factors of the detected inter-ethnic disparities of prevalence in Ghana are non-hereditary factors (Oldenburg et al., 2007).

Higher occurrence rates of breast cancer are subjected to greater prevalence of known risk factors associated with menstruation (early menarche age and later menopause age), fertility (null parity, late first birth age and less children), exogenous hormone intake and weight gain though breast feeding and physical activities are established protective factors that may be helpful (Brinton et al., 2018).

Lately, newer techniques and developments in the biomedical field have significantly enhanced the understanding of disease pathology and etiology, especially those arising from the genetic and molecular foundation. The use of Deoxyribonucleic Acid as a biomarker has contributed to the early diagnosis, prognosis, and disease risk assessment and monitoring status of a person’s tumour. However, in breast cancer, several signatures of gene expression have been created and can be used to evaluate the diagnosis of an individual based on tumour estimation. Circulating cell-free DNA is an example of the signatures of gene expression in breast cancer.

Circulating cell-free DNA released from normal and cancerous cells is a promising and highly capable tumour biomarker compared to traditional serum markers for cancer detection, diagnosis and personalized drug control. This biomarker is readily available, accurate and reproducible. Circulating cell-free DNA is a fragment of degraded DNA which is released into
the bloodstream from human cells and probably all eukaryotes. It is a mixture of fragments of extracellular nucleic acid derived from cell necrosis and apoptosis (Barták et al., 2018).

The most of circulating cell-free DNA belong to *Arthrobacter luteus* repetitive sequences comprising of both short and long interspersed nucleotide. *Arthrobacter luteus* sequences (ALU) are interspersed elements and are the most plentiful sequences in the human genome with 1.4 million copy number/genome (Lei et al., 2002). In addition, as a potential diagnostic and prognostic biomarker, cfDNA integrity (cfDI) which tests the degree of cfDNA fragmentation maybe exploited. cfDNA integrity for ALU repeats (247 and 115 bp) is determined as the ratio of longer fragments of DNA to the total fragments of DNA.

After cfDNA was discovered in 1948 by Mandel and Metias in human plasma, it was then recovered in 1966 (Tan & Kunkel, 1966) in autoimmune conditions and later by Leon (Leon et al., 1977) in cancers. Circulating cell-free DNA has also been contained in different body fluids, in addition to the blood circulation system such as urine (Illanes et al., 2006), saliva (Mithani et al., 2007), pleural fluid (Sriram et al., 2012) and cerebrospinal fluid (De Mattos-Arruda et al., 2015). To differentiate between necrotic cell death as well as apoptotic death, ALU 247 and ALU 115 are used. ALU 247 is known to be longer than ALU 115, which corresponds to the total DNA quantity. Since necrotic cell death is mainly linked to the progressive tumour process, a promising biomarker is planned to be the longer fragments of DNA (Jin & El-Deiry, 2005).

Several studies have shown that, in healthy individuals, the source of cfDNA is mainly by apoptosis which produces shorter (even) sized DNA fragments whiles necrosis, produces uneven longer DNA fragments in cancer patients in relation to the shorter ones from apoptosis, thus raising cfDNA concentrations in cancer patients (Delgado et al., 2013). As a better
biomarker for the presence of malignant tumour DNA, higher levels of longer DNA fragments in the blood have been targeted (Diehl et al., 2008).

1.2 Problem statement

Breast cancer is the most prevalent cancer in the world and the principal cause of death in females. In Africa, breast cancer has become a public health issue, with many more diagnosis awaiting in the coming years (Torre et al., 2015). It has been shown that, there are several significant breast cancer management problems in Africa, some of which include late diagnosis of the disease, late reporting to hospitals, lack of education for women and poorly resourced breast cancer health facilities. While breast cancer in both developed and developing countries (Africa) remains a major public health issue, majority of the incidence, mortality and morbidity lies in the developing countries (Kanavos, 2006).

Breast cancer is the leading cause of cancer for women with high mortality rates in Ghana. Though it remains the most common cancer in females with elevated mortality in Ghana, there is little understanding of the disease, leading to poor approaches to it with associated extreme results (Ferlay et al., 2019).

More pragmatic attempts must be made to contain, control and monitor the response of breast cancer care in light of these troubling trends since effective management and monitoring of breast cancer response, leads to higher survival rates in patients with early diagnosis. This however, requires vigorous studies into new and effective ways to control and monitor disease response as this is supposed to be comparatively cheaper, easier to introduce, effective and widely appropriate (Garcia-Murillas et al., 2015).

Despite the latest care and management choices for breast cancer patients such as radiotherapy, chemotherapy and surgery, morbidity and mortality rates are still high as breast cancer is still
causing the death of many females in Ghana. This is due to late diagnosis, poor management and most essentially poor monitoring response (Gupta et al., 2015). Therefore to determine the therapeutic impact, it is important to identify a biomarker which can be used to monitor response to treatment, especially chemotherapy since it is the most frequently used and the primary treatment for breast cancer.

Actually, for precise detection, management and monitoring response of breast cancer treatment, the use of biomarkers of inadequate accuracy such as cancer antigen (CA15-3) and carcinoembryonic antigen have been discouraged (van de Vijver, 2014). Therefore, there is a clear need for biomarkers which can improve present management and response to treatment. The use of circulating cell-free DNA to diagnose, control and also monitor the response to breast cancer treatment has been of great interest.

Degraded DNA fragments that are released from cells into the bloodstream of humans are called circulating cell-free DNA. The source of cfDNA in healthy individuals is solely by apoptosis producing evenly sized shorter DNA into the bloodstream, but the levels of cfDNA in the blood are increased in cancer patients because necrosis releases non-uniformly longer DNA fragments in addition to the shorter ones from apoptosis (Barták et al., 2018). Increased levels of longer fragments of DNA in the blood have therefore been targeted as a good biomarker for the presence of malignant tumour DNA and for monitoring the response to treatment/management. Therefore, the usefulness of cfDNA for the predicting response to breast cancer therapy treatment and monitoring is now the focus of many researchers around the world.

Leon et al. (1977) found a reduction in cfDNA concentrations following radiation therapy in one of the initial studies of cfDNA in cancer and found that, reduction in cfDNA concentrations was associated with improved clinical status (Leon et al., 1977). This has urged further
research into the use of cfDNA as a biomarker of treatment response. To evaluate the efficacy of the treatment modality, it is important to establish a reliable biomarker which can be used to assess and monitor response to chemotherapy in breast cancer.

1.3 Justification

Despite the availability of initiatives and treatment of breast cancer, morbidity and mortality rates are still high. The most prevalent cancer and the primary cause of death in females here in Ghana continues to be breast cancer. Cases of ineffective care and lack of monitoring of treatment response, in particular chemotherapy are very high. Several researchers have studied the use of cfDNA as a biomarker in breast cancer patients for early detection diagnosis and prognosis (Dawson et al., 2013; Garcia-Murillas et al., 2015; Olsson et al., 2015), but little evidence have been given on the treatment response in patient with breast cancer specifically those undergoing chemotherapeutic treatment.

Therefore, the evaluation of cfDNA as a biomarker can be used to monitor the response of breast cancer patients undergoing chemotherapy and it is expected that, the results of this research will make some contributions in the clinical settings as well as to increase our awareness and understanding of breast cancer treatment preferences.

This study includes intensive research into the impact of chemotherapy on serum concentrations of cfDNA in patients with breast cancer. Furthermore, the results or data obtained will assist the health sector in its efforts to establish a framework in which cfDNA will be considered to be a biomarker for successful treatment and monitoring of breast cancer response in order to minimize mortality and morbidity.
1.4 Aim

To evaluate circulating cell-free DNA as a blood biomarker for monitoring response to chemotherapy in breast cancer.

1.5 Specific objectives

i. To determine the serum concentrations of circulating cell-free DNA in newly diagnosed breast cancer patients and apparently healthy controls.

ii. To determine the effect of chemotherapy on serum cfDNA concentrations after the 3rd cycle of chemotherapy.

iii. To correlate serum cfDNA levels to the molecular subtypes of breast cancer.

iv. To correlate clinical pathological and anthropometric parameters (BMI and WHR) of breast cancer patients with serum cfDNA levels.

v. To correlate serum cfDNA levels with tumour parameters (stages and grades).

1.6 Null hypothesis

Serum concentration of cfDNA is not affected by chemotherapy and is not useful in monitoring response to chemotherapy in breast cancer.
CHAPTER TWO

LITERATURE REVIEW

2.1 Breast cancer

Cancer is recognized as a group of diseases with enhanced resistance to cell death, alterations of growth factors and metastases characterized by uncontrolled cell growth with the ability to enter or spread to body tissues (Tamimi et al., 2016). Ultimately, most cancer cells form a tumour mass and are also named for the part of the body where the tumour initiates. Others hardly ever develop tumour but blood and blood-forming organs are involved in these cancers and circulate through other tissues where they develop (Runowicz et al., 2016).

The risk of cancer for a person depends on several variables containing external factors such as tobacco use, hormonal treatment, infectious agents and internal factors like hereditary mutations, age, hormone imbalance, immune disorders and genetic make-up (Flavahan et al., 2017). It is called ‘Breast Cancer’ when this irregular cell growth happens in breast tissue.

Breast cancer is a disease that results from abnormal cell proliferation within the tissues of the breast. In general the breast consists of an endocrine gland made up of glandular tissues and ducts linking the lobules to the nipple and adipose tissue protected by a loose fibrous connective tissue structure (Jesinger, 2014).

2.2 Breast cancer statistics

In Africa and various parts of the world, cancer is a major public health issue. Over the years, clinical understanding of mortality from breast cancer has increased and the interest in its presentation, epidemiology and therapy of the disease has also increased (Ghoncheh et al., 2016). Breast cancer is the most frequently diagnosed cancer in women worldwide and a primary cause of cancer death (Ferlay et al., 2019). According to the 2018 estimation, breast
cancer has a prevalence of 11.6% among all cancers, accounting for 6.6% of mortality globally (Ferlay et al., 2019).

Breast cancer, with many more cases predicted in the coming years, is becoming a growing public health concern in Africa. In Africa, many problems have resulted in increased mortality from the disease including health facilities, inadequate vital registration, limited education on the disease awareness and late presentation to a health facility (Anderson et al., 2011). Moreover, it is common for women in a number of African countries to use conventional care before pursuing medical care at a health facility leading to delayed diagnosis (Pace et al., 2015).

Cancer of the breast in both developed and developing countries is a significant public health issue, considerably, its occurrence, mortality and morbidity resides in developing countries (Kanavos, 2006). With the cancer being the most commonly diagnosed among women in Africa, the illness has surpassed cervical cancer in a number of countries (Bray et al., 2018). Increased urbanization and the acceptance of certain lifestyles including smoking, drinking of alcohol have increased the occurrence of the disease in developing countries (Habib & Saha, 2010). The prevalence of breast cancer is lower in developing countries than in developed countries, however, the death rates from the disease are higher in developing countries compared to developed ones (Jemal et al., 2010).

In Ghana, the disease has been listed as one of the most common cause of mortality among women. This can be ascribed to absence of breast screening, late presentation and progressive diagnostic level (Bray et al., 2018).
2.3 Breast Anatomy

The human breast comprise mainly of three components namely; skin, subcutaneous tissue and breast tissue (parenchyma and stroma), which are a transformed cutaneous exocrine gland. The parenchyma consists primarily of ducts and lobules with fats interposed in a complex network of ligaments, nerves, arteries and veins as well as lymphatics in the supporting stroma (Figure 1). The parenchyma is made up of 15 to 20 lobes that are in a radial arrangement converge at the nipple. Each lobe comprise mainly of a lactiferous sinus, segmental collecting duct, sub segmental duct, ductile (terminal duct) and acini (terminal ductile) (Datta et al., 2017).

Figure 1: The anatomy of the breast (Adapted from: Amy and Sam Collins, 2015).
A lobe consisting of 20 to 40 lobules is drained by each duct with each lobule containing 10 to 100 alveoli or acini. Each lobule is made up of branching duct that break into sub-segmental structures, which end in the lobular unit of the terminal duct. The lobular terminal duct unit consist of the terminal duct and the acinus. The lobe ducts also converge into 6 to 10 significant collecting ducts that have nipple openings and attach to the outside. Each of these major ducts arises from the nipple and forms a lobe of glandular tissue which is then protected by the Cooper’s ligament originating from stromal tissue and attached to the prepectoral fascia and dermis which support and suspend the breast. The lactiferous sinus is seen beneath the nipple openings. A small opening of the ampulla part of the main duct is the lactiferous sinus. The skin of the breast also contains hair follicles, sebaceous glands and apocrine sweat glands. The skin over the breast binds to the underlying breast tissue through an anterior facial layer as well as via superficial fibrous extensions of Cooper’s ligaments (Bland et al., 2018).

Superiorly, the breast lies on top of the pectoralis major muscle, laterally the serratus anterior muscle and oblique upper abdominal muscle. The boundaries of the breast usually extend from the second rib superiorly to the sixth rib inferiorly with the medial sternum and the lateral midaxillary line in both males and females (Clemente, 2011). Female breast is bigger than that of males and therefore having bigger fibro glandular tissue volume compared to the fat seen in among males (Annonier, 2013).

2.4 The pathology of the breast tissue

Cancer of the breast is a condition in which cells in the breast grow out of control. It can also be seen as a heterogeneous disorder that involves a large spectrum of pathological units and a number of medical behaviours. These are strengthened by a complex spectrum of genetic changes affecting cellular processes at the molecular level (Reis-Filho & Lakhani, 2003).
Hundreds of genes in regular cells regulate cell division mechanism. In normal development, there is a balance in the activity of genes that promote increases in cells and genes that suppress it. It also relies on gene activities that indicate when apoptosis should be undergone by damaged cells. Cancerous cells act contrarily to the body’s regular cells and therefore these distinctions are associated to the action of cell division. Their replicative immortality can therefore divide severally than a regular body cell (VanDyke et al., 2016).

Generally, human cells undergo about 40 - 60 divisions until they stop dividing and hence grow old and ultimately die compared to cancer cells which divide far more times because, of the expression of the telomerase enzyme. This is contrary to the wearing down of chromosomes ends that typically occur during each division of cells (Artoni et al., 2017).

In other respects, those are not specifically cell-cycle related divisions since cancer cells are distinct from normal cells. These differences contribute to the growth, division and development of tumours. Cancer cells for example have the ability to spread to other areas of the body, a process called metastasis which facilitates the formation of new blood vessels, called angiogenesis that provides oxygen and nutrients to tumour cells (Muz et al., 2015). Under conditions where normal cells would undergo programmed cell death or apoptosis, cancer cells often fail to do so.

After mutations accumulate in different genes that regulate cell proliferation, cells become cancerous. Most cancer cells have 60 or more mutations according to research results from the Cancer Genome Project (Forbes et al., 2015). There are distinct mutational signatures in various types of cancers. Scientific comparisons of different forms of tumour have shown that some genes are more frequently changed in cancerous cells than others. Among the growth-promoting genes, signalling protein genes (Ras) are the most frequently mutated in cancer cells.
They are super-active and produce cells which are activated too strongly by developmental receptors (Blagosklonny, 2008).

Primarily, molecular processes associated with the development of cancers are not well known, however, initiation of breast cancer is commonly believed to results from unregulated cell proliferation and aberrant programmed cell death (apoptosis). This is due to accumulated genetic damage that leads to genetic changes and results in proto-oncogene activation and inactivation of tumour suppressor gene, whereby these are genes that normally inhibit the progression of the cell cycle (Labi & Erlacher, 2015).

Breast cancer cells located in solid tissues are called breast tumour and this tumour is either benign or malignant. Benign breast tumour is not cancerous and the cells tend to be near average, develop steadily and do not enter surrounding tissues (Girish et al., 2014).

Atypical hyperplasia, fibroadenomas, fat necrosis, cysts phyllodes tumour and sclerosing adenosis are examples of benign breast tumour. A tumour that develops out of control, is cancerous and this is termed a malignant breast tumour. They will gradually spread to other areas of the body outside the initial tumour. Most malignant breast tumours begin in the lactiferous duct and this is referred to as ductal carcinomas (Hüsemann et al., 2008). Basically two kinds of ductal carcinomas are present. They are ductal carcinoma in situ and invasive ductal carcinoma, respectively (Hüsemann et al., 2008).

The earliest stage in diagnosing the cancer is the Ductal Carcinoma in Situ (DCIS) which is an uncontrolled development of cells within the breast ducts and does not spread into the breast tissue exterior to the ducts. It is classified as stage zero and women with such diagnosis easily recover (Sumbaly et al., 2014).
The most common form of breast cancer is invasive ductal carcinoma (IDC), also called infiltrating ductal carcinoma and approximately 80% of all breast cancers are invasive ductal carcinomas (Huang & Fu, 2015). IDC refers to cancer that has penetrated through the milk duct wall and has started to enter the breast tissue. Invasive ductal carcinoma can spread to the lymph nodes and maybe to other areas of the body over time. Malignant breast tumours often begin in cells of the glandular tissue that have a secretory role and are referred to as adenocarcinoma (Weigelt & Bissell, 2008).

The development of a simple breast tumour is followed by many signs and symptoms. The most common warning sign is a painless lump signalling the development of the tumour into a more palpable size and is generally felt in the breast upper outer quadrants. Cancers that often cause painless lumps or swellings that may or may not be accompanied by nipple discharge are inflammatory breast cancers. Another relevant indicator of breast cancer is pain because, in addition to other signs, different symptoms of breast cancer have shown that, the risk of breast cancer is greater in patients with breast pain (Macias & Hinck, 2012).

2.5 Molecular and biological classification of breast cancer

Breast cancer is a heterogeneous disease characterized by numerous molecular alteration, cellular composition and clinical manifestation which offers particular phenotype to each breast tumour used as a molecular target for personalized therapy (Toss & Cristofanilli, 2015). The tumour size, histological grade and hormone receptor status are the most widely used prognostic markers of breast cancers. Other parameters including mitotic index, Antigen KI-67 (Ki-67) rate and involvement of axillary lymph node have also been evaluated. These biological markers are essential in breast cancer to predict prognosis and make successful treatment decisions that are typically made based on knowledge derived from them (Nishimura et al., 2014).
One type of knowledge that is critical for making successful treatment decision is the molecular profiling of breast cancer. Each breast cancer has a molecular profile and this can help decide the most appropriate method of treatment. Molecular breast cancer profiling is also focused on the presence or absence of breast tumour receptors for Estrogen Hormone Receptor (ER), Progesterone Hormone Receptor (PR) and Human Epidermal Growth Factor-2 (HER2-) (Nishimura et al., 2014).

The estrogen hormone receptor is a member of the nuclear steroid receptor family and serves as a transcriptional regulator mediated by the 17p-estradiol estrogen hormone (Zwart et al., 2011). In the late 1950s, it was first studied in breast cancer and became the first molecular marker for prognosis and reactions to therapy. ER is the most significant biologic marker of response to hormonal therapy in breast cancer, in addition to its prognostic importance (Keller et al., 2010). It also has a function in cellular development, proliferation and differentiation (Kabel et al., 2016).

The progesterone hormone receptor is a member of the nuclear hormone receptor family that binds directly to progesterone and is encoded on chromosome 11q22 by a single gene named the Progesterone Receptor (PR) gene (Jacobsen & Horwitz, 2012). The receptor of progesterone is also one of the active tumour markers that effectively predict hormonal responsiveness in breast cancer (Shah et al., 2014).

Bacus and associates initially discovered the Human Epidermal Growth Factor Receptor 2 (HER2-) in 1990 (Bacus et al., 1990). It is localized to chromosome 17q and encodes a transmembrane tyrosine kinase receptor protein that is a member of the epidermal growth factor receptor (EGFR) or HER family (Ritter & Arteaga, 2003). HER-2 and its ability to predict response to hormonal and cytotoxic therapies have been considered as a prognostic factor in thousands of preclinical and clinical studies (Ross et al., 2009).
Five key intrinsic or molecular subtypes of breast cancer are available based on the categorization mentioned above and are based on the genes that cancer expresses (Prat et al., 2015). These subtypes are as follows;

2.5.1 Luminal A

Luminal A is a molecular subtype of breast cancer with estrogen receptor and/or progesterone receptor being positive and HER-2 negative with low levels of Ki-67 (Eliyatkın et al., 2015). Ki-67 is a nuclear protein associated with cell proliferation and was initially recognized in the early 1983s, using a mouse monoclonal antibody from a Hodgkin’s lymphoma-descended cell line directed against a nuclear antigen (Gerdes et al., 1983). As they prepare to differentiate into new cells, Ki-67 increases. For the care and follow-up of patients, it is necessary to take the Ki-67 index value into account (Nishimura et al., 2014). In addition to breast cancer, the outcome of less than 10% is considered low, 10-20% borderline, and strong for Ki-67, if more than 20%.

Luminal A breast cancers are low grade and due to the low levels of Ki-67 which is an indication of how quickly cancer cells grow, they appear to grow slowly. Approximately 80% of breast cancers in Luminal A are ER + and about 65% of these are also PR + (Iqbal & Buch, 2016). Breast cancer with Luminal A also has the highest prognosis.

2.5.2 Luminal B

For Luminal B breast cancer, the cancer tumour is positive for estrogen and/or progesterone receptor and has either a negative or positive HER-2 receptor with elevated Ki-67 levels (Regan et al., 2015). Studies have shown that breast cancers with elevated Ki-67 levels are correlated with the worst results (De Azambuja et al., 2007).
The Ki-67 index is higher than 20% and they appear to develop slightly faster than Luminal A cancers because they have higher Ki-67 levels and their prognosis is slightly worse showing a more violent phenotype, including the worst outcome in patients (Sørlie et al., 2001). Luminal B cancers are significantly different than Luminal A cancers at the molecular level and can be treated with hormone therapy as well as HER-2 receptor targeting and medication.

2.5.3 Human epidermal growth factor receptor 2 (HER 2-Enriched)

The human epidermal growth factor receptor (HER 2) is the second member of the epidermal growth factor receptor family (EGFR) and is an oncogene that encodes a transmembrane tyrosine kinase receptor. HER 2 proteins are receptors found on breast cells that help regulate the development, division, and repair of healthy breast cell itself (Krishnamurti & Silverman, 2014). If the HER 2- gene does not function properly and makes too many copies of itself, it is called amplification or overexpression of HER 2- gene. However, in many distinct forms of human cancers, including breast, ovarian, lung, gastric, and oral cancers, the HER 2-gene is overexpressed. The HER 2-gene functions inappropriately and generates a number of copies of itself in about 10% to 20% of breast cancers (Hayes, 2015), contributing to so many problems.

HER 2-positive are labelled breast cancers with HER 2 gene amplification or HER 2 protein overexpression. However, both estrogen and progesterone receptors are negative for HER 2 enriched form of breast cancer with HER 2 being positive (Pusztai et al., 2010). HER 2-positive breast cancers appear to develop more quickly than luminal cancers and are more likely than HER 2-negative breast cancers to spread and recur (Singh et al., 2018). They often have a weaker prognosis but are mostly treated effectively with targeted HER 2 protein therapies such as Herceptin, Nerlynx and Kadcyla. There are also some methods used to find out whether or
not breast cancer is HER 2-positive. The immunohistochemistry test (IHC) and Fluorescence in Situ Hybridization (FISH) are two of the most common test (Lal et al., 2004).

2.5.4 Triple-negative

The first mention of Triple-negative breast cancer was in October 2005 and the phrase has appeared in more than 600 publications since then (Brenton et al., 2005). Triple-negative breast cancer is also a breast cancer subtype that lack estrogen receptor (ER), progesterone receptor (PR), and HER 2 expression. Popular therapies such as hormone therapy and medicines targeting estrogen, progesterone and HER 2 are unsuccessful since tumour cells lack the required receptors. Several educations have shown that triple-negative breast cancers are related to bad prognosis as a group, not like HER 2-positive breast cancers which were associated with poor diagnoses before trastuzumab targeted antibody treatment came into use although no triple-negative biologic” therapy is accessible (Pal et al., 2011).

Triple-negative breast cancers are more possible to metastasize to viscera, mostly to the lungs and brain, and are less expected to metastasize to the bone (Dent et al., 2009). In addition, more than 75% of tumours that arise in women with BRCA1 mutation have a triple-negative phenotype (Rakha et al., 2008) and account for around 15% of all invasive breast cancers, that typically have high histological grade and no special type of invasive carcinoma (Reis-Filho & Tutt, 2008). In young black and Hispanic women, however, triple-negative breast cancers occur more commonly as compared to young women from other racial or ethnic groups (Bauer et al., 2007).

Females with triple-negative breast cancer will not benefit from endocrine therapy or have been treated with trastuzumab. Chemotherapy is still the cornerstone of systemic medical care, but triple-negative disease patients, have a worse outcome after chemotherapy than breast cancers patients of other subtypes when considered as a group (Liedtke et al., 2008).
2.5.5 Normal-like breast cancer

It is identical to the Luminal A breast cancer subtype. With HER 2 being negative, estrogen hormone-receptor and/or progesterone hormone receptors are positive. The protein Ki-67 also has low levels, which helps to regulate how rapidly cancer cells develop. However, though normal-like breast cancer has a better prognosis, its prognosis is somewhat worse than the prognosis of Luminal A cancer (Eliyatkin et al., 2015).

2.6 Breast cancer staging

The stage of breast cancer is an indicator of how much the cancer has progressed and spread. When treating and predicting prognosis, the stage is very critical (Giuliano et al., 2017). Two key groups for the staging of breast cancer are given by the American Joint Committee on Cancer. They are the Anatomic Stage and Prognostic Stage (Weiss et al., 2018).

The Anatomic Stage is centered on the degree of breast cancer as defined by the size of the tumour (T), that is how far the cancer cells have spread inside the breast and adjacent tissues, the status of the lymph node (N) which also extends to the surrounding lymph nodes and the spread to distant organs by distant metastasis (Benson, 2003).

Anatomic Tumour, Node and Metastasis (TNM) staging, tumour grade and biomarker status comprising of human epidermal growth factor receptor 2, estrogen receptor, and progesterone receptor are included in the Prognostic Stage (Wang et al., 2018). For patient treatment, the prognostic stage is chosen and its phases are split into clinical and pathological groups.

As the initial treatment for breast cancer, the Pathological prognostic stage refers to patients who have undertaken surgery and comprises of all data used for clinical staging plus surgical outcomes and pathological findings from surgical resection (Cserni et al., 2018). For patients who have undergone neoadjuvant therapy, the pathological prognostic” process does not apply.
The clinical prognostic stage is identified by imaging studies as well as by clinical evaluation and has features that are highly suspect of malignancy or suspended pathologic macro metastasis based on cytological examination of Fine Needle Aspiration biopsy (Cserni et al., 2018).

2.7 Causes and risk factors of breast cancer

There is no full and exhaustive awareness of the causes of breast cancer. Nevertheless, investigators have identified elements that increase or decrease the probability of developing breast cancer and they are referred to as risk factors. Risk factor is described as something that affects the chance of having a disease for individuals, in this situation breast cancer. Breast cancer risk factors can increase the chances of a woman developing the illness. Having one or extra risk factors does not inherently mean that, the disease would evolve in a woman (Feng et al., 2018).

Breast cancer is a complicated ailment, as no single cause can describe any particular circumstance of the disease adequately. Numerous risk factors for breast cancer exist. These comprise of age, gender, hereditary features, nutrition, gynaecological intervention, hormone replacement therapy, endogenous hormonal levels, behaviours (extreme consumption of smoking and alcohol), absence or non-breastfeeding on infants, early menarche and late menopause, exposure to radiation and ecological contaminants (Suba, 2013).

2.7.1 Gender

Gender is by far the largest breast cancer risk factor. Being a woman is the key risk factor for breast cancer as this illness is somewhere around 100 times more possible to happen in females than in males (Richie & Swanson, 2003). The lifetime exposure of a female to estrogen is also a contributing aspect to this risk.
Hence, the longer the amounts of estrogen flowing in the lady’s body, the higher the risk of breast cancer development (Samavat & Kurzer, 2015). In addition, pregnancy is thought to reduce the risk of breast cancer by 7% since ovaries generate fewer estrogen throughout the time of pregnancy at this stage (Britt et al., 2007). Breast cancer risk is decreased every 12 months by 4.3% during breastfeeding, but the reasons for these are not well known.

2.7.2 Age

As demonstrated by the fact that majority (80%) of breast cancers are detected in women aged 50 years and older (Key et al., 2001), ageing eventually raises one’s risk of developing breast cancer. The incidence of breast cancer is known to increase significantly until 55 years of age after which the prevalence decreases (McGuire et al., 2015). Compared with older women, young women have a significantly different picture of breast cancer.

2.7.3 Genetic factors

As a risk factor for breast cancer, genetics play a small but equally significant role. In Western countries, approximately 10 out of every 100 breast cancer cases are as a result of hereditary predisposition (Jansen-van der Weide et al., 2010). Generally, around 5-10% of breast cancers are related to gene mutations inherited from a parent and an inherited mutation called Breast Cancer Associated Gene 1 or 2 (Colditz et al., 2012) is the common cause of inherited breast cancer. The genes BRCA 1 and BRCA 2 are present on chromosomes 17a and 13q individually and are considered to be accountable for a large ratio of very high risk of breast cancer families (Metcalfe et al., 2004). In normal cells by synthesizing proteins that retard abnormal cell development, these genes help to prevent cancer.

Statistically, women with a BRCA1 mutation have a 55-65% lifetime risk of developing breast cancer while the lifetime risk is 45% for women with a BRCA2 mutation. As well as the risk
of developing cancer in both breasts, women with one of these two mutations are also more likely to be diagnosed with breast cancer at a younger age (Allison, 2012). In comparison, BRCA1 mutations are less commonly observed in male breast cancers (Kamińska et al., 2015).

2.7.4 Family history

Having a healthy family history of the disease is one of the most commonly known risk factors for breast cancer. Less than 15% of people with breast cancer have a family member with this disease and there is a greater chance of developing the disease among people who have close blood relatives with breast cancer (Kamińska et al., 2015). For example, having a first-degree relative with breast cancer nearly doubles the risk of a woman getting the sickness, whereas having two first-degree relatives with the disease rises the risk of the woman having the disease by about 3 times (Veronesi et al., 2005).

Additionally, females with a father or brother who have breast cancer also have an increased risk of breast cancer. Within the background of a person, the risk of developing a new cancer in the other breast or in another section of the same breast is greater for a woman with cancer in one breast (Barnard et al., 2015).

2.7.5 Birth control and contraceptives

Many procedures for birth control use hormones that can raise the risk of breast cancer. Women who use oral contraceptives have a marginally higher risk of breast cancer than women who have never used them, but once the regimen is discontinued, the risk tends to return to normal over time. It has also been shown that an injectable form of progesterone called Depo-Provera has an increased risk of breast cancer, but it appears to be no increased risk for women five years after they quit taking the shots (Westhoff, 2003).
Usually, birth control implants, intrauterine devices (IUDs), skin patches, and vaginal rings also use hormones and can, therefore, in theory, raise the risk of breast cancer risk (Westhoff, 2003).

2.7.6 Hormone replacement therapy (HRT) after menopause

To ease menopausal symptoms, the hormone estrogen that is also paired with progesterone has been used (Vogel, 2008). Nevertheless, for ladies who have undergone a hysterectomy, estrogen might also be used on its own. In this way, postmenopausal combined hormone therapy raises the risk of breast cancer as well as the possibility of dying from breast cancer and the probability that the breast cancer will only be diagnosed at a more progressive stage (Chlebowski et al., 2003).

With as little as two years of use, this rise is commonly seen. However, the elevated risk of combined HRT is reversible and its risk extends only to current and recent users, as within five years of quitting HRT. The risk of breast cancer of a woman tends to revert to that of the general population (Watkins, 2007). After menopause, short term usage of estrogen only does not appear to significantly raise the risk of breast cancer. However, to raise the risk of breast cancer, long-term use of estrogen therapy (e.g., >15 years) has been confirmed.

2.7.7 Excessive alcohol consumption

Epidemiologic studies have shown that moderate to high alcohol intake may increase the risk of getting breast cancer (Key et al., 2001; Byrne et al., 2002; Fagherazzi et al., 2015; White et al., 2017). Beer, alcohol and liquor have been established as a positive relationship separately (Byrne et al., 2002). The increased risk of breast cancer is specifically associated with the amount of alcohol consumed. For example, relative to women who don’t drink alcohol, women who have two to three drinks a day have around 20% higher risk of breast cancer. And there is a very small increase in risk among women who have only one alcoholic drink per day (Kwan
et al., 2010). It has also been shown that, alcohol intake affect plasma estrogen as it interferes in several ways with estrogen pathways and can increase breast cancer through its effect on estrogen at least in parts (Dumitrescu & Shields, 2005).

2.7.8 Obesity and overweight

Females’ ovaries create much of the body’s estrogen before menopause (premenopausal), while fat tissue produces just a small amount, so when the ovaries stop producing estrogen after menopause (postmenopausal), much of the estrogen of a woman comes from fat tissue. Thus, after menopause (postmenopausal), having more fat tissue (obese) can increase estrogen levels and raise breast cancer risk. The risk of postmenopausal breast cancer is roughly 1.5 times greater in women who are overweight and around 2 times greater in women who are obese (La Vecchia et al., 2011).

In addition, being overweight and obese appears to lead to higher levels of blood insulin, and some cancers, including breast cancer since they are related to higher levels of insulin (Gunter et al., 2015). Obesity is a risk factor for type II diabetes that in postmenopausal women who have also been associated with an augmented risk of breast cancer (Tsilidis et al., 2015). Analysis of forty works settled that, the risk of breast cancer was 16% greater in females with obesity independent type II diabetes (Boyle et al., 2012).

2.7.9 Not having children or not breastfeeding

This is a significantly higher average risk of breast cancer in females who have not had kids or who have their first kid after age 30 (Brinton et al., 2018). Conversely, breast cancer risk is decreased by having several pregnancies and/or being pregnant at an early age (Lyons et al., 2009). Breastfeeding has also been suggested to reduce the risk of breast cancer significantly, especially if it lasts for 1.5 to 2 years and a potential reason for this consequence is that, breastfeeding decreases the overall number of menstrual cycles for women (Minh et al., 2004).
2.7.10 Early menarche and late menopause

If females start menstruating early, especially before age 12, they will have more menstrual cycles and therefore have a longer lifetime exposure to the hormones estrogen and progesterone leading to a slightly higher risk of breast cancer. Similarly, if they go through menopause later, especially after age 55, women will have more menstrual cycles and have a longer lifetime exposure to estrogen and progesterone with an increased risk of breast cancer (Cancer, 2012).

2.7.11 Environmental pollutants and factors

Majority of works have been placed into identifying potential environmental pollutants in the production of breast cancer. Laboratory research provides evidence that, by damaging DNA, development of tumour growth, or increasing susceptibility by altering mammary gland production, environmental contaminants can contribute to breast cancer risk (Rudel et al., 2007). Approximately 250 chemicals that mimic or interfere with estrogen have been detected in in-vitro assays (Brophy et al., 2012), and these promote the production in laboratory studies of estrogen-sensitive breast cancer cells and seemingly underlies various of the known risk factors for breast cancer (Bernstein, 2002).

Studies have also indicated that females working on night shifts have a higher risk of breast cancer because of fluctuations in the circadian physiology of the body that are influenced by light exposure (Lunn et al., 2017).

2.8 Treatment of breast cancer

Treatment aims to prevent breast cancer-related mortality and avoid life-long problems. The treatment choices are highly customized and are made together by the patient and physician after contemplation of the tumour subtype, comprising of the status of the hormone receptor (ER, PR) and HER2, the stage of the tumour, the existence of identified mutations in genes of hereditary breast cancer, such as BRCA1 or BRCA2, age of the patient, universal health and
menopausal status, preferences and the risks and benefits related with each choice (Long et al., 2014). For breast cancer, many management options are available and they comprise surgery, chemotherapy, radiation therapy, target therapy and hormone therapy (Schnipper et al., 2015).

2.8.1 Surgery
Breast cancer surgery’s primary objectives are to remove the tumour and any underlying healthy tissue throughout the procedure and to test the adjacent axillary lymph nodes underneath the arm (Reigle & Zhang, 2018). Lumpectomy, mastectomy, sentinel lymph node biopsy and axillary lymph node dissection (Gera et al., 2018) are among the types of breast cancer surgery done.

2.8.2 Radiation therapy
The use of high-energy x-rays or other elements to kill cancer cells in radiation therapy. This aids to minimize the risk of breast cancer recurrence (Curigliano et al., 2017). In fact, in the 10 years following treatment, recurrence rates in the breast are now less than 5% with modern surgery and radiation therapy. Its endurance is also similar to lumpectomy or mastectomy (Leachman & Galloway, 2016). Radiation therapy can also be given to the same side of the neck or underarm near the breast or chest wall if cancer is found in the lymph nodes under the arm. Depending on the characteristics of the tumour, patients who have a mastectomy do not require radiation therapy (Hoang et al., 2017). If the patient has a bigger tumour, cancer in the lymph nodes, cancer cells outside the lymph node capsule, or cancer that has spread into the skin or chest wall, or for other causes, radiation therapy might be prescribed after mastectomy (Goutos & Ogawa, 2017).

Radiation therapy may exhibit some side effects, including weakness, enlargement of the breast, redness of the skin, and pain in the skin where the radiation is focused. Radiation therapy may affect a small amount of the lung, triggering pneumonitis, engorgement of the lung tissue
associated with radiation tissue. This risk depends on the size of the region receiving radiation therapy, and with time, this appears to heal.

There are many different forms of radiation therapy including external-beam radiation therapy, intra-operative radiation therapy and brachytherapy (Zhao et al., 2017). After or before surgery, radiation therapy can be performed. It is called neoadjuvant when administered before surgery and adjuvant after surgery.

2.8.3 Chemotherapy

It is a systemic therapy which uses drugs to kill cancer cells, usually by preventing more cells from developing, dividing, and making cancer cells. It can be given to shrink a big tumour before surgery, promote surgery, and minimize the risk of recurrence, named neoadjuvant chemotherapy (Mijwel et al., 2018). It can also be given after surgery called adjuvant chemotherapy to lower the risk of recurrence. A chemotherapy regimen typically involves a combination of medications given over a fixed period of time in a particular number of cycles (Leinert et al., 2017). Based on what performed best in clinical trials with that particular form of regimen, chemotherapy can be given on several different schedules and it can be given once a week, once every 2 weeks, once every 3 weeks, or even once every 4 weeks (Dang et al., 2015).

Various kinds of chemotherapy medications are used to treat breast cancer. Some of the common medications include capecitabine, carboplatin, cisplatin, cyclophosphamide, docetaxel, fluorouracil and paclitaxel (Bishayee et al., 2013).

A patient can take one medicine at the same time or a mixture of different medicines given at the same time. Research has indicated that combinations of some drugs are more powerful than single adjuvant drugs (Ando et al., 2014). As an adjuvant treatment for early and locally
advanced breast cancer, the following drugs or combinations of drugs can be used (Diéras et al., 2017). They are AC (doxorubicin and cyclophosphamide), EC (epirubicin, cyclophosphamide), CEF (cyclophosphamide, epirubicin, and 5-FU), and CMF” (cyclophosphamide, methotrexate, and 5-FU).

Nonetheless, with chemotherapy for HER2-positive breast cancer, therapies targeting the HER-2 receptor can be administered and the antibody trastuzumab is an example (Fang et al., 2020). Therefore, early-stage HER-positive breast cancer combination regimens might include: AC-TH (doxorubicin, cyclophosphamide, paclitaxel or docetaxel, trastuzumab), TCHP (paclitaxel or docetaxel, carboplatin, trastuzumab, pertuzumab) and TH (paclitaxel, trastuzumab) (Swain et al., 2020).

The side effects of chemotherapy may include exhaustion, risk of infection, nausea, vomiting, hair loss, loss of appetite, diarrhoea, constipation, numbness and tingling, early menopause, weight gain, and chemo-brain, depending on the person, the drug(s) used, the schedule and dose (Yeo et al., 2020). These side effects can also be avoided or controlled effectively throughout supportive drug therapy, and they frequently go away after the end of therapy.

Long-term side effects such as heart damage, irreversible nerve damage, or secondary cancers such as lymphoma and leukemia, can rarely occur.

2.8.4 Hormonal therapy

Hormonal therapy, is a successful treatment that test positive for either estrogen or progesterone receptors. This kind of tumour use hormones to fuel its development, so when used either alone or after chemotherapy, blocking the hormones can help prevent a cancer recurrence and death from breast cancer (Nekhlyudov et al., 2011). In order to shrink a tumour, make surgery simpler, and minimize the risk of recurrence, hormonal therapy can be offered
before surgery and this is referred to as neoadjuvant hormonal therapy (Nekhlyudov et al., 2011). To lower the risk of recurrence, it may be given after surgery named adjuvant hormonal therapy. Tamoxifen, ovarian suppression and aromatase inhibitors are some of the types of hormonal therapy.

2.8.5 Targeted therapy

Targeted therapy is a procedure that addresses the particular genes, proteins, or tissue environment of the cancer that contributes to the growth and survival of cancer. These therapies work differently than chemotherapy and are very focused (Perez-Herrero & Fernandez-Medarde, 2015). This form of treatment prevents cancer cells from growing and spreading while minimizing damage to healthy cells. Not all tumours have similar targets. Your physician can run tests to recognize the genes, proteins, and other features in your tumour to determine the most effective treatment. Hormonal therapies were the first approved targeted therapies for breast cancer, and HER2-targeted therapies were later accepted to treat HER2-positive breast cancer (Loibl & Gianni, 2017).

2.9 Imaging modalities for evaluating treatment response of breast cancers

Different imaging modalities are used to determine the efficacy of breast cancer care, but the patients are sadly exposed to radiations which then affects their health status. Magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), PET/CT, and bone scintigraphy are some of those included.

2.9.1 Positron emission tomography (PET)

Positron emission tomography is one of the most detailed imaging methods for assessing the tumour response to breast cancer treatment as both metabolic and morphologic data are taken in account (Humbert et al., 2015). PET is a molecular imaging test that can monitor the response of breast cancer to treatment earlier than the shrinking tumour found with traditional
imaging (Schwarz et al., 2005). Positron emission tomography enables non-invasive visualization of many biological processes that are modulated during breast cancer treatment and quantitative evaluation. Of these, F-fluorodeoxyglucose (FDG) glucose metabolism assessment are the most commonly used and have an emerging role in the treatment of breast cancer (Groheux et al., 2013).

In addition, the ability of FDG-PET to predict treatment response in individual patients has been an active area of research for many years, particularly in the neoadjuvant environment since glucose metabolic changes occur earlier than tumour shrinking (Groheux et al., 2013). In addition, since glucose metabolism is increased in breast cancer, for early prediction, monitoring of the metabolic response with FDG-PET has been proposed. PET uptake test should include an ongoing response predictor and brings data beyond the conventional dichotomous measurements commonly used in other response evaluation (Wahl, Jacene, Kasamon, & Lodge, 2009).

### 2.9.2 Bone scintigraphy (BS)

Bone scintigraphy (BS) is one of the imaging modality for assessing the response of tumour to breast cancer treatment. Bone scintigraphy is an important instrument for detecting and evaluating cancer tumour response that has spread (metastasized) from the original position of the tumour to the bone, such as the breast (Shie et al., 2008). Usually small quantities of radioactive materials called radiotracers that are injected into the bloodstream. Bone scintigraphy helps diagnose and analyze a number of bone diseases and conditions. The radiotracer passes through the area being investigated and gives off radiation in the form of gamma rays that are detected to generate photographs of your bones by a special gamma camera and a device (Ravikanth, 2017).
Instead of directly imaging the tumour, bone scintigraphy measures changes in bone structure and can take up to 6 months to show the reaction to therapy (Al-Muqbel & Yaghan, 2016). While bone scintigraphy (BS) is susceptible to detecting bone metastasis, there are slow changes in the appearance of bone lesions with successful treatment, making it difficult to evaluate treatment (Al-Muqbel & Yaghan, 2016). Since BS represents the osteoblastic reaction of the bone to metastatic disease that increases as the disease responds to treatment, it is inadequate for the response assessment of bone metastases.

2.9.3 Computed tomography (CT)

In determining the response of patient’s to cancer therapy, computed tomography has a specific role. CT is also a molecular imaging test that can monitor the response of breast cancer therapy before traditional imaging shows the tumour shrinking (Schwarz et al., 2005).

2.10 Screening of breast cancer

Before any signs arise, particularly in asymptomatic patients, screening is one of the most common methods of identifying breast cancer (Panieri, 2012). It also assists in the disease’s early detection. Early detection is important in order to enhance breast cancer outcomes and survival, so screening is one of the methods for primary discovery of breast cancer (Harper et al., 2009). As breast cancer screening tools, different approaches have been assessed and they are mammography, clinical breast exam, magnetic resonance imaging and breast self-examination (Society, 2008).

2.10.1 Mammography

The only approach actually deemed suitable for mass screening of asymptomatic women is mammography screening (Coleman, 2017). To detect anomalies in the breast, it uses low-energy x-rays and also enables visualization of the interior structures of the breast. In high-resource environment, mammography has been indicated to minimize breast cancer death by
Mammography screening is structured as a multi-aspect program which represents a measure that facilitates early detection of breast cancer early in order to minimize mortality and increase therapeutic decisions. It is related to suitable side effects for the requested people, generates reproducible outcomes and may be extended to the people at satisfactory societal costs (Heywang-Köbrunner et al., 2011).

The World Health Organization (WHO) Position Paper on mammography screening established that if pre-specified criteria for program implementation are met, women aged 50-69 should undergo coordinated, population-based mammography screening in well-resourced settings (Organization, 2014). Mammography is not cost-effective in limited resource settings with poor health systems. Early detection should concentrate on reducing the diagnostic stage through increased understanding (Dey, 2014). WHO recommends systematic mammography screening only in the sense of comprehensive studies and in well-resourced settings for women aged 40-49 years or 70-75 years.

As mammography is associated with a limited amount of radiation, radiation exposure is one of the drawbacks of mammography screening (Heywang-Köbrunner et al., 2011). Therefore, the possibility of radiation is irrelevant due to the early detection, given the fact that mammography can save lives. False alarm risk is also a downside of mammography in that, around 90% of the breast which is susceptible to mammograms signifies that, there is roughly 10% possibility of finding a small tumour but not present and this is termed false negative (Rafferty et al., 2013).

2.10.2 Clinical breast examination (CBE)

Clinical breast examination is one of the constituents of the triple evaluation conducted for the assessment of breast lumps during the first meeting of the patient with a qualified health professional (Zafar, 2014). It is an examination by a qualified health professional of both
breasts. It is mostly done during routine medical check-up. The National Comprehensive Cancer Network (NCCN) advises that, for any changes or anomalies (such as a lump), a skilled health expert need to cautiously sense your breasts, underarms and the area just beneath your clavicle (Chen et al., 2006). When you sit up, the professional health practitioner will visually inspect your breasts and physically test your breasts when you lie down. For low resource environment, CBE seems to be a promising solution and this could be implemented based on the evidence from ongoing studies. Three to forty-five percent of breast cancer missed by screening mammography can be identified by CBE alone (Feigin et al., 2006).

In several trials, CBE has been called unreliable, and has been revealed to have poor sensitivity and specificity to detecting breast cancer. Other CBE objections are insufficient evidence to allocate any substantial advantage, absence of standardization, lack of self-assurance of doctor’s in their CBE abilities and their lower precision than that of mammography.

2.10.3 Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI), is a technique that generates accurate cross-sectional pictures of the inside of the body using magnets and radio waves and is much delicate (Crook & Robinson, 2009). MRI doesn’t use X-rays and doesn’t require any disclosure to radiation. Breast MRI has a variety of diverse applications for breast cancer comprising of screening for high-risk females, collecting much knowledge about a mammogram suspicion region or ultrasound testing for post-treatment recurrence (Monticciolo et al., 2018). While it is usually considered more vulnerable than mammography for picking up breast cancer, certain cancers that would be found through mammography can also be missed. Breast MRI is therefore, only recommended in conjunction with other examination, such as mammogram or ultrasound (Berg et al., 2012).
Breast MRI is not commended for females who are at usual risk of developing breast cancer as a screening tool. A big drawback however, is that breast MRI screening leads to further false positives results or in other words, the test detects something that primarily appears alarming but turns out not to be cancer (Sardanelli & Podo, 2007). Also, majority of females would end up undergoing needless biopsies and other examinations, not to mention anxiety and depression, if breast MRI were implemented as a screening tool for all. MRI is often more costly than mammography and it is not commonly available to and dedicated breast MRI screening equipment.

You must have a contrast solution (dye) introduced into your arm through an intravenous line before the MRI test (Pouw et al., 2015). As the dye can damage the kidneys, before the contrast solution is administered, clinicians often conduct kidney function tests. The solution will allow any breast tissue that is potentially cancerous to show up more clearly. During the infusion of the contrast solution, more individuals experience temporary discomfort (Asdourian et al., 2016).

However, in order to develop, cancers need to increase their blood flow and so on a breast MRI, the contrast appears to become more concentrated in cancer growth portions appearing on an otherwise dark background as white areas (Kuhl, 2007). This allows the radiologist to assess which parts may be cancerous.

2.10.4 Breast self-examination

Breast self-examination is an effective way of detecting breast cancer early, or frequently inspecting your breasts on your own, as it is more possible to be handled successfully (Öztürk et al., 2000). Although, no single test can diagnose all breast cancers early, it is thought that the chances of early detection can be improved by conducting breast self-examination in conjunction with other screening methods (Lee Champion, 1985). Breast self-examination is
a simple, cost free method that can be used on daily basis and at any age. As part of their general breast cancer screening plan, it is recommend that, most females regularly conduct breast self-examination.

Unfortunately, as a screening technique for females with an average risk of breast cancer, the American Cancer Society no longer acclaims breast self-examination since it may not have a different effect on survival rates of breast cancer and can even cause mischief by causing pointless biopsies (Oeffinger et al., 2015).

2.11 Anthropometric measurement

Anthropometric measurements are a collection of quantitative human body measurements in terms of muscle, bone, and adipose tissue dimensions used to determine the body’s composition (Di Sebastiano & Mourtzakis, 2012). Height, weight, body mass index, body circumferences (waist, hip, and limbs), and skin fold thickness are the main components of anthropometry (Sebo et al., 2017). These measurements are significant since it is stated that people with higher values are at increased risks for hypertension, diabetes mellitus, cardiovascular illness, gallstones, arthritis, cancer and many more (Kidy et al., 2017). In addition, anthropometric dimensions can be used as a physical fitness baseline and to assess fitness improvement.

Epidemiological evidence has shown that in the development of breast cancer, anthropometric parameters such as BMI and WHR are involved (Van den Brandt et al., 2000). The correlations among anthropometric parameters (BMI and WHR) and BC in both pre- and postmenopausal females have been investigated by numerous research and meta-analyses. Consistent positive associations between BMI and WHR and the risk of BC among postmenopausal women have been identified overall (Chow et al., 2005). In addition, in
postmenopausal women, WHR that correlate with central (abdominal) obesity was associated with higher risk of BC  (Wu et al., 2007), but not in premenopausal females.

2.11.1 Body mass index (BMI)

The Body Mass Index is a word firstly devised by Ancel Keys in 1972 (Keys et al., 1972). It is the most popular method of obesity measurement and detection. The Body Mass Index is an estimation of the fat of the body based on the weight in kilograms divided by the square of height in meters. BMI does not explicitly calculate body fat but uses an equation to make an estimate instead (Rush et al., 2007). BMI value < 18.5 is underweight, between 18.5 and 24.9 is normal weight, between 25.0 and 29.9 is pre-obesity and ≥ 30 obesity according to WHO (Lau et al., 2007).

A high BMI value may be a high body fat indicator whereas, a low BMI may be a low body fat indicator. BMI may be used to test for classes of weight that can lead to health issues like breast cancer but an individual’s body fat or health is not diagnosed.

In the past few years, much attention is given to the relationship between BMI and breast cancer risk but the results have still been controversial (Reeves et al., 2007). Nevertheless, every 5 kg/m² increase in BMI leads to a 2% rise in women’s risk of breast cancer, so increased BMI may be a defensive factor for women’s risk of breast cancer (Liu et al., 2018).

2.11.2 Waist to hip ratio (WHR)

The dimensionless ratio of the circumference of the waist to that of the hips is the waist-to-hip ratio. It is also known as the circumference of the waist divided by the circumference of the hip. As an alternative indicator of body fat distribution, WHR was proposed (Janssen, 2010). The proportion can be calculated more accurately than skin folds, and both subcutaneous and intra-abdominal adipose tissue indexes are given (Luppino et al., 2010). A greater
independent risk factor than BMI is tended to be WHR (Janssen et al., 2004). A WHR value of $\leq 0.80$ is low for females and $\leq 0.95$ is low for males, between $0.81$ to $0.85$ is normal for females and $0.96$ to $1.0$ is normal for males, and $\geq 0.86$ is higher for females and $\geq 1.0$ is higher for males according to WHO (Lau et al., 2007).

However, studies have indicated that, an increased risk of breast cancer is associated with a higher WHR value (Connolly et al., 2002). The elevated WHR value is also reported as an indicator of breast cancer mortality (Borugian et al., 2003).

### 2.12 Cancer biomarkers

A biomarker is a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process of a disorder or disease such as cancer according to the National Cancer Institute (Hasanzadeh et al., 2017). It may also be a series of modifications, such as signatures of gene expression, proteomic, and metabolomics. Usually, biomarkers identify an infected person from an individual without the illness (Henry & Hayes, 2012). A variety of factors, including germ line or somatic mutations, transcriptional changes, and posttranslational modifications, may be responsible for these differentiations (Henry & Hayes, 2012).

There are vast variations of biomarkers including proteins, nucleic acids, antibodies and peptides (Paoletti & Hayes, 2014). In the circulation (whole blood, serum, or plasma), excretions or secretions (stool, urine, sputum, or nipple discharge) biomarkers can be observed. These may simply be examined non-invasively and serially, or may be tissue-derived and need either biopsy or superior imaging (Paoletti & Hayes, 2014).

Biomarkers can however, be used in various medical settings for patient assessment, comprising of estimating disease risk, screening for occult primary cancers, differentiating
benign from malignant outcomes or one form of malignancy from another, evaluating
diagnosis, prediction for cancer patients and monitoring of disease status, either to identify
recurrence, determine response or progression to therapy  (Henry & Hayes, 2012).

In order to evaluate an individual’s risk of developing cancer, biomarkers have also been
identified (Hanash et al., 2011). A female with a clear family history of breast cancer, for
example, could be genetically screened to decide if she is a carrier of a germline mutation, such
as BRCA1, which raises her risk of developing breast cancer. If so, in order to minimize her
risk of developing malignancy, she could opt for more rigorous screening, tamoxifen
chemoprevention or prophylactic bilateral mastectomy. Biomarkers can help determine the
prognosis or probability of disease recurrence independent of treatment in patients who have
been diagnosed with cancer (Ludwig & Weinstein, 2005).

For a particular treatment, or to assess which treatment is likely to be most successful,
biomarkers may be used as response modifiers, or predictive factors, (Hayes, 2015). However,
HER2- gene expression or gene amplification in breast cancers predicts response to anti-HER2 agents such as trastuzumab whiles estrogen receptor expression in breast cancer
predicts response to anti-endocrine treatments such as tamoxifen. It may also be used to
monitor response to treatment in the metastatic setting (Barault et al., 2018). For monitoring
response to palliative therapy in metastatic colorectal, prostate, ovarian, breast, and pancreatic
cancers, circulating soluble protein tumour markers such as CEA, PSA, CA125, the MUC-1
antigens, CA15-3, CA27.29 and CA19-9 are recommended (Boeckx et al., 2018).

More recently, to determine the prognosis of an individual’s tumour, newer approaches are
being used. For instance, there are varieties of signatures of gene expression in breast cancer
that have been established and may be used to evaluate diagnosis for an individual patient based
on tumour evaluation and circulating cell-free DNA is an example (Chin et al., 2019).
Circulating tumour cells have been revealed to be predictive for overall survival in metastatic breast cancer setting. CfDNA also serves as prognostic and diagnostic marker in cancers particularly in patients with breast cancer. However, little knowledge has been produced on the usefulness of cfDNA for predicting response to treatment and monitoring breast cancer treatment. It is now the priority of majority of investigators across the nation.

2.13 History of circulating cell-free DNA (cfDNA)

Circulating cell-free DNA (cfDNA) was first discovered in 1948 by Mandel and Metais in the human plasma of normal healthy individuals (Mandel & Metais, 1948). In 1977, Leon identified that in cancer patients circulating cell-free DNA also occurred (Leon et al., 1977). This finding was first made in patients with Lupus and later it was identified that cfDNA concentrations are higher in more than half of patients with cancer (Leon et al., 1977). Molecular study of cfDNA has also led to a significant discovery that cancer patients’ blood plasma DNA includes tumour-associated mutations and may be used for cancer diagnostics and follow up. In noninvasive cancer detection, the capacity to derive cfDNA from the human plasma and serum has led to enormous advances (Aarthy et al., 2015). It has contributed, most importantly, to what is now identified as liquid biopsy. A concept similar to tissue biopsy is liquid biopsy. It is a scientific way of detecting the cells and free DNA that enter body fluids to study the nonsolid biological tissue (Ranuncolo, 2017).

Liquid biopsy denotes to the real-time checking of the identification of circulating tumour cells, cfDNA and exosomes and also complex disease alterations. As a method for primary disease detection, real-time monitoring and development, curative observation and assessment outcome, prognosis evaluation, metastasis risk enquiry, using biomarkers & cancer cells in the blood as a way of detecting cancer form and stage. This technique has great application value (Aarthy et al., 2015).
2.14 Circulating cell-free DNA (cfDNA)

Circulating cell-free DNA is a degraded DNA fragments released into the bloodstream of humans from cells and possibly all eukaryotes. In the blood, these degraded DNA fragments circulate. CfDNA has also been found in different body fluids, in addition to the blood circulation method, comprising of urine (Illanes et al., 2006), saliva (Mithani et al., 2007), pleural fluid (Sriram et al., 2012) and cerebrospinal fluid (De Mattos-Arruda et al., 2015). A combination of extracellular nucleic acid fragments resulting from cell necrosis, apoptosis, and energetic release of DNA is cfDNA (Stroun et al., 2000). The modal length of cfDNA corresponding to the unit size of a nucleosome has been determined as around 160–180 bp.

Nevertheless, Stroun and associates detected in 1989 that, cfDNA comprises of tumour DNA derived from the cancer cells in the plasma of cancerous people, thus increasing the quantity of circulating cell-free DNA. Higher number of genetic and epigenetic changes in cfDNA have subsequently been identified (Schwarzenbach et al., 2011). Additionally, the amount of cfDNA in the state of infection (De Vlaminck et al., 2015), tissue injury (Rainer & Lam, 2006) or cancer (Leon et al., 1977) may be increased.

Recent research have shown that, cfDNA has possible biological purposes such as cell transformation stimulation and recipient cell tumourigenesis (Garcia-Olmo et al., 2010) and the initiation of neutrophil discharge into the blood to clear infected bacteria (Gould et al., 2016).

Liquid biopsy has gained increasing attention compared to solid biopsy. It is easy, noninvasive and easily repeated by detection of cfDNA. The identification of cfDNA as a therapeutic biomarker has greater benefits.
The benefits of cfDNA as a clinical biomarker are that, it is possible to identify intra-abnormalities in cfDNA at an earlier level, allowing quick detection of the illness. In contrast to traditional serum markers, circulating cell-free DNA is also a very capable tumour biomarker for cancer detection and monitoring, prediction evaluation, and personalized drug control. And cfDNA identification makes regular sampling possible for the disease progression, drug response and prognostic monitoring.

2.15 Origin of circulating cell-free DNA

Circulating cell-free DNA is a fragment of degraded DNA which is released into the bloodstream and circulates in the human blood. Through apoptosis and necrosis, circulating cell-free DNA is thought to derive from dead cells (Dobrzycka et al., 2010). The source of circulating cell-free DNA however, is divided into groups. They are the cfDNA origins and causes of the release of cfDNA.

2.15.1 Sources of circulating cell-free DNA

In humans and all eukaryotes, there are many sources of circulating cell-free DNA. Necrosis, apoptosis, pyroptosis, and netosis are some of them.

2.15.1.1 Necrosis

Necrosis is one of the origins of cfDNA. Necrosis happens as an involuntarily death of cells during which the cellular capacity for energy production and renewal is gone. It result in swelling of cells and organelles, fragmentation, clumping of nuclear chromatin and non-specific digestion that causes DNA to be released into the blood (Holdenrieder & Stieber, 2009). Other types of controlled or programmed necrosis occur as well. They are necroptosis (this form of necrosis is triggered by death receptors, containing tumour necrosis factor (TNFR) and first apoptosis signal receptors (Fas), with necrosis-like cell fragmentation and pyro necrosis (which is triggered) (Yang et al., 2009).
Necrosis happens more quickly than apoptosis, but necrotic cell removal is slower and the resulting fragments of DNA released into the bloodstream from necrotic cells are usually much larger than apoptotic fragments of DNA (Suzuki et al., 2008). In-vitro experiments have showed that the release of necrotic DNA fragments into human’s bloodstream involves phagocytic clearance (Choi et al., 2005).

2.15.1.2 Apoptosis

One of the origin of circulating cell-free DNA is apoptosis. Numerous studies show that in both normal and diseased tissues, apoptosis acts as the key source of cfDNA (Delgado et al., 2013).

However, the internucleosomal cleavage of chromatin through caspase-activated DNase (CAD) involves a fundamental apoptotic process mechanisms that contribute to the presence of cfDNA which analytically cleaves chromosomal DNA into multiples nucleosomal fragments of 160 to 180 bp. In addition, after electrophoresis and sequencing of cfDNA, a distinct ladder pattern close to that of apoptotic DNA fragments, is evident, suggesting too many that, apoptosis could be the key source of cfDNA (Del Re et al., 2019).

Conversely, even in tissues with high cellular turnover due to highly efficient clearance mechanisms, the presence of apoptotic cells is short-lived (Hochreiter-Hufford & Ravichandran, 2013). In addition, the discovery of newly synthesized DNA’s active release also disputes in contradiction of the principal source of cfDNA being apoptosis (Nahata et al., 2013). Apoptotic cell phagocytosis is regarded as a non-immunogenic and non-inflammatory mechanism with limited recruitment of neutrophils requiring recognition sufficient acknowledgement, clearance and fragmentation of apoptotic cell content to sustain a defensive environment against unrestrained inflammation and potential autoimmunity (Hochreiter-Hufford & Ravichandran, 2013).
Over time, apoptotic cells that are not eliminated by phagocytosis leak cell content, leading to inflammation, self-antigen and intolerance to disturbance (Hochreiter-Hufford & Ravichandran, 2013). Autoimmune reactions may result in the release of self-antigens with increased immunogenicity through the cleavage, cross-linking and/or relocation of proteins during apoptosis and if apoptosis is the primary source of cfDNA then, persistent symptoms in cancer and any other conditions with increased levels of cfDNA will therefore be inflammation and autoimmune reactions (van der Vaart & Pretorius, 2007). Moreover, inflammation and autoimmune reactions in pregnant mothers or in subjects undergoing routine exercise are not frequent or common symptoms indicating that apoptosis is not likely to be the key contributor to cfDNA (Aucamp et al., 2018).

In addition, despite treatment-induced cell death, tumour radiation therapy does not increase levels of cfDNA (van der Vaart & Pretorius, 2007). Therefore, this lack of increased cfDNA levels may be due to radiation therapy causing tumour cell proliferation to be hindered rather than killing the cells (Deligezer et al., 2008). With a concomitant increase in cfDNA, some cancer cells can resist apoptosis and continue to proliferate (van der Vaart & Pretorius, 2007). In several human pathologies where augmented levels of cfDNA are observable, including autoimmune disorders, respiratory diseases, cardiovascular diseases, atherosclerosis, sepsis and septic shock, neurological conditions and cancers defects in apoptotic cell clearance and elevated cell death are assumed to play a role signifying that apoptosis may be a more probable cause of cfDNA in some diseases (Hochreiter-Hufford & Ravichandran, 2013).

### 2.15.2 Causes of Circulating Cell-Free DNA Release

There are numerous causes of cfDNA release into the bloodstream, some of which are DNA release from dead or dying cells mediated by phagocytosis, autophagy, aging, and DNASES action, oxidative stress, exercise and cancer.
2.15.2.1 Phagocytosis-mediated DNA release from dead or dying cell

In vivo studies have revealed that, unless macrophages are present, DNA is not detectable in blood samples with apoptotic and/or necrotic cells, implying that a blood DNA response allows dead and dying cells to associate with phagocytes (Pisetsky, 2012). The phagocytes either digest the cells and liberate fragments of cleaved low molecular weight DNA or fail to do so, causing the phagocytes to die and discharge into the bloodstream their DNA and the DNA of the ingested cells (Pisetsky, 2012). The necrotic behaviour or presence of apoptotic cells results in changes accompanied by increased DNA release in the interactions of these cells with macrophages (Choi et al., 2005). The clearance of necrotic cells through macrophages results in cfDNA release.

2.15.2.2 Autophagy (clearance of cell degradation)

Macroautophagy is the sequestering into double-membrane vesicles of portions of the cytoplasm that merge with lysosomes to facilitate degradation of their contents (Rello-Varona et al., 2012). It is a mechanism primarily reserved for the regulation of the consistency and turnover of cytosol or cytoplasmic organelles in mammalian cells, while at the same time sparing nuclei. Autophagic nuclei removal can be lethal to cells or lead to euploidy, while multinuclear cell nuclear macro autophagy is yet to be established. By autophagy (mitophagy) however, mitochondria can be removed, which may result in the release of mtDNA that also forms part of cfDNA into the plasma (Oka et al., 2012). As with NETosis, in the early stages of tumourigenesis, autophagy can play a tumour-suppressive role and a tumour-supporting role in later stages (White et al., 2015). Throughout the later phases of tumourigenesis, inhibition of autophagy can end in cell death via apoptosis and tumour regression. However, on the other hand, autophagy suppression in healthy tissues impairs cell survival under metabolic stress that promotes augmented DNA damage, amplification of gene and aneuploidy which can promote tumourigenesis in turn (de Bruin & Medema, 2008). Therefore, autophagy cannot
actually be a source of cfDNA by direct nucleus degradation, but may indirectly facilitate the release of cfDNA by either destroying cells with damaged organelles and proteins, shielding existence tumour from stressful situations or by helping the production of tumour and DNA damage when suppressed in healthy cells.

2.15.2.3 Aging

It was found that total repeat levels of cfDNA, unmethylated cfDNA, RNase P-coding cfDNA and ALU repeats differ between nonagenarians and young controls. Thus, with increased cellular senescence and death, as well as decreased clearance and phagocytic capabilities, cfDNA levels can increase with age (Jylhava et al., 2011). Though, cfDNA function and pathological roles are still vague, higher concentrations of cfDNA can increase blood viscosity and become immunogenic that are prevalent in elderly individuals (Jylhava et al., 2012).

Excitingly, Jylhava et al. (2012) showed that, total and non-methylated cfDNA levels represented overall frailty. Lower body strength, cognitive impairment and reduced daily functioning and mobility capabilities were found to be correlated with higher total cfDNA levels and unmethylated cfDNA (Zhang et al., 2019).

2.15.2.4 Action of DNAses

High levels of DNase activity are followed by low cfDNA levels in healthy subjects and vice versa in patients with cancers and inflammatory illnesses (Velders et al., 2014). In healthy subjects after training, substantially increased cfDNA levels are also subsequently decreased by changes in the behavior of endogenously expressed DNase to restore homeostasis (Velders et al., 2014). However, the DNase source will modify the results from cfDNA clearance to the release of cfDNA. In bacteria, fungi and other pathogens, NET-resistance is mediated by extracellular release of DNase (exDNase) (Buchanan et al., 2006). ExDNase results in the breakdown of the NETs DNA structure, not only enabling the release and
systemic dispersal of the pathogens (Hawes et al., 2015) but also contributing to the release into circulation of NET-DNA fragments. Therefore, NET-resistant pathogens in patients with infections can function as both exogenous and endogenous sources of cfDNA. A number of factors have not yet been determined with regard to the cfDNA-clearing functions of DNase, whether endogenous DNA fragments resulting from DNase activity (e.g. NET-DNA fragments) have physiological or damaging effects.

Whether cfDNA from endogenous sources other than cellular breakdown mechanisms (apoptosis, necrosis) and ETs may also be degraded by DNase release, particularly actively released DNA with possible physiological purposes and whether the breakdown of functional DNA segments may have indirect negative effects (Gahan & Stroun, 2010). The resolutions of these enquiries can provide valuable perceptions of cfDNA biological purposes. DNase-mediated degradation may be unlikely, with regard to actively released DNA since the complexation and encapsulation of this DNA segment may avoid DNase-mediated fragmentation (Ignatiadis et al., 2015).

2.15.2.5 Oxidative stress

In several pathologies, comprising of cardiovascular illness, cancer, neurological conditions, diabetes, arthritis, aging and sepsis oxidative stress has been involved (Galley, 2011). In various pathological conditions, it may serve as a source of cfDNA release in the form of both normal and oxidized nuclear, mitochondrial and apoptotic DNA fragments. Oxidative stress-mediated destruction to mtDNA lead to a cycle of development of reactive oxygen species (ROS) and further damage, causing apoptosis, and subsequent mitochondrial disaster or toxic oxidative stress called cell death. Oxidative stress in the mitochondria leads inside the NET-like structures of both released genomic DNA and mtDNA (Lood et al., 2016).
2.15.2.6 Exercise

A leukocyte inflammatory reaction, mechanical and metabolic muscular damage and DNA damage can be associated with high levels of physical activity due to oxidative stress that raises the levels of cfDNA. Tug et al., (2017) indicated that cfDNA release could also be independent of inflammatory markers such as leukocyte oxidative burst, and leukocyte or muscle cell apoptosis throughout apoptosis. These flaws can suggest that, as seen in a comparison of chronic and acute exercise, different types of exercise cause dissimilar methods of cfDNA release (Helmig et al., 2015). The large and speedily inconsistent concentrations of cfDNA resulting from acute exercise provides essential insights into the production of cfDNA study sample-collection guidelines (Frühbeis et al., 2015).

It is obvious that, ambient temperatures and levels of physical activities or energy (e.g. exercising before hospital appointment, walking and/or climbing stairs, and whether the subject has enough energy prior to blood sampling for physical activities) will influence the levels and characteristics of cfDNA collected (Frühbeis et al., 2015). This may lead to cfDNA background levels resulting from cell injury and/or immunological actions.

2.15.2.7 Cancer

In vitro DNA synthesis pattern (induced by carcinogenic drug or chemical exposure) of cfDNA was determined by Stroun et al. (1989) from seven malignant patients. In vitro DNA synthesis patterns typical of neoplastic DNA were increased in five patients, while the remaining two cases showed no improvement in synthesis. This absence may be due to the presence of DNA generated in response to the malignant cells from non-malignant host cells from the immune system. Additional, cfDNA levels were also found to vary between metastatic patients and localized tumour patients (Gahan et al., 2008). However, in cancer patients,
cfDNA can originate from two sources and tumour cells and the surrounding tissue cells are the sources (Alix-Panabieres & Pantel, 2016).

Cell senescence is considered a normal cancer defense mechanism that facilitates oncogenic cell immune clearance (Chandler & Peters, 2013). In-vitro studies have shown that cancer cell genetic alterations can allow cells to escape senescence (Watanabe et al., 2017). Increased cfDNA levels in cancer patients, may be involved in the evasion of senescence and growth of polyploidy cancer cells due to certain genetic changes. Additionally, knockout of retinoblastoma protein tumour suppressors enables some of the cells to continue synthesizing DNA into cells undergoing RAS-induced senescence (Chandler & Peters, 2013).

The cells did not however, increase in number and were polyploidy, suggesting the existence of other tumour proliferation barriers. Senescence can paradoxically encourage tumourigenesis, perhaps through the secretion of growth factors and cytokines by matrix metalloproteases (MMPs,) (Aravinthan, 2015). In cancer “patients, there are also conflicting findings concerning the sources or causes of higher circulating cell-free DNA concentrations, specifically with regards to cell death/degradation involvement.

These inconsistencies are probably due to the complexities of the pathophysiology involved in multiple cancers. Instead of treating each type of cancer as a separate pathological disease, researchers are attempting to generalize cfDNA release mechanisms to cancer in general. For instance, cancers that vary in tissue origin, malignancy and whether or not metastases may be involved, produce distinct levels of cfDNA from either the cancer cells themselves or the host cells that are not malignant” (Haller et al., 2018).

Contradictions between cancers must therefore be predicted. Finally, because of lack of existing standard operating procedures like choice of sample (e.g. serum versus plasma),
sample collection, storage and extraction, processing, cfDNA categorization and experimental
differences, discrepancies between research methods could seriously complicate the
elucidation of the origins and functional functions of cfDNA (Bronkhorst, Aucamp, &
Pretorius, 2016).

2.16 Clearance of circulating cell-free DNA (cfDNA)

A balance between DNA release and DNA clearance processes influences the amount of
extracellular DNA in the circulation. In the home tissue, blood or other body fluids and
organs, such as the liver, spleen, kidney, or lymph nodes, circulating cell-free DNA clearance
can occur (Leung et al., 2016). As apoptotic cells and cfDNA are easily cleared, healthy
individuals have low levels of circulatory cfDNA. Clearance is inadequate for malignancies,
chronic inflammation, or excessive cell death, and cfDNA accumulates. The association
between high levels of cfDNA and pathological conditions may be clarified by inadequate
clearance. The exact mechanisms of accumulation of cfDNA remain unclear, but it can be
hypothesized that the clearance system may be overloaded by excess of dying and that, surplus
cell content is released into the medium.

In circulating blood, the approximate half-life of cfDNA ranges from several minutes to 1–2
hours (Celec et al., 2018). Excitingly, clearance from maternal blood of fetal DNA happens
in a bi-phasic way. Primarily, with a mean half-life of ~10 minutes to 1 hour a rapid phase
takes place, then a second slow phase takes place with a mean half-life of ~13 hours. In a pre-
clinical rabbit model of head and neck cancer, the half-life of circulating tumour DNA stage
after surgical tumour resection was 23–52 minutes (Muhanna et al., 2017). Sequences of
analysis of ctDNA in colorectal cancer person demonstrated a half-life of 114 minutes (Diehl
et al., 2008). However, for real-time studies of cfDNA, the short half-life of cfDNA is
convenient, enabling treatment response assessments and complex tissue status assessments in different pathophysiological situations such as, tissue damage or regeneration.

Circulating cell-free DNA half-life relies on different aspects, containing its interaction with molecular complexes that avoid quick fragmentation of cfDNA, the form and stage of the tumour and the modality of treatment (Thierry et al., 2016).

CfDNA “degradation in the blood is basically passed out by circulating enzymes like DNase I, protease activating plasma factor VII and factor H (Martin et al., 2016). The amount of DNase I was inversely associated with cfDNA concentrations in cancer patients (Cherepanova et al., 2008).

In the liver, spleen, and kidney, cfDNA removal occurs (Butler, Spellman, & Gray, 2017). For nucleosome clearance, the liver is the key organ. Within 10 minutes, 71.0% to 84.7% of nucleosomes are eliminated from circulation (Gauthier et al., 1996). It has been shown that, Kupffer cells in the liver and spleen macrophages are accountable for trapping and clearing DNA and nucleosomes (Clos, 1999). Kidney extracellular DNA elimination studies have shown that, the clearance rate of naked DNA through glomeruli is dependent on the size of the DNA. Also, after 24 hours, short fragments (160–200 bp) were present in the kidney but longer fragments (2–6 kb) were not identified (Celec et al., 2018).

The data indicated that the DNA clearance of the kidney might be selective, but that study didn’t fully reflect the in vivo situation, as cfDNA is predominantly double-stranded, coiled around histones, or bound to other multimolecular complexes. Furthermore, animal studies have revealed that chronic renal failure is connected with low cfDNA absorption and low cfDNA plasma levels, indicating that the kidney only partially participated in cfDNA clearance (Celec et al., 2018). Additionally, the kidney has moderate-to-high deoxy
ribonuclease and urine has the uppermost enzyme activity. Such properties may explain why urine samples have low concentrations of DNA and high fragmentation of DNA.

The three main components of cfDNA life are presented: release, biological activity, and clearance. Although naked nuclear cfDNA is studied in most clinical settings, it moves in body fluids in different forms: free within exosomes, bound to histones (nucleosomes), covered by transcriptional factors or as part of immune-related components (such as NETs). Circulating cell-free DNA is not a passive pathophysiological biomarker, but plays an active role in various procedures such as inflammation, immunomodulation and promotion of tumour formation.

2.17 Circulating cell-free DNA in cancers

Alternative approaches to gain understanding into the molecular structure of cancer cells are the capturing and examination of circulating biomarkers (Rapisuwon et al., 2016). Leon et al. (1977) first demonstrated the correlation of circulating cell-free DNA and cancer whereby cfDNA was enhanced in cancer patients, after several researchers performed in-depth studies of this biomarker (cfDNA). Cancer cells leads to uncontrolled cell proliferation as lots of cells are formed within a short period of time. Therefore, a percentage of cfDNA in cancer originates from tumour cells, known as circulating-tumour DNA, increasing the rates of cfDNA in them (Wan et al., 2017).

Several investigators have reported that, in cancer patient’s tumour, circulating cell-free DNA is obvious and their levels are also increased. Circulating cell-free DNA profiling has recently become a field of growing clinical interest in oncology, in particular due to advances in molecular biology technique sensitivity, as this enables the detection and monitoring of tumour mutations in a non-invasive way (Stewart & Tsui, 2018). This has also opened up new opportunities to track the growth of tumour and to gain resistance, predictive or diagnostic method as well as to direct management choices when tumour biopsy tissue is ineffective or
inaccessible (Perakis et al., 2017). In oncology, clinically validated cfDNA tests are starting to appear, and this is likely to develop extensively for monitoring patient’s response and molecular stratification (Stewart & Tsui, 2018). There are several types of cancers where there is elevated cfDNA in them. Some of which are endometrial cancer, ovarian cancer, hepatocellular cancer and breast cancer.

2.17.1 Endometrial cancer (EC)

The most prevalent malignancy in the female genital tract is endometrial carcinoma, making it the fourth most common cancer in women (Ronnett et al., 2002). When epithelial cells lining the myometrium begin to multiply unusually, this tumour then initiates in the inner layer of the uterus (Van den Bosch et al., 2012). Though the diagnosis of EC is usually strong, numerous advanced stage cases are detected. These cases are typically high-grade carcinomas that are eventually more probable to persist and linked with increase mortality (Tsikouras et al., 2013). Nevertheless, in EC patients, surgery is the main therapy. In addition, patients also undergo adjuvant radiotherapy, along with minimal chemotherapy. Relative to other cancers, chemotherapy and radiotherapy procedures are costly and less effective (Morice et al., 2016).

This review describes the clinical encounter of discovering fresh molecular targets and biomarkers that can assists in early detection, choosing individual management regimen, disease monitoring and therapy response as useful resources to enhance our endometrial cancer management (Muinelo-Romay et al., 2018). Circulating cell-free DNA (cfDNA) was one of the biomarkers tested to see whether, it can assist in the early detection, monitoring and reaction to EC therapy as it was raised in patients with endometrial cancer (Cabel et al., 2018).

One of the investigators, whose work concentrated on cfDNA in endometrial cancer was Dobrzycka. He proposed that, the utility of cfDNA could aid as a biomarker for monitoring, predicting EC diagnosis and choosing individual endometrial cancer treatment schedules
(Dobrzycka et al., 2010). Tananka et al. (2012) also investigated cfDNA in EC patients and noticed that, variations of cell-free DNA in patient may be a prognostic EC biomarker and his statement was supported by Cicchillitti et al. (2017) who stated that patient’s data suggested that, cfDNA concentrations in blood sera can aid clinical management and prognosis in patients with endometrial cancer. As a prognostic factor in endometrial cancer, cfDNA levels also have potential (Casas-Arozamena et al., 2020). Circulating cell-free DNA offers a more suitable method for monitoring the progress of disease, helping to determine the response of EC patients to treatment.

2.18 Circulating cell-free DNA in breast cancer

Breast cancer is the most frequently diagnosed cancer accounting for 23% of overall cancer cases and 14% of cancer deaths and the leading cause of cancer death in women worldwide (Bray et al., 2018). Despite the rising incidence, over the past decade, mortality from breast cancer has decreased. A large percentage of the decline in mortality is due to early diagnostic techniques, such as modern digital mammography. Mammography misses many breast cancers and doubtful lesions over diagnosis, resulting in needless biopsies and emotional torment (Jørgensen & Gøtzsche, 2009). 13% of breast cancers are often unnoticeable by mammography and are affected by patient’s tumour size and age. Presently, biomarkers of unsatisfactory accuracy such as cancer antigen CA15-3 and carcinoembryonic antigen (CEA), have been discouraged for precise diagnoses of breast cancer (Jørgensen & Gøtzsche, 2009).

As such, markers that can improve current screening protocols and differentiate between benign and malignant disease are strongly required to avoid overtreatment. There has been a great deal of interest in separating benign from malignant disease by using cfDNA. New technologies with increased sensitivity and precision for the detection and diagnosis of breast cancer are also in serious demand. The discovery of alternations in cfDNA present in patients
with breast cancer has resulted to multitude of research analyzing the genetic and epigenetic character of these alterations. Several studies have focused on quantifying the levels of cfDNA and using elevated levels to differentiate between benign and malignant, given that cfDNA levels increase in cancer. Some researchers have reported substantially increased levels of cfDNA in breast patients (Kohler et al., 2009; Skvortsova et al., 2006).

All of these studies have identified meaningfully higher levels of cfDNA in breast cancer patients and as a result, numerous works have discussed the possible utility of circulating cell-free DNA assays as a repeatable and non-invasive liquid biopsy for breast cancer to see if it can be used in breast cancer patients for prognostic and diagnostic use. One of the researches performed by Yu et al., 2019 showed that, cfDNA concentration has a possible first diagnostic value for breast cancer and could be a good source of breast cancer detection (Yu et al., 2019). Another study suggested that, for breast cancer, cfDNA has diagnostic and prognostic utility (Arko-Boham et al., 2019). Hussein et al. (2019) suggested that, preoperative prognostic markers for breast cancer appear to be cfDNA (Hussein et al., 2019).

Moreover, after surgery and radiation therapy, more recent studies in breast cancer patients have also found a decrease in cfDNA concentrations (Lehner et al., 2013). A decrease in level of cfDNA after radiation therapy and a decreases in the level of DNA is associated with better clinical condition (Leon et al., 1977). This has incited further research into the use, as defined, of cfDNA as a marker of treatment response. The use of cfDNA as a biomarker of treatment response in patients with breast cancer has been further studied by some researchers (Olsson et al., 2015).

Even, despite curative therapy, numerous patients with breast cancer still relapse and how to identify those with a high risk relapse remains a severe issue. While early breast cancer is a curable disease, after treatment, up to 40% of such patients still relapse (Davies et al., 2011).
And now investigators have studied how to differentiate patients with high relapse risk of early breast cancer from those with low relapse risk. A study found that, prior to neoadjuvant therapy, the presence of circulating cell-free DNA associates with poor prognosis and ctDNA can also help classify high-risk patients for treatment intensification (Li et al., 2020).
CHAPTER THREE

METHODOLOGY

3.1 Study design

A prospective case-control study was carried out at the Breast Unit of the Department of Surgery at the Korle-Bu Teaching Hospital (KBTH).

3.2 Study site

The study was conducted at the Breast Unit of the Department of Surgery at the KBTH. KBTH lies in the Greater Accra region between longitude 0° 13’ 23.20” East and latitude 5° 32’ 9.71” North. It is the core of excellence for healthcare. It is also the University of Ghana’s leading teaching hospital and several health bodies in the Greater Accra region of Ghana. It serves as the country’s leading national referral center and is currently Africa’s third largest hospital. There is also a Breast Unit at the Surgical Department at the KBTH. It is a new well-equipped testing facility situated at the epicenter of the hospital where patients with breast cancer receive care. Accessible data from the KBTH Breast Unit of the Surgical Department showed that, an average of four newly diagnosed breast cancer patients are referred to the Breast Unit on a weekly basis. Patients of different socio-economic and ethnic background are received by the unit.

3.3 Study participants

Patients with breast cancer were randomly recruited from the Breast Unit of the Department of Surgery at the KBTH. Apparently healthy women without breast cancer were recruited as controls for each breast cancer patient recruited.

3.4 Inclusion criteria

- Female breast cancer patients willing to partake in the research.
• Newly diagnosed patients with breast cancer who were naïve to chemotherapy, radiotherapy and surgical treatment.

• Breast cancer patients who were due for chemotherapy.

3.5 Exclusion criteria

• Breast cancer patients who are undergoing radiotherapy, surgery and other adjuvant treatments.

• Breast cancer patients who are diagnosed with other types of cancer.

• Breast cancer patients that are still on cancer chemotherapy at recruitment time.

3.6 Ethical approval

Approval for the conduct of the study was given by the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana (Reference Number: EPRC/DEC/2019, Protocol Identification Number: CHS-Et / M2-5.3 / 2019-2020). Participation of this study was made absolutely voluntary and participants were granted the right to withdraw from the study without consequences at any time. Before their inclusion in the study, informed consent was obtained from the participants both verbally and in writing.

3.7 Sample size

The sample size was determined according to the following formula (Berkowitz & Lynch, 2015).

\[ N = \frac{2(za + z\beta)^2}{\Delta^2} \]

For \( \alpha = 0.005 \), \( za = 1.96 \); for \( \beta = 0.20 \), \( z\beta = 0.84 \). Hence \( 2(za + z\beta)^2 = 2(1.96 + 0.84)^2 = 15.68 \) (\( \beta \) as the probability of making a Type II error and \( \alpha \) as a probability of making a Type I error).
$z_\alpha =$ A standardized standard deviate value that relates to a level of statistical significance equivalent to 1.96.

$z_\beta =$ Probability of detection or power (80%).

$N =$ Entire number of participants.

$\Delta =$ Effect Size

Effect sizes are small, moderate and large (0.2, 0.5 and 0.7 for two-group comparisons) as a guide by Jacob Cohen- Statistical Power Analysis for the Behavioral Sciences, Revised Edition).

$N = 15.68/ (0.7)^2 = 32$

$N = 32$ per group, hence a total of 64 subjects were required for the study.

3.8 Participant selection

Participants for the study were recruited by simple random sampling method at the Breast Unit of the Department of Surgery at the KBTH. Newly diagnosed breast cancer patients attending the Breast Unit of the KBTH between December, 2019 and March, 2020 who agreed to be part of this study were included. Participants who were first diagnosed with breast cancer based on relevant diagnostic and laboratory tests were newly diagnosed patients and had not yet been put on any sort of medical treatment. The Breast Unit is visited every week by an average of four newly diagnosed breast cancer patients. In order to determine their age, health and breast cancer status upon approval, identified potential women were screened through an interview guide. This research enrolled 32 patients with breast cancer.
In all, thirty-two (32) apparently healthy controls were selected for the study. Participants were only recruited after they had consented by completing an informed consent form in Appendix III.

3.9 Anthropometric parameters

3.9.1 Waist to hip ratio (WHR)

Waist-to-hip ratio is defined as the ratio of the circumference of the waist to that of the hip. In order to ensure the reliability and reproducibility of the measurements, special care was taken. For the waist circumference, a calibrated tape measure was used to measure the approximate midpoint between the lowest rib and the iliac crest in a horizontal plane. The calibrated tape was snug but not pulled so tightly around the body that, it was constricting. Participants (patients with breast cancer and apparently healthy controls) stood with feet placed closed together with weight distributed evenly across the feet.

The applicants also stood with feet positioned closed together and weight spread across the feet for the hip measurement. The calibrated tape was snug for the hip circumference at the widest region of the hip at the greatest protuberance of the buttocks. By using the formula (Waist circumference (cm)/ Hip circumference (cm)), the WHR was determined.

3.9.2 Body mass index (BMI)

Body Mass Index is an estimation of the fat of the body based on the weight in kilograms divided by the square of height in meters. A calibrated scale was put at a zero mark on a leveled and uncarpeted surface with the calibrated scale. Participants (both patients with breast cancer and apparently healthy individuals) were asked to do away with their shoes and any additional heavy outer wear. On the calibrated scale, they stood motionless at the middle with their feet slightly apart and body weighted evenly distributed on both feet. Arms were relaxed and
loosely hanged at the sides of their bodies and on the calibrated scale, the weight was read and documented.

Participants stood with their shoes off and their feet together next to a stadiometer. The head was leveled to the external auditory meatus below an imaginary line from the lower boundary of the eye orbit. The participants stood straight and recorded their heights. The BMI was calculated by using the formula $(\text{Weight (kg)}/ \text{Height (m}^2))$.

![Image of measurements](image)

*Figure 2: A: Measurement of the waist circumference; B: Measurement of the hip circumference.*

### 3.10 Blood sample collection and serum separation

Venous blood samples were taken at two different time points from the breast cancer patients. Time point one was before the commencement of treatment when the breast cancer patients were not exposed to chemotherapy. Time point two was the twelve weeks after the commencement of chemotherapy treatment. This period coincides with the third chemotherapy
cycle and it is usually the point when clinicians begin to assess patient’s response to treatment or the efficacy of the chemotherapeutic agent. Three months were used to recruit 32 newly diagnosed breast cancer patients without bias. Venous blood samples were also collected from the apparently healthy volunteers as controls.

At each time point, 5 mls of venous blood was collected from the median cubital vein of each participants into serum (gel) separator tubes. Fifteen minutes thereafter, each samples was centrifuged at 1000 g for 15 minutes. After centrifugation, a sterilized pipette was used to pipette the sera into sterilized Eppendorf tubes. The sera in the Eppendorf tubes were stored at -20°C until use.

3.11 Clinical-pathological parameters
Clinical-pathological information such as the age, grade and stage, menarche and menopausal status, molecular and histopathological subtypes of the breast cancer were obtained from the hospital folders of the breast cancer patients. Age, menarche and menopause status were also taken from the apparently healthy individuals. Other demographic data such as health history of the breast cancer patients were also obtained through questionnaires in Appendix II.

3.12 DNA concentration and estimation
3.12.1 Materials and reagents
Luna Universal qPCR Master Mix (# M3003S, Lot number: 10056087, Amount: 200 reactions), ALU 115, ALU 247 and B-Actin Primers (Both forward and reverse) (all from Inqaba Biotec Ltd), Nuclease Free or Distilled Water, Vortex, Sterile Pipette Tips, Pipettes, Multichannel Pipette, Template DNA, 96 Sterile Well Plates, Optically Transparent Films, Centrifuge, Heating Block, Quant Studio 5®, Tween-20, Tris Buffer, Ethylenediaminetetraacetic acid (EDTA), Proteinase K, Eppendorf Tubes and Beaker.
3.12.2 Sample preparation for quantitative PCR (qPCR)

At room temperature on ice, frozen serum samples were thawed. According to the technique described by Iqbal et al. (2016), the sample preparation for qPCR was performed. A preparation buffer was formed containing 2.5% Tween-20, 50 mmol/L Tris and 1 mmol/L Ethylenediaminetetraacetic acid (EDTA). Twenty (20) µl of the preparation buffer was applied to each 20 µl of the serum sample pipetted with sterilized micropipette to deactivate proteins bound to the DNA template that could invalidate the results of qPCR.

Subsequently, 20 µg of Proteinase K (Inqaba Biotec, South Africa) was added to the mixture for protein digestion. Brief centrifugation was performed on the mixture to make sure that, every solution was at the bottom of the Eppendorf tube. The mixture was then placed in a heating bath for 56 °C for 50 minutes, followed by heat inactivation at 95 °C for 5 minutes. The mixture was centrifuged at 10,000 g for 5 minutes to obtain the supernatant. 0.5µl of the supernatant was used as template in the reaction mixture for each sample.

3.12.3 Quantitative PCR (qPCR) conditions and quantification of ALU fragments

For each direct qPCR, the reaction mixture consisted of 0.5 µl of template sequence, 0.5 µl of forward and reverse primers each, for ALU115 and ALU247, 6.25 µl of the master mix (LUNA MASTERMIX) and 4.75 µl of distilled water together making up a total reaction volume of 12.5 µl. The reaction mixture was performed in a sterile 96 well plate. Each reaction mixture was performed in triplicate in the sterile 96 well plate. All the procedures were performed on ice. After pipetting the constituents stated above into the 96 well plates, the plates were covered with an optical sterile transparent film (Figure 3). This was done properly by sealing the plate’s edges and corners to prevent artifacts caused by evaporation. The plates were centrifuged at 2,500 rpm for 1 minute in order to remove bubbles and collect liquid at the bottom of the wells of the plate.
Real-time qPCR amplification was performed on each plate with an initial denaturation at 95 °C for 60 seconds, followed by 45 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds and extension for 60 °C for 30 seconds using QUANTSTUDIO 5® REAL-TIME PCR. One hour, thirty minutes was the time used to run the real-time qPCR for each sterile 96 well plate. A standard curve with serial dilutions (10 ng – 0.01 pg) of gently prepared genomic DNA collected from peripheral blood leukocytes of healthy donor volunteers determined the absolute equivalent amount of DNA in each sample. The goal of using a healthy donor’s DNA from peripheral blood leukocytes was to act as an external standard. For each plate, a negative control (sample without targetable DNA) was performed.

A qPCR assay that uses primer sets was applied to amplify the consensus ALU sequences (ALU115 and ALU247) to achieve the highest sensitivity for DNA quantification. Two sets of
ALU primers were designed: the 115 bp amplicon (ALU115) primer set both amplified shorter (apoptosis truncated) and longer DNA fragments, while the 247 bp amplicon (ALU247) primer set amplified only longer DNA fragments. ALU115 primer sequences were forward: 5′-CCTGAGGTCAGGAGTTCGAG-3′ and reverse: 5′-CCCGAGTAGCTGGGATTACA-3′; ALU247 primers were forward: 5′-GTGGCTCACGCCTGTAATC-3′ and reverse: 5′-CAGGCTGAGTGCAGTGG-3′. For all qPCR assays, β-actin (endogenous or reference control) was used as normalizer. Forward: 5′-GACCTCTATGCCAACACAGT-3′ and reverse: 5′-AGTACTTGCGCTCAGGAGGA-3′ were the sequences of β-actin primers used. Mean values were calculated from triplicate reactions.

3.12.4 ALU 115 and ALU 247 concentrations

ALU 115 and ALU 247 concentrations were first determined by QUANTSTUDIO 5® REAL-TIME PCR. The concentrations were presented as Cycle Threshold (CT) values. CT is defined as the PCR cycle at which an arbitrarily placed threshold is crossed by the fluorescent signal of the reporter dye. It ensures that the PCR is in the exponential stage by presenting data as CT, and that the numerical value of the CT is inversely proportional to the quantity of amplicon in the reaction (i.e., the lower the CT, the greater the amount of amplicon). The CT values for both ALU 115 and 247 were normalized with β-Actin. The concentrations of the β-Actin was subtracted from both ALU 115 and 247 to get the normalized concentrations for both the cases and controls. The normalized concentrations for both ALU 115 and 247 were presented as delta CT (ΔCT). ΔCT is defined as the difference between the gene of interest (ALU 115 and 247) and the reference gene (β-Actin).

The ALU (115 and 247) concentrations were not normally distributed (non-parametric). Natural logarithm was performed on each of the ALU (115 and 247) concentrations values (ΔCT) to normal distributed values (parametric values).
3.12.5 Circulating cell-free DNA integrity determination.

The ratio of ALU247 concentration to ALU115 concentration was measured as the circulating cell-free DNA Integrity. CfDNA integrity characterizes the fragmentation patterns of the DNA. The values of ALU115 represent the total amount of free serum DNA that amplifies both shorter (apoptosis-truncated) and longer fragments of DNA. The ALU247 values are the total amount of DNA released from non-apoptotic cells, amplifying only longer fragments of DNA.

3.13 Statistical analysis

Demographic and clinical parameters were captured and validated using Microsoft Excel. Results of both ALU 115 and 247 were log transformed before undertaking any statistical analysis. This was because the untransformed gene expression values was not normally distributed, nonparametric and heavily skewed.

The results were then analyzed statistically using Statistical Package for Social Sciences version 20 statistical software for windows. All parametric data were expressed as mean and standard deviation. Statistical significance of the differences between three or more group means were performed by using one-way analysis of variance (ANOVA). Paired Student t-test was used to compare the differences between repeated measures (before chemotherapy and after the 3rd cycle of chemotherapy of the breast cancer patients). Differences in the study parameters between groups (breast cancer patients and apparently healthy controls) were assessed using unpaired student t-test. Spearman’s rank correlation coefficient was used to find association between ALU 115 and ALU 247 concentrations with age, BMI and WHR.
CHAPTER FOUR

RESULTS

4.1 Socio-demographic characteristics of study population

A total of 64 females, 32 breast cancer patients (cases) and 32 apparently healthy individuals (controls) were recruited into the study. The mean ages of the case and the control groups were found to be $62.38 \pm 13.32$ years and $49.31 \pm 12.74$ years respectively ($p = 0.0002$). The menarche index was statistically higher in the healthy individuals as compared to the breast cancer patients with mean ± SD of $16.38 \pm 1.31$ and $12.63 \pm 2.20$ respectively ($p < 0.0001$).

As indicated in Table 1, 5 (15.6%) out of the 32 breast cancer patients routinely drunk alcohol whereas 3 (9.4%) of the apparently healthy individuals did. Furthermore, 21 (65.5%) out of the 32 breast cancer patients and 9 (28.1%) out of the 32 healthy individuals were hypertensive. Twenty (62.5%) out of the 32 breast cancer patients and 5 (15.6%) out of the 32 healthy individuals were also diabetic. Breast cancer patients were found to show greater proportion of family history of breast cancer than the controls ($p < 0.00001$) (Table 1). Both groups recorded no smoking among participants.
### Table 1: Socio-demographic characteristics of study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Breast Cancer Patients (N=32)</th>
<th>Controls (N = 32)</th>
<th>95% CI of difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>62.38 ± 13.32</td>
<td>49.31 ± 12.74</td>
<td>6.56 – 19.58</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Menarche (yrs)</td>
<td>12.63 ± 2.20</td>
<td>16.38 ± 1.31</td>
<td>-4.65 – (-2.85)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Menopause (yrs)</td>
<td>47.45 ± 4.27</td>
<td>47.33 ± 4.55</td>
<td>-2.01 – 2.33</td>
<td>0.4172</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Breast Cancer Patients (N=32)</th>
<th>Controls (N=32)</th>
<th>Chi-square ($\chi^2$)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol intake:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5 (15.6%)</td>
<td>3 (9.4%)</td>
<td>0.5714</td>
<td>0.4497</td>
</tr>
<tr>
<td>No</td>
<td>27 (84.4%)</td>
<td>29 (90.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>No</td>
<td>32 (100.0%)</td>
<td>32 (100.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertensive:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>21 (65.6%)</td>
<td>9 (28.1%)</td>
<td>7.592</td>
<td>0.005*</td>
</tr>
<tr>
<td>No</td>
<td>11 (34.4%)</td>
<td>23 (71.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2DM:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20 (62.5%)</td>
<td>5 (15.6%)</td>
<td>12.866</td>
<td>0.0003*</td>
</tr>
<tr>
<td>No</td>
<td>12 (37.5%)</td>
<td>27 (84.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family History BC:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18 (56.3%)</td>
<td>1 (3.1%)</td>
<td>19.163</td>
<td>&lt;0.00001*</td>
</tr>
<tr>
<td>No</td>
<td>14 (43.7%)</td>
<td>31 (96.9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$N =$ study population, data is presented as mean ± standard deviation. Chi-square ($\chi^2$). *p-value is statistically significant, T2DM= Type2 Diabetes Mellitus, BC=breast cancer, CI=Confidence Interval

### 4.2 Anthropometric data of study population

As illustrated in Table 2, the BMI was statistically higher in the breast cancer group than the control group with mean ± SD of 31.03 ± 7.52 and 24.35 ± 5.19 respectively. The mean ± SD
of WHR was not statistically significant when the breast cancer patients were compared with the healthy individuals (control).

Table 2: Anthropometric data of study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Breast Cancer Patients (N=32)</th>
<th>Controls (N=32)</th>
<th>95% CI of difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>31.03 ± 7.52</td>
<td>24.35 ± 5.19</td>
<td>3.45 – 9.90</td>
<td>0.0001*</td>
</tr>
<tr>
<td>WHR</td>
<td>0.86 ± 0.06</td>
<td>0.85 ± 0.02</td>
<td>-0.01-0.03</td>
<td>0.3746</td>
</tr>
</tbody>
</table>

N = study population, data is presented as mean ± standard deviation. BMI=Body Mass Index, WHR= Waist-to-Hip Ratio. CI= Confidence Interval

4.3 Clinical characteristics of breast cancer patients

As indicated in Table 3, 29 (90.6%) out of the 32 breast cancer patients had invasive ductal carcinoma of no special type, whereas 3 (9.4%) had metastatic carcinoma. Furthermore, 18 (56.3%) out of the 32 breast cancer cases were cancer grade III while 12 (37.5%), 1 (3.1%) and 1 (3.1%) were cancer grade II, grade IV and grade I, respectively.

Out of the 32 breast cancer cases, 25 (78.1%), 4 (12.5%) and 3 (9.4%) were in stage III, II and IV. Of the 32 patients, 10 (31.2%), 9 (28.1%), 7 (21.8%) and 6 (18.7%) cases were Luminal B, Triple Negative, HER2-Enriched and Luminal A molecular subtypes of breast cancer respectively.

Also, 18 (56.2%) and 14 (43.8%) out of the 32 breast cancer patients had left and right unilateral breast cancer respectively. Majority of the patients 26 (81.3%) were within the 0-6 months duration since the diagnosis of the breast cancer, 3 (9.3%) within the 7-12 months and more than 12 months of the duration since diagnosis respectively (Table 3).
**Table 3: Clinical characteristics of breast cancer patients**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration since diagnosis (months)</strong></td>
<td></td>
</tr>
<tr>
<td>0 – 6</td>
<td>26 (81.3)</td>
</tr>
<tr>
<td>7 – 12</td>
<td>3 (9.3)</td>
</tr>
<tr>
<td>&gt;12</td>
<td>3 (9.3)</td>
</tr>
<tr>
<td><strong>Tumour stages</strong></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4 (12.5)</td>
</tr>
<tr>
<td>III</td>
<td>25 (78.1)</td>
</tr>
<tr>
<td>IV</td>
<td>3 (9.4)</td>
</tr>
<tr>
<td><strong>Tumour grades</strong></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>1 (3.1)</td>
</tr>
<tr>
<td>G2</td>
<td>12 (37.5)</td>
</tr>
<tr>
<td>G3</td>
<td>18 (56.3)</td>
</tr>
<tr>
<td>G4</td>
<td>1 (3.1)</td>
</tr>
<tr>
<td><strong>Molecular subtype</strong></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>6 (18.8)</td>
</tr>
<tr>
<td>Luminal B</td>
<td>10 (31.3)</td>
</tr>
<tr>
<td>HER-2 -Enriched</td>
<td>7 (21.8)</td>
</tr>
<tr>
<td>Triple Negative</td>
<td>9 (28.1)</td>
</tr>
<tr>
<td><strong>Histopathological classification</strong></td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>29 (90.6)</td>
</tr>
<tr>
<td>Metastatic carcinoma</td>
<td>3 (9.4)</td>
</tr>
<tr>
<td><strong>Location of cancer</strong></td>
<td></td>
</tr>
<tr>
<td>Left breast</td>
<td>18 (56.2)</td>
</tr>
<tr>
<td>Right breast</td>
<td>14 (43.8)</td>
</tr>
</tbody>
</table>

*N* = breast cancer patients, *HER-2 enriched= Human epidermal growth factor receptor-2*
4.4 Comparison of circulating cell-free DNA concentrations and cfDNA integrity among the breast cancer patients and apparently healthy controls

The levels of ALU 115 and 247 at T1 (before chemotherapy) were significantly higher in the breast cancer patients than the controls (Table 4). The serum cfDNA integrity was also higher in the breast cancer patients than the controls but was not statistically significant.

Table 4: Comparison of circulating cell-free DNA concentrations and DNA integrity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Breast cancer (N=32)</th>
<th>Controls (N=32)</th>
<th>95% CI of mean</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALU 115 (ng/ml)</td>
<td>2.24 ± 0.80</td>
<td>1.83 ± 0.65</td>
<td>-0.77 – (-0.05)</td>
<td>0.028*</td>
</tr>
<tr>
<td>ALU 247 (ng/ml)</td>
<td>2.73 ± 0.11</td>
<td>1.96 ± 0.85</td>
<td>-1.07 – (-0.47)</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>cfDNA integrity</td>
<td>1.22 ± 0.14</td>
<td>1.07 ± 1.31</td>
<td>-0.62 – 0.31</td>
<td>0.522</td>
</tr>
</tbody>
</table>

Circulating cell-free DNA (cfDNA) levels of breast cancer patients at baseline (before chemotherapy) were compared with apparently healthy control group. *p-value ≤ 0.05 is statistically significant. N = Number of participants, CI = Confidence Interval

4.5 Circulating cell-free DNA concentrations and cfDNA integrity among the breast cancer patients at time points one and two

The concentrations of ALU 115 and ALU 247 significantly decreased in the breast cancer patients after the third cycle chemotherapy (T2). The cfDNA integrity however, was higher at T2 but was not statistically significant (Table 5).

Table 5: Circulating cell-free DNA concentrations and DNA integrity

<table>
<thead>
<tr>
<th>Parameter (ng/ml)</th>
<th>Breast cancer patients</th>
<th>95% CI of mean</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALU 115</td>
<td>2.24 ± 0.80</td>
<td>-0.94 – (-0.21)</td>
<td>0.003*</td>
</tr>
<tr>
<td>ALU 247</td>
<td>2.73 ± 0.11</td>
<td>-0.86 – (-0.36)</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>cfDNA integrity</td>
<td>1.22 ± 0.14</td>
<td>-0.32 – 0.42</td>
<td>0.788</td>
</tr>
</tbody>
</table>

T1: before commencement of chemotherapy; T2: after the 3rd cycles of chemotherapy. *p-value ≤ 0.05 is statistically significant. CI: confidence interval.
4.6 Circulating cell-free DNA concentrations among tumour stages in the breast cancer patients at time points one and two

It was observed that, the concentrations of ALU 247 decreased at time point two for all the stages. The concentrations of ALU 115 significantly decreased at stage II (Table 6).

Table 6: Circulating cell-free DNA concentrations among tumour stages in the breast cancer patients at time points one and two

<table>
<thead>
<tr>
<th>Parameters ng/ml</th>
<th>Stage II (n = 4)</th>
<th>Stage III (n = 25)</th>
<th>Stage IV (n = 3)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALU 115 (T1)</td>
<td>1.69 ± 0.82</td>
<td>2.05 ± 0.37</td>
<td>1.77 ± 0.34</td>
<td>0.230</td>
</tr>
<tr>
<td>ALU 115 (T2)</td>
<td>1.64 ± 0.69</td>
<td>1.67 ± 0.46</td>
<td>1.52 ± 0.79</td>
<td>0.893</td>
</tr>
<tr>
<td>p-value</td>
<td>0.929</td>
<td>0.002</td>
<td>0.641</td>
<td>-</td>
</tr>
<tr>
<td>ALU 247 (T1)</td>
<td>2.22 ± 0.53</td>
<td>2.23 ± 0.88</td>
<td>2.01 ± 0.47</td>
<td>0.909</td>
</tr>
<tr>
<td>ALU247 (T2)</td>
<td>1.81 ± 0.69</td>
<td>2.03 ± 0.70</td>
<td>1.34 ± 1.85</td>
<td>0.388</td>
</tr>
<tr>
<td>p-value</td>
<td>0.361</td>
<td>0.378</td>
<td>0.576</td>
<td>-</td>
</tr>
<tr>
<td>cfDNA integrity</td>
<td>1.08 ± 0.42</td>
<td>1.10 ± 1.00</td>
<td>0.88 ± 0.43</td>
<td>0.927</td>
</tr>
<tr>
<td>(T1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cfDNA integrity</td>
<td>1.22 ± 0.66</td>
<td>1.31 ± 0.65</td>
<td>1.14 ± 0.72</td>
<td>0.895</td>
</tr>
<tr>
<td>(T2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.733</td>
<td>0.383</td>
<td>0.619</td>
<td>-</td>
</tr>
</tbody>
</table>

Results were presented as mean ± SD: *p-value ≤ 0.05 is considered significant: T1 = before commencement of chemotherapy; T2 = after the 3rd cycle of chemotherapy
4.7 Circulating cell-free DNA concentrations among tumour grades in the breast cancer patients at time points one and two

ALU 115 and ALU 247 levels decreased at time point two for all the tumour grades. Nevertheless, the cfDNA integrity was higher at time point two for all the tumour grades (Table 7).

Table 7: Circulating cell-free DNA concentrations among tumour grades in the breast cancer patients at time points one and two

<table>
<thead>
<tr>
<th>Parameters (ng/ml)</th>
<th>G1 (n = 1)</th>
<th>G2 (n=12)</th>
<th>G3 (n = 18)</th>
<th>G4 (n = 1)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALU 115 (T1)</td>
<td>1.88</td>
<td>1.74 ± 0.68</td>
<td>2.41 ± 0.48</td>
<td>1.39</td>
<td>0.018</td>
</tr>
<tr>
<td>ALU 115 (T2)</td>
<td>1.8</td>
<td>1.56 ± 0.88</td>
<td>1.83 ± 0.43</td>
<td>0.6</td>
<td>0.262</td>
</tr>
<tr>
<td>p-value</td>
<td>-</td>
<td>0.581</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ALU 247 (T1)</td>
<td>2.05</td>
<td>2.24 ± 0.29</td>
<td>2.97 ± 0.87</td>
<td>2.48</td>
<td>0.054</td>
</tr>
<tr>
<td>ALU 247 (T2)</td>
<td>1.93</td>
<td>1.86 ± 0.94</td>
<td>2.48 ± 0.81</td>
<td>1.5</td>
<td>0.235</td>
</tr>
<tr>
<td>p-value</td>
<td>-</td>
<td>0.195</td>
<td>0.089</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cfDNA integrity (T1)</td>
<td>1.07</td>
<td>1.19 ± 1.07</td>
<td>1.23 ± 0.53</td>
<td>1.78</td>
<td>0.905</td>
</tr>
<tr>
<td>cfDNA integrity (T2)</td>
<td>1.09</td>
<td>1.29 ± 0.43</td>
<td>1.36 ± 0.55</td>
<td>2.5</td>
<td>0.159</td>
</tr>
<tr>
<td>p-value</td>
<td>-</td>
<td>0.767</td>
<td>0.475</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results were presented as mean±SD: *p-value ≤0.05 is considered significant: T1= before commencement of chemotherapy; T2= after the 3rd cycle of chemotherapy

4.8 Circulating cell-free DNA concentrations among the molecular subtypes in the breast cancer patients at time points one and two

The concentrations of ALU 115 and ALU 247 decreased at time point two for all the molecular subtypes of breast cancer. However, the cfDNA integrity was higher at time point two for all the molecular subtypes (Table 8).
Table 8: Circulating cell-free DNA concentrations among the molecular subtypes in the breast cancer patients at time points one and two

<table>
<thead>
<tr>
<th>Parameters (ng/ml)</th>
<th>Luminal A (n = 6)</th>
<th>Luminal B (n = 10)</th>
<th>HER2-Enriched (n = 7)</th>
<th>Triple negative (n = 9)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALU 115 (T1)</td>
<td>2.16 ± 0.53</td>
<td>1.64 ± 0.61</td>
<td>1.80 ± 0.88</td>
<td>1.84 ± 0.28</td>
<td>0.429</td>
</tr>
<tr>
<td>ALU 115 (T2)</td>
<td>1.70 ± 0.15</td>
<td>1.61 ± 0.52</td>
<td>1.60 ± 1.12</td>
<td>1.77 ± 0.49</td>
<td>0.942</td>
</tr>
<tr>
<td>p-value</td>
<td>0.068</td>
<td>0.907</td>
<td>0.717</td>
<td>0.715</td>
<td>-</td>
</tr>
<tr>
<td>ALU 247 (T1)</td>
<td>2.26 ± 0.78</td>
<td>2.00 ± 0.79</td>
<td>2.21 ± 0.27</td>
<td>2.19 ± 0.55</td>
<td>0.844</td>
</tr>
<tr>
<td>ALU 247 (T2)</td>
<td>2.19 ± 0.37</td>
<td>1.94 ± 1.08</td>
<td>1.62 ± 1.20</td>
<td>1.96 ± 0.66</td>
<td>0.730</td>
</tr>
<tr>
<td>p-value</td>
<td>0.846</td>
<td>0.888</td>
<td>0.228</td>
<td>0.433</td>
<td>-</td>
</tr>
<tr>
<td>cfDNA integrity (T1)</td>
<td>1.04 ± 0.68</td>
<td>1.20 ± 0.48</td>
<td>1.01 ± 0.93</td>
<td>1.11 ± 0.74</td>
<td>0.948</td>
</tr>
<tr>
<td>cfDNA integrity (T2)</td>
<td>1.28 ± 0.41</td>
<td>1.22 ± 0.77</td>
<td>1.23 ± 0.31</td>
<td>1.19 ± 0.51</td>
<td>0.992</td>
</tr>
<tr>
<td>p-value</td>
<td>0.476</td>
<td>0.945</td>
<td>0.563</td>
<td>0.792</td>
<td>-</td>
</tr>
</tbody>
</table>

Results were presented as mean±SD: p-value ≤0.05 is considered significant: T1= before commencement of chemotherapy: T2= after the 3rd cycle of chemotherapy: HER2-Enriched=Human Epidermal Growth Factor Enriched Receptor 2

4.9 Circulating cell-free DNA concentrations among the histopathological classification in the breast cancer patients at time points one and two

The concentrations of ALU 115 and ALU 247 also decreased at time point two for all the histopathological classification of breast cancer. However, the cfDNA integrity was higher at time point two for all the molecular subtypes as compared to time point one (Table 9).
Table 9: Circulating cell-free DNA concentrations among the histopathological classification in the breast cancer patients at time points one and two.

<table>
<thead>
<tr>
<th>Parameters (ng/ml)</th>
<th>Invasive ductal carcinoma (n = 29)</th>
<th>Metastatic carcinoma (n = 3)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALU 115 (T1)</td>
<td>1.84 ± 0.58</td>
<td>1.77 ± 0.34</td>
<td>0.840</td>
</tr>
<tr>
<td>ALU 115 (T2)</td>
<td>1.69 ± 0.66</td>
<td>1.38 ± 1.84</td>
<td>0.525</td>
</tr>
<tr>
<td>p-value</td>
<td>0.331</td>
<td>0.736</td>
<td>-</td>
</tr>
<tr>
<td>ALU 247 (T1)</td>
<td>2.13 ± 0.71</td>
<td>2.01 ± 0.47</td>
<td>0.778</td>
</tr>
<tr>
<td>ALU 247 (T2)</td>
<td>2.01 ± 0.72</td>
<td>1.52 ± 0.79</td>
<td>0.273</td>
</tr>
<tr>
<td>p-value</td>
<td>0.525</td>
<td>0.408</td>
<td>-</td>
</tr>
<tr>
<td>cfDNA integrity (T1)</td>
<td>1.16 ± 0.82</td>
<td>1.10 ± 0.18</td>
<td>0.901</td>
</tr>
<tr>
<td>cfDNA integrity (T2)</td>
<td>1.19 ± 0.92</td>
<td>1.32 ± 0.73</td>
<td>0.815</td>
</tr>
<tr>
<td>p-value</td>
<td>0.896</td>
<td>0.639</td>
<td>-</td>
</tr>
</tbody>
</table>

Results were presented as mean±SD: *p-value ≤0.05 is considered significant: T1 = before commencement of chemotherapy: T2 = after the 3rd cycle of chemotherapy.

4.10 Spearman’s correlation of circulating cell-free DNA concentrations and DNA integrity with age, BMI and WHR in breast cancer patients at time points one and two

The Spearman’s correlation analysis showed that, there was no significant correlation of circulating cell-free DNA (ALU 115 and 247) and serum cfDNA integrity with age, BMI and WHR at time points one and two (Table 10 and 11).
Table 10: Spearman’s correlation of circulating cell-free DNA concentrations and DNA integrity with age, BMI and WHR in breast cancer patients at time point one

<table>
<thead>
<tr>
<th>Parameters (ng/ml)</th>
<th>Age</th>
<th>BMI</th>
<th>WHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALU 115</td>
<td>r = 0.06</td>
<td>r = 0.98</td>
<td>r = 0.17</td>
</tr>
<tr>
<td></td>
<td>p = 0.77</td>
<td>p = 0.56</td>
<td>p = 0.37</td>
</tr>
<tr>
<td>ALU 247</td>
<td>r = 0.11</td>
<td>r = 0.18</td>
<td>r = 0.19</td>
</tr>
<tr>
<td></td>
<td>p = 0.53</td>
<td>p = 0.18</td>
<td>p = 0.32</td>
</tr>
<tr>
<td>DNA integrity</td>
<td>r = -0.18</td>
<td>r = 0.23</td>
<td>r = 0.05</td>
</tr>
<tr>
<td></td>
<td>p = 0.33</td>
<td>p = 0.21</td>
<td>p = 0.79</td>
</tr>
</tbody>
</table>

*Spearman’s correlation is denoted as r and p-value < 0.05 represents significance. BMI; Body Mass Index, WHR; Waist-Hip Ratio.*

Table 11: Spearman’s correlation of circulating cell-free DNA concentrations and DNA integrity with age, BMI and WHR in breast cancer patients at time point two

<table>
<thead>
<tr>
<th>Parameter (ng/ml)</th>
<th>Age</th>
<th>BMI</th>
<th>WHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALU 115</td>
<td>r = 0.17</td>
<td>r = -0.05</td>
<td>r = -0.02</td>
</tr>
<tr>
<td></td>
<td>p = 0.36</td>
<td>p = 0.79</td>
<td>p = 0.90</td>
</tr>
<tr>
<td>ALU 247</td>
<td>r = 0.03</td>
<td>r = -0.02</td>
<td>r = 0.17</td>
</tr>
<tr>
<td></td>
<td>p = 0.87</td>
<td>p = 0.94</td>
<td>p = 0.38</td>
</tr>
<tr>
<td>DNA integrity</td>
<td>r = -0.24</td>
<td>r = -0.11</td>
<td>r = 0.07</td>
</tr>
<tr>
<td></td>
<td>p = 0.20</td>
<td>p = 0.54</td>
<td>p = 0.71</td>
</tr>
</tbody>
</table>

*Spearman’s correlation is denoted as r and p-value < 0.05 represents significance. BMI; Body Mass Index, WHR; Waist-Hip Ratio.*
CHAPTER FIVE

DISCUSSION

5.1 General

The main focus of this study was to evaluate circulating cell-free DNA (cfDNA) as a blood biomarker in patients with breast cancer to monitor the response to chemotherapy. Previous research has demonstrated that in the serum of breast cancer patients, circulating cell-free DNA was elevated and can be used as a diagnostic and prognostic biomarker for breast cancer (Arko-Boham et al., 2019).

Consequently, DNA integrity which is the ratio of ALU 247 to ALU 115 has been researched for its utility in cancer diagnosis and prognosis. The values of ALU115 reflects the total amount of free serum DNA that amplifies both shorter (apoptosis-truncated) and longer fragments of DNA. The ALU247 values reflect the total amount of non-apoptotic released DNA that amplifies only longer fragments of DNA. It has been suggested to be increased in cancer patients and also found to predict tumour progression, and regional lymph node metastases in primary breast cancer patients (Fleischhacker & Schmidt, 2007).

Therefore, this study sought to investigate the effect of chemotherapy on serum cfDNA concentrations and if cfDNA can be useful in monitoring response to chemotherapy in breast cancer.
5.2 Demographic and anthropometric characteristics of breast cancer patients and apparently healthy controls

In this study, age was found to be an important risk factor of breast cancer as has been found in other studies (McPherson et al., 2000), with majority of the breast cancer patients assessed in this study aged between the ages of 60 and 65 years. The mean age of the breast cancer patients was 62.38 ± 13.32 years (Table 1). These were consistent with existing pattern that 81% of all female breast cancers occur in women 50 years of age and older (Key et al., 2001) as aging inevitably increases the risk of breast cancer. This reason is not fully understood but possible reason is the shortening of the telomere in older people. Shortening of telomere is one of the many reasons that may be well suited for this study.

Telomeres are regions at the end of chromosomes that are composed of the repeated DNA sequence which are mediated by the enzyme telomerase, along with associated proteins that protect the blunt ends of DNA and prevent the cell from triggering a DNA damage response (Kalmbach, 2015). The theory is that, any time a cell divides, chromosomes are decreased by around 25-200 per replication (Ciocan & Martinescu, 2015). However, since the ends are secured by telomeres, the only part of the chromosome that is missing, is the telomere and the DNA is remains undamaged. Without telomeres, essential DNA would be lost anytime a cell divides (usually between 50 to 70 times) (Ciocan & Martinescu, 2015).

Telomere length shortens with age. Telomere shortening happens at each DNA replication, and if it persist, leads to chromosomal degradation and cell death. It has been associated with increased incidence of diseases and poor survival. Telomere shortening has been proposed as a potentially useful biomarker of human ageing and age-related morbidity and mortality (Wang et al., 2018). The rate of telomere shortening can indicate the pace of aging. However, several studies have indicated that, shorter telomeres are a risk factor for cancer and have been
associated with increased incidence of diseases (Carvalho et al., 2019). Individuals with shorter telomeres seem to have a greater risk for development of breast, lung, bladder, renal cell, gastrointestinal, and head and neck cancers (Wu et al., 2003). This tends to affect the health and lifespan of an individual.

The mean menarche for the breast cancer patients and the controls were 12.63 ± 2.20 and 16.31 ± 1.31 respectively. It was found that, most of the breast cancer patients had their menarche at an early stage unlike the controls who had theirs at a later stage. It is well known that early menarche increases the risk of breast cancer development (Duarte et al., 2014). Women with early onset of menarche have more menstrual cycles particularly before 14 years of age, and thus would have a longer lifetime exposure to the hormones estrogen contributing to a slightly higher risk of breast cancer (Leung et al., 2008).

Exposure to estrogen is a significant determinant of the risk of breast cancer because, estrogens or their catechol metabolites are carcinogens in different tissues, including the kidneys, liver, uterus, and mammary glands (Yager & Davidson, 2006). Also the hormone estrogen acts upon other cells in order to increase their proliferative capacity or cellular hyperplasia, speeding up and increasing the production of any present mutant cancerous cells (Macias & Hinck, 2012).

Statistically speaking, there was no significant difference between the age of onset of menopause for both the breast cancer patients and apparently healthy individuals, with the recorded ages of early onset of menopause at 47.45 ± 4.27 and 47.33 ± 4.55 respectively (Table 1).

The mean BMI of the breast cancer patients was 31.03 ± 7.52 kg/m² and that of the controls was 24.35 ± 5.19 kg/m² (Table 2). Majority of the breast cancer patients were obese whereas the apparently healthy individuals had normal BMI. The relationship between BMI and the risk of
breast cancer has gained a great deal of attention in the recent years, although the results have still been controversial (Reeves et al., 2007). Several studies have indicated that, higher BMI (obese) raises the risk of breast cancer since every 5 kg/m² increase in BMI correlates to a 2% increase in the risk of breast cancer in females (Liu et al., 2018). Overweight and/or obesity are typically expressed in the body mass index (BMI) and is a risk factor for breast cancer growth (Sweeney, Blair, Anderson, Lazovich, & Folsom, 2004). Being obese can increase one’s chance of developing breast cancer by raising estrogen levels. Estrogen acts upon other cells to increase their proliferative capacity, speeding up and increasing the production of any present mutant cancerous cells (Macias & Hinck, 2012).

According to WHO, a WHR value which is ≤ 0.80 is considered low, between 0.81 to 0.85 is normal and ≥ 0.86 is considered as high (Lau et al., 2007). In this study, the breast cancer patients were within the ≥ 0.86 category and so, they had higher WHR values (Table 2). Research has shown that, higher WHR value is associated with an increased risk of breast cancer (B. S. Connolly et al., 2002) and this supports the results stated above. It is well known that, WHR is a measure of central adiposity and is increasingly used as a measure of etiologically relevant obesity, which is considered to be more closely linked to pathology (Tchernof & Després, 2013). In addition, the metabolic changes that follow central adiposity include peripheral hyperinsulinemia, hyperglycemia and glucose intolerance (la Fleur, Luijendijk, Van Rozen, Kalsbeek, & Adan, 2011). These changes, particularly in early adulthood, may be of fundamental importance in the development of breast cancer (la Fleur et al., 2011)

In this study, we could not accurately ascertain the effect of alcohol since a negligible percentage of the study participants routinely drunk alcohol (Table 1). Majority of the breast cancer patients were hypertensive. Hypertension is a common chronic disease and major risk
factor for chronic kidney disease. It has also been implicated as a risk factor for breast cancer (Han et al., 2017).

Most of the breast cancer patients were diabetic as compared to the controls (Table 1). Diabetes is a health condition that causes an increase in blood glucose levels and is one of the most significant chronic disorders worldwide (Morabito et al., 2020). It has been implicated as a risk factor for breast cancer. This reason may probably be due to the fact that, increased level of insulin (hyperinsulinemia) increases the risk of developing cancer in any organ including the breast since it stimulates mammary carcinogenesis (Ferguson et al., 2012). Moreso, research has shown that, up to 10% to 20% of patients with breast cancer have type 2 diabetes and the substantial risk factors for type 2 diabetes are old age and obesity, which are both risk factors for breast cancer (Wolf, Sadetzki, Catane, Karasik, & Kaufman, 2005). Studies have also shown that, there is a small increase in the risk of developing breast cancer among women with type 2 diabetes (Ban & Godellas, 2014).

Eighteen (18) out of the 32 breast cancer patients had family history of breast cancer. According to Collins et al. (2006), family history of breast cancer is a recognized risk factor for breast cancer and is used to classify women at higher risk, although the effect of risk factors for breast cancer in women with family history is not well established (Collins et al., 2006).

5.3 Clinical pathological characteristics of breast cancer

Majority (78.1%) of the breast cancer patients were in their late stage (stage III) (Table 3). This was consistent with established trend that, most (≥ 50%) of the Ghanaians with breast cancer report to the hospital at an advanced stage of the disease (Clegg-Lamptey, Edusa, Ohene-Oti, & Tagoe, 2007) due to the lack of awareness and absence of screening programs. Therefore, the burden of breast cancer is growing and threatens to exact heavy morbidity and mortality (Aabdein et al., 2017).
Furthermore, most (31.3%) of the breast cancer patients were diagnosed of Luminal B molecular subtype of breast cancer and (90.6%) of invasive carcinoma of no special type (Table 3). According to most researchers, invasive ductal carcinoma of no special type constituted the majority of the breast cancers diagnosed although the reason, is still not known (Elgaili et al., 2010). Luminal B cancers show a more aggressive phenotype including significantly poorer outcomes in patients and are genetically and genomically altered to a greater extent (De Azambuja et al., 2007).

Most of the patients had the cancer in their left breast (Table 3). Left breast cancer occurs more frequently than right breast cancer and it has been concluded that cancer of the left breast was about 11% more common than that of the right breast (Sardaro et al., 2012).
5.4 Circulating cell-free DNA concentrations and cfDNA integrity

The serum cfDNA levels (ALU 115 and 247) and cfDNA integrity were determined in the breast cancer patients and apparently healthy individuals. ALU 115 represents the total serum circulating cell-free DNA and ALU 247 represents the absolute amount of only longer fragments of serum DNA, supposedly released from non-apoptotic dead cells. The breast cancer patients showed slightly higher levels of concentrations of serum cfDNA and cfDNA integrity as compared to the apparently healthy controls in all instances.

The pattern of serum cfDNA levels and cfDNA integrity among cancer patients and healthy controls, was consistent with earlier studies (Hussein et al., 2019; Iqbal & Buch, 2016; Stötzer et al., 2014). This is most likely the result of increased amounts of DNA released into the bloodstream arising from apoptotic and tissue necrotic activities of the body tissues in the breast cancer patients. This increases the concentrations of both longer and shorter DNA fragments in the breast cancer patients as compared to the apparently healthy controls (Arko-Boham et al., 2019).

However, the lower concentrations of serum cfDNA and cfDNA integrity in the apparently healthy controls may probably be due to low necrotic activity in body tissues, thereby lowering the concentration of longer DNA fragments in the bloodstream (Arko-Boham et al., 2019).
5.5 Circulating cell-free DNA concentrations and cfDNA integrity in breast cancer patients at time points one and two

Chemotherapy is a systemic therapy that uses drugs to destroy both normal and cancer cells, usually by preventing the cancer cells from growing, dividing, and making more of themselves (Mijwel et al., 2018). However, normal cells are easily restored and replaces. Chemotherapy is one of the treatment methods used to treat breast cancer and typically consists of a mixture of medications delivered over a fixed period of time in a set number of cycles (Leinert et al., 2017).

In this present study, the concentration of ALU 115 and ALU 247 significantly decreased at time point two unlike the cfDNA integrity which increased at time point two but was not statistically significant (Table 5). This observation may probably be as a result of the effect of the chemotherapy and the circulating cell-free DNA clearance system. The effect of the chemotherapy caused the cancer cells to be killed and this resulted in the release of longer uneven fragments of DNA from the cancer cells into the bloodstream (Swystun et al., 2011).

The circulating cell-free DNA (cfDNA) clearance system may have possibly cleared the released DNA from the bloodstream thereby, resulting in the decreased concentrations of the cfDNA (Aucamp et al., 2018). However, due to the excessive death of the cancer cells by the chemotherapy, longer fragments of DNA (necrotic DNA) were released into the bloodstream which increased the concentrations. These elevated levels of the necrotic DNA (longer DNA fragments) may have overloaded the clearance system (Leung et al., 2016) causing the accumulation of the longer uneven fragments of DNA in the blood therefore, leading to higher levels of circulating cell-free (cfDNA) integrity.
5.6 Circulating cell-free DNA concentrations and cfDNA integrity among the tumour stages, grades, histopathological and molecular subtypes in breast cancer patients at time points one and two

The data from this study indicates that, the concentrations of both ALU 115 and 247 decreased after the 3rd cycle of chemotherapy (T2) among the tumour grades, stages, histopathological and molecular subtypes of breast cancer (Table 6, 7, 8 and 9). However, the cfDNA integrity increased at time point two (after the 3rd cycle of chemotherapy) for all the parameters stated. These observations might perhaps be due to the effect of chemotherapy and the cfDNA clearance system as stated earlier.

Chemotherapy induces the release of DNA from the cancer cells as a result of the tissue necrotic activities into the bloodstream and this increased the levels of cfDNA in the blood (Swystun et al., 2011). However, the increased concentration of the circulating cell-free DNA (specifically the longer DNA fragments) by the chemotherapy may have probably activated the cfDNA clearance system to clear off the released DNA from the bloodstream (Aucamp et al., 2018). Thus effectively decreasing the concentrations of cfDNA in the blood.

Due to the excessive death of the cancer cells by the chemotherapy, more necrotic DNA (longer DNA fragments) were released into the bloodstream. This may have overloaded the cfDNA clearance system (Leung et al., 2016) causing the accumulation of the necrotic DNA resulting in higher cfDNA integrity among the tumour grades, stages, histopathological and molecular subtypes in the breast cancer patients.

5.7 Circulating cell-free DNA concentrations and DNA integrity with age, BMI and WHR in breast cancer patients at time points one and two

There was no significant correlation of the levels of circulating cell-free DNA (ALU 115 and 247) and cfDNA integrity with age, BMI and WHR in the breast cancer patients at time points
one and two (Tables 10 and 11). Meaning the cfDNA concentrations and cfDNA integrity were not affected by age, WHR and BMI. According to other reports, there was no significant association between BMI, age and sperm DNA integrity (Bandel et al., 2015).

5.8 Key findings

i. The circulating cell-free DNA (ALU 115 and 247) concentrations and cfDNA integrity was higher in the breast cancer patients as compared to the apparently healthy controls. This findings may probably be as a result of increased amounts of DNA released by both apoptotic and tissue necrotic activities into the bloodstream of breast cancer patients thereby increasing the concentrations of both the longer and shorter DNA fragments as compared to the apparently healthy controls.

ii. The concentrations of both ALU 115 and 247 significantly decreased in the breast cancer patients after the 3rd cycle of chemotherapy (T2) unlike the cfDNA integrity which increased at time point two (T2). This may be attributed to the effect of chemotherapy which destroyed the cancer cells due to the high necrotic activity causing the release of long uneven fragments of DNA into the bloodstream. These increased the levels of cfDNA (ALU 115 and 247) in the blood. This observation maybe hinge on my speculation that, the cfDNA clearance system cleared the released DNA from the blood causing the decreased concentrations of cfDNA (ALLU 115 and 247). However, due to the excessive death of the cancer cells by the chemotherapy, more necrotic DNA was released into the blood and this overloaded the clearance system. Accumulation of longer uneven fragments of DNA occurred and this resulted to higher cfDNA integrity at time point two.

iii. The concentrations of both ALU 115 and 247 decreased among the tumour grades, stages, histopathological and molecular subtypes in the breast cancer patients after the 3rd cycle of chemotherapy (T2) whiles the cfDNA integrity increased at time point two
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(T2). This observation is similar to what has been specified earlier stating the effect of the chemotherapy and cfDNA clearance system.

iv. There was no significant correlation of the cfDNA concentrations and cfDNA integrity with age, WHR and BMI at both time points one and two. This signifies that circulating cell-free DNA is not affected by age, WHR and BMI.

5.9 Conclusion

The objective of this research was to evaluate circulating cell-free DNA as a blood biomarker to monitor the response of chemotherapy in breast cancer. The study has shown that, circulating cell-free DNA may be used as a vague biomarker to monitor response to chemotherapy in breast cancer.

5.10 Limitation of this study

i. Patients recruited for this study had to withdraw due to the high cost involved during chemotherapy. This resulted to a smaller sample size.

ii. Measurement of the tumour volume of the breast cancer patients after the 3rd cycle of chemotherapy was not performed due to clinical reasons. And so, further studies should be done on the tumour volume of the breast cancer and correlate it with the circulating cell free DNA levels in breast cancer patients undergoing chemotherapy.

5.11 Recommendation

Further studies must be done on the circulating cell-free DNA clearance system in breast cancer patients undergoing chemotherapy.
REFERENCES


screening MRI to mammography in women with elevated breast cancer risk. *Jama, 307*(13), 1394-1404.


Lancet, 365(9472), 1727-1741.


APPENDIX I

Information sheet

Participant number: …………………………

CONSENT TO PARTICIPATE IN A RESEARCH PROJECT

Before you agree to participate in this research study, it is important that you read and understand the following study. This statement describes the aim, procedure, risks, discomforts and precautions as well as your right to withdraw from the study at any time.

You are being invited to participate in a research project title: ‘CIRCULATING CELL-FREE DNA AS A BLOOD BIOMARKER FOR MONITORING RESPONSE TO CHEMOTHERAPY IN BREAST CANCER’.

Breast cancer is a malignant disease which results from the abnormal proliferation of cells within the breast tissue. The breast is made up of gland and ducts which connects the lobules to the nipple of the breast. Breast cancer is the commonest cancer among women worldwide making it a major problem that needs to be looked at. There are several molecules in the blood of a breast cancer patient including circulating cell-free DNA (cfDNA). This cfDNA are degraded DNA fragments that are released into the bloodstream during normal physiological and pathological conditions. Elevated cfDNA is associated with cancer development and its aggressiveness where breast cancer is not an exception. And so, this study is to investigate if this cfDNA can be used as a biomarker for monitoring response to chemotherapy in breast cancer patients. Also to find out, if the treatment administered to them is really working or not. If the treatment isn’t working, then it will be changed.

The aim of this study is to assess the utility of circulating cell free DNA as a biomarker for monitoring response to chemotherapy in breast cancer.
Explanation of procedures

The approach of the research is through the use of information obtained from the hospital folders of the breast cancer patients. Also, body measurements such as BMI and WHR will be measured using a calibrated tape measure. Afterwards, about a table spoon full of a blood sample will be taken from your vein for the laboratory analysis.

Possible risks and discomforts

You will not be at any risk when participating in this research, though you may experience a minor discomfort during the blood sample collection due to the prick of the needle used. The blood sample will be used for this study only and not for further studies.

Confidentiality

All the information that will be obtained from you and from the analysis of your blood sample will be handled confidentially and used for the purpose stated for the study only. Your identity as a participant will not be disclosed to any unauthorized persons; only the researchers will have access to the research materials, which will be kept under lock. Participants will be coded with the code known only to the principal investigator.

Costs and compensation for participation in research

This is a purely voluntary participation that is required of you and no monetary compensation is available. There will also be no costs for participating in the research.

Withdrawal from project

Participation in this study is voluntary; refusal to participate will not involve any penalty. You are free to withdraw consent and discontinue participation in this project at any time without you being a victim of the research team. Withdrawal or refusal to participate will not affect the
care you receive from the Oncology Unit of the Department of Surgery. Also you can contact the following people if there is any form of misconduct or withdrawal.

- Ethical and Protocol Review Committee

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- Evelyn Adusei

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**Informed consent form**

Principal Researcher: Evelyn Adusei

Name of Institution: Department of Anatomy, University of Ghana Medical School, College of Health Sciences, University of Ghana

**Supervisory team**

Dr. Benjamin Arkoh-Boham

Dr. John Ahenkorah

Dr. Nii Ayite Aryee

Dr. Nii Adu-Aryee
Project Title: ‘CIRCULATING CELL-FREE DNA AS A BLOOD BIOMARKER FOR MONITORING RESPONSE TO CHEMOTHERAPY IN BREAST CANCER’

I have been invited to take part in this study for the research titled above. My role in this study is to obtain information from the hospital folders of the breast cancer patients. Also, body measurements such as BMI and WHR will be measured using a calibrated tape measure. Afterwards, a blood sample will be taken from your vein for the laboratory analysis.

I acknowledge that the purpose, research procedures, risk and discomforts as described above have been explained to me fully and that any questions that I have asked have been explained to my satisfaction.

I have been informed of the alternatives to participation in this study including the right to not participate. I also understand that I may not benefit directly from this research and that my participation is totally voluntary and I have also been given enough time and opportunity to consider taking part in this study.

I have also been informed that the confidentiality of the information I will provide will be safeguarded and that my privacy and anonymity will be ensured in the collection, storage, and publication of the research material.

I, ………………………………………………………………….. have fully understood the aim, methods and conditions to participate in this study, I therefore consent to my participation.

……………………………………...……………………………………………………………………
Participant’s signature/Thumb-print Date

……………………………………...……………………………………………………………………
Researcher’s signature Date
APPENDIX II

QUESTIONNAIRE FOR BREAST CANCER PATIENTS

Please kindly answer the following questions. Please note that, information provided here will be kept confidential.

1). Your age please. Tick a range. 18-30 ☐ 31-40 ☐ 41-50 ☐

51-60 ☐ 61-70 ☐ above 70 ☐

2). Duration since diagnosis. 2 weeks ago ☐ a month ago ☐ above 1 month ☐

3). Have you had any treatment since you were diagnosed? Yes ☐ No ☐

4). What type of treatment please? Radiotherapy e.g. Treatment with ionizing radiation
Chemotherapy e.g. Cancer drugs ☐ Surgery ☐ Hormone therapy ☐

5). Have you been diagnosed of any other chronic condition before? E.g. Hypertension, diabetes ☐ Yes ☐ No ☐

6). If yes, please state……………………………

7). The stage of breast cancer. Stage 0 ☐ Stage I ☐ Stage II ☐ Stage III ☐ Stage IV ☐

8). Grade of breast cancer. Grade 1 ☐ Grade 2 ☐ Grade 3 ☐

9). Molecular sub-types of breast cancer. Triple negative breast cancer ☐ Luminal A ☐
Luminal B ☐ HER2- enriched ☐ Normal-like breast cancer ☐
APPENDIX III

QUESTIONNAIRE FOR APPARENTLY HEALTHY INDIVIDUALS

Please kindly answer the following questions. Please note that, information provided here will be kept confidential.

1). Your age please. Tick a range. 18-30 □ 31-40 □ 41-50 □ 51-60 □ 61-70 □ above 70 □

2) Occupation of individual. Please state………………………………..

3). Have you been diagnosed of any chronic condition before? E.g. Hypertension, diabetes etc.

   Yes □ No □

4). If yes, please state…………………………..