CHAPTER ONE

1.0 INTRODUCTION

Breast cancer is one of the most common types of cancer that affects millions of women around the world with a noticeable fatality rate (Msolly *et al*., 2011). In Nigeria, the incidence which has been reported to be on the increase has been attributed to changes in demography, socio-economic status and epidemiological risk factors (Adebamowo and Ajayi, 2000; Privalsky, 2002). Several hormone-related factors, such as age at menarche, parity and age at menopause, are associated with breast cancer (Helzlsouer *et al*., 1994). Moreover, high levels of endogenous sex hormones, especially oestrogens, are believed to increase breast cancer risk (Ho *et al*., 2009).

17β–oestradiol (E_2) is the most potent natural oestrogen and it is secreted by the granulosa and theca cells of the ovaries (Rotstein, 2011). This is under the control of the pituitary hormones, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). FSH stimulates the growth and recruitment of immature ovarian follicles in the ovary (Zhou *et al*., 2013). This is in addition to the fact that it regulates aromatase activity, whereas LH is responsible for the actual production of androgens in the ovarian theca cells, thus providing the substrate for aromatization to oestrogens in the granulosa cells (Powell *et al*., 2003; Rotstein, 2011). The principal function of the oestrogens is to cause cellular proliferation and growth of the tissues of the sex organs. This includes the development of the stromal tissues of the breast. It is thought that in promoting the growth of breast's end buds, oestrogens may contribute to an increase in cells that become prone to cancerous growth later in life (Russo and Russo, 1998; Brisken, 2008). Reports from animal studies and cultured human breast cells also suggest the induction of mammary tumours by oestrogens (Drabsch *et al*., 2007). Moreover, FSH has been linked with breast cancer cell proliferation and an increased risk of breast cancer development in females who have undergone infertility treatments (Zreik *et al.*, 2010).

Progesterone, a sex hormone is primarily produced by the granulosa-lutein cells of the *corpus luteum* during the luteal phase of the menstrual cycle as well as the syncytiotrophoblast of the placenta during pregnancy. It enhances breast's lobular-alveolar development in preparation for milk secretion and facilitates implantation and maintenance of early pregnancy (Al-Asmakh, 2007). Although, the role of progesterone in breast cancer is controversial, it is suggested that its

activity of opposing oestrogenic stimulation of the breast, decreases breast cancer risk (Ho *et al*., 2009). Conversely, it is thought that the risk of breast cancer is increased because breast mitotic rates are highest in the luteal phase (with high progesterone levels) of the menstrual cycle (Ho *et al*., 2009; Wang *et al*., 2009).

Thyroid hormones are the only iodine-containing substances of physiologic significance in vertebrates (Bello and Bakari, 2012). Thyrotropin Releasing Hormone (TRH) acts on the pituitary thyrotropes to stimulate both the synthesis and release of Thyroid Stimulating Hormone (TSH). Thyroid stimulating hormone controls the size and number of thyroid follicular cells. It stimulates the thyroid gland to produce thyroxine (T_4). Thyroxine, a prohormone, is converted to triiodothyronine (T_3), the active form of thyroid hormone in the peripheral tissues by 5'-deiodination (Krassas *et al*., 2010; Bello and Bakari, 2012). It is postulated that the thyroid gland interacts with the breast tissues, based on the common property of the mammary and thyroid epithelial cells to concentrate iodine by a membrane active transport mechanism. Additionally, TSH receptors in fatty tissues which are abundant in mammary gland have been reported to be a possible reason for this interaction (Turken *et al*., 2003; Ali *et al*., 2011). Thus, thyroid hormones appear to stimulate breast's lobular development, contributing to the differentiation of normal breast tissue (Lai *et al*., 2002; Neville *et al*., 2002). However, the relationship between breast cancer and thyroid hormone is controversial (Saraiva *et al*., 2005; Ali *et al*., 2011).

Hormone receptors are ligand-activated proteins that regulate transcription of selected genes. Oestrogen Receptor (ER) and Progesterone Receptor (PR) play important roles in the growth and differentiation of breast cancers making them important prognostic markers (Patel *et al*., 2013; Ramsey *et al*., 2015). The biologic, prognostic and predictive importance of assessment of ER expression in breast cancer is well established. However, the assessment of PR appears controversial in some regions of the world (Hefti *et al*., 2013; Qiao *et al*., 2013). Most evidence regarding the prognostic role of PR is based upon the assumption that its expression indicates a functioning ER pathway (Ravdin *et al*., 1992). Results from observational studies showed that loss of PR expression was associated with worse prognosis among ER+ breast cancer (Dunnwald *et al*., 2007; Prat *et al*., 2013). These results suggests that evaluation of PR status in ER+ breast cancer might be helpful in identifying those most likely to benefit from hormonal therapy (Hefti *et al*., 2013). Moreover, patients with ER- and/ or PR- breast cancer have been reported to have

higher mortality compared with those with ER+ and/ or PR+ (Anderson *et al*., 2001). Human Epithelial Receptor 2 (HER 2) also known as ErbB2-neu, located on chromosome 17q21 is also considered to be closely associated with the occurrence and development of breast cancer (Gown, 2008). HER 2 is inactive under normal physiological conditions but upon activation, it may enhance tumour invasion and metastasis (Guo and Bai, 2008). Hence, HER 2 status is important in the treatment of patients, particularly those with metastatic tumours who respond to Herceptin (Olayioye, 2001; Khokher *et al*., 2013). The knowledge of the different expression patterns of ER, PR and HER 2 is essential in planning the management of the disease (Low *et al*., 1992; Sacks and Baum, 1993).

Recent findings have suggested the contribution of environmental factors to the high incidence of breast cancer (Ragab *et al*., 2014). The current industrial revolution has brought about an increased use of various metals and compounds in industry, agriculture and medicine (Antila *et al*., 1996; Caserta *et al*., 2008). This is coupled to the wide spread environmental pollution which has been reported in Nigeria (Anetor *et al*., 2005). Environmental pollution has led to an increased exposure not only to occupationally exposed workers but also to consumers of the various products and the general public at large (Adachi and Tainosho, 2004). Some of these metals and compounds have been reported to adversely affect the endocrine signaling system and are referred to as endocrine disruptors (EDs).

Endocrine disruptors (EDs) may mimic, block or modulate the synthesis, release, transport, metabolism and binding or elimination of natural hormones. Even though EDs may be present in the environment at only very low levels, they may still cause harmful effects, especially when several different compounds act on one target (IPCS, 2002). EDs are widespread in food chains and in the environment. They include arsenic (As), cadmium (Cd), lead (Pb), bisphenol-A (BPA) and polychlorinated biphenyls (PCBs). Once in the environment, they are almost impossible to eliminate, because they do not decompose. These EDs are absorbed into the human system through different routes. Arsenic and cadmium compounds as well as PCBs are lipophilic, hence, they readily penetrate cell membranes (Carpenter *et al*., 2005). On the other hand, cadmium can bind to protein to form a complex, cadmium-metallothionein which is actively taken into the cell by endocytosis (Antila *et al*., 1996). Lead may be absorbed by passive diffusion while BPA is

absorbed upon ingestion of BPA contaminated food and water (Karmakar and Jayaraman, 1988; Kang *et al*., 2006).

Arsenic is a metalloid that is ubiquitous in the environment. Human exposure includes ingestion of contaminated food and water, inhalation of contaminated air and by dermal contact. Arsenic compounds are lipid soluble and within 24 hours of absorption are distributed throughout the body where they can bind to sulfhydryl (-SH) groups on proteins. Arsenic may also replace phosphorus in bone tissue and be stored for years (Bartolome *et al*., 1999). Methylation efficiency in humans appears to decrease at high arsenic doses and studies show that aging is associated with a diminishing capacity to methylate inorganic arsenic, resulting in increased retention of arsenic in soft tissues (Tseng *et al*., 2005) including breast tissues. Interaction of arsenic compounds with thyroid hormone has been reported. Arsenite (AsO_3^{3-}**)** was reported to inhibit in vitro binding of triiodothyronine to its nuclear receptor at relatively high (millimolar) concentrations (Takagi *et al*., 1990). Chronic exposure to arsenic compounds has been associated with several types of cancer (Frumklin *et al*., 2001). Arsenite blocks the binding of E_2 to ER-alpha (ERα), acts as a ligand for ER thus, activating it in the absence of the hormone, suggesting that the metal interacts with the hormone binding domain of the ER. It increases cell growth and mimicked the effects of E_2, decreases the amount of ERα and increases the expression of the progesterone receptor (Stoica *et al*., 2000a). However, there is paucity of information on arsenic in breast cancer patients in Nigeria.

Cadmium ranks close to lead as a metal of current toxicological concern (ATSDR, 2005). It occurs in nature in association with zinc and lead. Extraction and processing of these metals often lead to environmental contamination with Cd (Klaassen, 1996). Although, smoking is a well established source of cadmium exposure, the major route of cadmium exposure is ingestion of shellfish and certain food, particularly root vegetables, potatoes and grains (rice and wheat) grown on cadmium-rich soils. (McLaughlin *et al*., 1997). Cadmium is a known cumulative toxicant with a biological half-life of more than 10 years in humans. Cadmium accumulation occurs in the adipose tissue, liver and kidneys (Sivrikaya *et al*., 2013). Only a small fraction of inhaled or ingested Cd is excreted, resulting in increased body burden over time (Fujishiro *et al*., 2012; Tekin *et al*., 2012). Women tend to have higher Cd levels than men presumably because of lower iron stores, which increase Cd absorption (Olsson *et al*., 2002; Reeves and Chaney, 2008).

Thus, comparable environmental exposures to Cd may disproportionately affect women compared to men (Reeves and Chaney, 2008). Chronic low Cd exposure will eventually result in accumulation to toxic levels (Sivrikaya *et al*., 2013).

The ability of cadmium to induce cell proliferation, differentiation, apoptosis and signal transduction by enhancement of protein phosphorylation, activation of transcription and translation factors suggests its ability to induce breast cancer (Siewt *et al.,* 2010). Moreover, cadmium has the potential to disrupt endocrine function by behaving like sex hormones (Stoica *et al*., 2000b). At low concentrations, the metal mimics the effects of oestradiol and binds with high affinity to the hormone-binding domain of ERα. This binding involves several amino acids, suggesting that cadmium activates the receptor through the formation of a complex with specific residues in the hormone-binding domain (Johnson *et al*., 2003; Stoica *et al*., 2000b). Circulating concentrations of pituitary hormones such as LH, FSH and TSH were altered in female rats exposed to Cd and Pb (Martin and Stoica, 2002).

Lead has been reported as a metal that can be found in drinking water, which is of great public health concern (ATSDR, 2005). Lead contamination in the environment, resulting in toxicity in several body organs and systems has been documented (Rothenberg *et al*., 1994). This is in spite of the fact that Pb in gasoline, food cans and in paints was banned in the United States between 1980 and 1990. Recent reports showed that enamel paints with very high levels of Pb were sold freely in Nigeria (Clark *et al*., 2007; Kessler, 2014). Lead adversely affects steroidogenesis by substituting for zinc in the DNA binding zinc (Zn^{2+})-finger motif of steroidogenic enzymes. These enzymes are Steroidogenic Acute Regulatory Protein (StAR), Cytochrome P450 side chain cleavage enzyme (CYP450cc) and 3 beta hydroxysteroid dehydrogenase (3β HSD). This results in decrease in the expression of the enzymes. (Huang *et al*., 2002; Lutzen *et al*., 2004). The reported mechanisms of Pb carcinogenesis are: direct DNA damage as a result of oxidative stress, inhibition of DNA synthesis and repair, and clastogenicity (Martin *et al.,* 2003; Anetor *et al*., 2005; Ragab *et al.,* 2014). The results of epidemiologic studies investigating the association of Pb exposure with cancer are inconsistent and vary according to the type of cancers reported (Steenland *et al*., 1992; Wong and Harris, 2000).

Bisphenol-A (BPA) also known as (BPA, 2, 2-bis (4-hydroxyphenyl) propane is a component of a variety of commonly used household items. It is used primarily in the manufacture of

polycarbonate plastic, epoxy resins and as a non-polymer additive to other plastics (Peretz *et al.*, 2014). There is a wide spread and well documented human exposure to BPA. This is due to its extensive use in the manufacture of consumer goods and products, including polycarbonate food containers and utensils, dental sealants, protective coatings, some flame retardants, and water supply pipes (Calafat *et al*., 2005; Kang *et al*., 2006; Brody *et al*., 2007). Studies on the safety of BPA are inconsistent (Oehlmann *et al*., 2009). It is thought that BPA binds to the oestrogen receptors with almost the same strength as the oestradiol thereby eliciting oestrogenic effects (Welshons *et al*., 2006; Stahlhut *et al*., 2009). Moreover, BPA may also elicit a rapid response by binding non-classical membrane oestrogen receptors (ncmERs) (Alonso-Magdalena *et al*., 2005) or oestrogen-related receptors (ERRs) (Ben-Jonathan *et al*., 2009). BPA exposure increases adipose mass in rats by activating a key adipogenic regulator Peroxisome Proliferator Activated Receptor gamma (PPARγ), thus contributing to adiposity, a known breast cancer risk factor (Somm *et al*., 2009; van Kruijsdijk *et al*., 2009; Kwintkiewicz *et al*., 2010). There is currently paucity of information on the association of environmental exposure to BPA and the risk of breast cancer in Nigerian women.

Polychlorinated biphenyls (PCBs) are members of a chemical family that were widely used in the past in industry as lubricants, coatings and insulation materials for dielectric equipment like transformers and capacitors (Iyengar, 2005; Gray *et al,* 2009). The release of PCBs to the environment has been reported to be through poorly maintained hazardous waste dumps and city landfills, illegal or improper dumping of hydraulic fluids/coolants, leaks from electrical transformers and other equipment, burning of medical, industrial or city waste from older consumer goods like televisions (Gray *et al.*, 2009). Human exposure to PCBs is through inhalation of contaminated air (outdoor or indoor), ingestion of contaminated food or non-food items, and dermal contact of contaminated surfaces. The primary route of exposure to PCBs is through consumption of contaminated lipid-enriched foods (e.g. fish and cooking oils) as PCBs can accumulate in these and other foodstuffs (Van-Emon *et al*., 2013). Studies on the association of PCBs and breast cancer aetiology are currently inconsistent. Some data indicate that high levels of PCBs are associated with oestrogen- negative tumours, which are more aggressive and have a faster rate of progression (Muscat *et al*., 2003 Kerdivel *et al*., 2013). One of the mechanisms involved include the induction of cytochrome P450 1A1 (CYP1A1) gene. An increased risk for breast cancer was reported for women with the highest blood levels of PCBs

who also possessed CYP1A1 variant (Muscat *et al*., 2003). Conversely, other studies did not find association between PCBs and breast cancer aetiology (Sapozhnikova *et al*., 2004; Martinez *et al*., 2010).

There is a growing interest in understanding whether exposure to toxic metals and chemicals contribute to the increasing number of breast cancer cases worldwide. Unfortunately, relatively few studies have investigated the impact of these environmental chemicals on general human health and even fewer have addressed the roles endocrine disruptors may play in the initiation, promotion and progression of breast cancer (Martin *et al*., 2003; Parkin and Fernandez, 2006). This present study was designed to identify the possible relationships of endocrine disruptors with pituitary, gonadal, thyroid hormones and selected receptors (ER, PR, HER 2) in Nigerian women with breast cancer.

1.1 Research Questions

1. Do participants with breast cancer have altered levels of reproductive and thyroid hormones?
2. What is the pattern of expression of ER, PR and HER2 in women with breast cancer?
3. Do participants with breast cancer have increased levels of EDs?
4. Are the alterations in hormone levels due to increased levels of EDs?
5. Are there relationships among the EDs, hormones and the receptors (ER, PR and HER2)?

1.2 Rationale for the Study

The incidence of breast cancer in Nigeria has been reported. 13.5 per 100,000 in the 1980s, 33.3 per 100,000 in 1992, 116 per 100,000 in 2001(Adebamowo *et al*., 2003). In spite of the numerous theories that have been proposed, the exact aetiology of breast cancer has not been clearly defined (Ijaduola and Smith, 1998; Adebamowo and Ajayi, 2000; Omar *et al*., 2003).

Triple negative breast cancer is aggressive. It has been reported to be peculiar to African-americans and suggested to be common in African young women (Huo *et al*., 2009). Studies aimed at determining the pathogenesis of the molecular subtypes that disproportionately affect young women of African ancestry are currently sparse.

High levels of endogenous sex hormones particularly oestrogens are thought to increase the risk of breast cancer (Ho *et al*., 2009). However, the role of progesterone and gonadotropins in the aetiology of breast cancer is controversial.

Thyroid hormones appear to stimulate breast's lobular development, thereby contributing to normal breast tissue differentiation (Neville *et al*., 2002). The role of thyroid hormones in the aetiology of breast cancer has not been systematically studied in indigenous women of Sub-saharan Africa.

Environmental exposure to EDs has been implicated in the aetiology of breast cancer (Ragab *et al*., 2014). However, there is currently paucity of information on the serum concentration of these EDs; lead, cadmium, arsenic, bisphenol-A, polychlorinated biphenyls.

There are reports of interactions of the EDs with the hormone signalling pathways (Caserta *et al*., 2008). There is paucity of information on the interactions of EDs with sex and thyroid hormones in Nigerian women with breast cancer.

1.3 Aim

This present study was designed to identify the relationships of endocrine disruptors with pituitary, gonadal, thyroid hormones and selected receptors (ER, PR, HER 2) in Nigerian women with breast cancer.

1.4 Objectives

a. To determine the contribution of reproductive and thyroid hormones to the pathogenesis of breast cancer.
b. To identify the pattern of expression of hormone receptors in Nigerian women with breast cancer.
c. To understand the role of endocrine disruptors in breast carcinogenesis.
d. To find possible relationships among endocrine disruptors, hormones and some receptors in Nigerian women with breast cancer.

1.5 Research Hypothesis

Exposure to Pb, Cd, As, BPA and PCBs which may result in altered serum levels of reproductive, thyroid hormones and expression of ER, PR and HER 2 could be associated with breast cancer development.

1.6 The Significance of the Study

Examining the roles of lead, cadmium, arsenic, bisphenol-A, polychlorinated biphenyls, reproductive and thyroid hormones in participants with breast cancer might help in the prevention, early diagnosis and treatment of breast cancer.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of Cancer

Cancer has been reported as the most dreaded non-communicable disease in developing countries, where it is invariably fatal. This is due to lack of adequate preventive and curative services. This is unlike the developed countries that have policies, strategies and programmes for cancer prevention and management (WHO, 2002; Thun, 2010; Nnodu, 2010; Kolawole, 2011). Although, the incidence of cancer is rising globally, the developing countries account for about 52% of this increase and about 70% of cancer deaths (Parkin, 2003; Kolawole, 2011) while possessing only 5% of global funds for cancer control and very few human and material resources (Jones, 1999). Cancer is the second most common cause of death constituting about 12% of all deaths after cardiovascular disease. Globally, cancer kills more people than tuberculosis, Human immune virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) and malaria combined (WHO 2006a, 2006b). In 2007, there were 11 million cancer cases, 7 million cancer deaths and 25 million people living with cancer globally. This is projected to increase to 27 million cases, 17 million deaths and 75 million people living with cancer in 2050 (WHO, 2002; WHO, 2005). Africa carries an increasing cancer burden, 75% of the 650,000 annual cases present late and are at younger ages and about 510,000 deaths occur (Ngoma, 2006). In Nigeria, there are about 100,000 new cancer cases annually (Durosinmi, 2008). The incidence of cancer in Nigerian men and women by 2020 will be 90.7/100,000 and 100.9/100,000 respectively and the deaths rates will be 72.7/100,000 and 76.0/100,000, respectively (WHO, 2008). Cancer accounts for 4.4% of all deaths and is likely to increase to 6.8% in 2030. Out of 89,000 cancer deaths in 2005, 54000 were individuals younger than 70 years (WHO, 2008). Cancers will yet pose significant challenge to Nigeria and other developing countries which currently have insufficient cancer control programs directed at reducing cancer incidence and mortality and to improve quality of life (Kolawole, 2011). The aetiology of many cancers are still unknown, however there are risk factors which are either modifiable or non-modifiable.

2.2 Incidence, Morbidity and Mortality of Breast Cancer

Breast cancer is caused by the development of malignant cells in the breast and has been reported as a major health burden worldwide (Wang *et al.,* 2009). It is the most common type of cancer among women in both high-resource and low-resource settings. It is responsible for over one million of the estimated ten million neoplasms diagnosed worldwide each year in both genders (Ferlay *et al.,* 2001). It is also the primary cause of cancer death among women globally and was responsible for about 375,000 deaths in the year 2000 (Ferlay *et al*., 2001). As a consequence of changing exposures to reproductive and nutrition-related determinants over time, women are at increasingly high risk of breast cancer, with incidence rates increasing in most countries and regions of the world in the past few decades (Bray *et al.,* 2004). The most rapid rises in incidence rate are seen in developing countries, where breast cancer risk has historically been low relative to industrialized countries (Adebamowo *et al*., 2003). In Nigeria, the incidence of breast cancer has been reported to be on the increase (Adebamowo and Ajayi, 2000). It increased from 13.8-15.3 per 100,000 in the 1980s to 33.6 per 100,000 in 1992 and 116 per 100,000 in 2001 (Adebamowo *et al.,* 2003). The increasing trends of breast cancer in the developing countries are often considered the result of the 'westernization' of lifestyles such as; delay in childbearing, dietary habits and exposure to exogenous oestrogen, towards a distribution closer in profile to that of women in industrialised countries (Barton *et al*., 1999; Bray *et al*., 2004).

An increase in the occurrence of breast cancer in premenopausal women in recent times has been reported (Abdulkareem, 2009). A report from the Niger Delta region of Nigeria showed that 65% of breast cancer cases occurred at 50 years and below; 50% occurred between ages 30 and 45 years (Sule, 2011). This was similar to other local reports in which premenopausal women accounted for between 57% and 67% of breast cancer cases (Adesunkanmi, 2006; Okobia, 2006; Kene, 2010) while postmenopausal women accounted for 20% of cases in certain studies (Oluwatosin and Oladepo, 2006). These reports illustrate the prominence of premenopausal breast cancer in Nigeria (Okonofua *et al*., 1990; Sule, 2011). Local investigators have attributed the higher incidence of premenopausal breast cancer to population demographics (Adebamowo and Ajayi, 2000). This was put in perspective with a life expectancy at birth in Nigeria of 51.56 years in the year 2000 and 46.94 years in the year 2009 (CIA, 2009). Higher life expectancy at 75 and 82 years was reported for United States and Britain respectively (CIA, 2009). Thus,

women in this country (Nigeria) may not live long enough for postmenopausal breast cancer. Emerging reports holds that the incidence of premenopausal breast cancer is higher in African–Americans than in their Caucasian counterpart in spite of a life expectancy at 74 and 80 years respectively (Pinheiro, 2005). The reason for this is not yet clear.

Approximately 10-15% of patients with breast cancer has the aggressive type (tumour with triple negative hormone receptors i.e. ER-, PR- and HER2-) and develops distant metastasis within three years after the initial detection of the primary tumour (Gakwaya *et al*., 2008). This appears to be peculiar to Blacks (Thompson, 2006). This also results in unpredictable disease in which some patients present with relatively early stage disease and die of wide spread metastasis within six months and one year, while others present with advanced disease and yet survive longer (Gakwaya *et al*., 2008). The predominant feature of late presentation of breast cancer had been reported over three decades in Nigeria (Lawani *et al*., 1973; Khwaja *et al*., 1980; Chiedozie, 1985; Ihekwaba, 1992; Adebamowo and Adekunle, 1999; Okobia *et al.*, 2006). This has been observed to be coupled with attendant poor outcome (Abdulkareem, 2009). Lack of established national screening program for breast cancer has been reported as one of the reasons adduced to late presentation with advanced breast cancer. Other factors are low social economic level (poverty), fear of mastectomy and ignorance (Ajekigbe, 1991; Elumelu *et al.*, 2011). Poverty coupled with ignorance does not only impede access to health care system, but is associated with other co-factors that can relatively affect outcomes such as co-morbidity and lack of breast health awareness (Oluwole *et al.*, 2003).

Metastasis is the leading cause of mortality in patient diagnosed with breast cancer (Schoppmann *et al.*, 2002). Most breast cancer deaths are due to advanced cancer diagnosed when metastases have already disseminated to lymph nodes or distant organs (Autier *et al.*, 2009). Despite many advances in diagnosis and screening, the disease is frequently discovered after it has spread to regional lymph node or even after dissemination of distant metastasis (Maki and Grossman, 2000). It has been reported that about 20 to 30% of patients with breast cancer will experience relapse with distant metastatic disease (Popoola *et al.*, 2012).

In Nigeria, the most common histological type of breast cancer is invasive ductal carcinoma. This accounts for about 73-80% of cases (Sule, 2011). It was observed that the peak age of breast

cancer in Nigerian women is about a decade earlier than Caucasian women (Okobia *et al*., 2006). The survival rate of breast cancer in Nigeria is low (about 10%) when compared with survival rate of 50% in East Africa. Moreover, a survival rate of 85% was reported for Americans (Olopade, 2004; Adetifa and Ojikutu, 2009).

2.3 Risk Factors of Breast Cancer

The two primary risk factors of breast cancer are increasing maternal age and female gender. Other risk factors are; longer reproductive span, exposure to exogenous hormones, socio-economic status, obesity, abnormal genes (BRCA 1, BRCA2 genes); less than 10% of all breast cancers can be attributed to genetic factors (Brisken., 2008). Other risk factors include; Obesity, lower levels of physical activity, diet, smoking, alcohol, previous breast lesion with atypical changes. Unoccupational exposure to endocrine disruptors (lead, cadmium, arsenic, bisphenol-A, polychlorinated biphenyls) in the aetiology of breast cancer has attracted little attention in the developing world like Nigeria.

2.3.1 Longer Reproductive Span: Early age at menarche, nulliparity, late age at first birth, late age at any birth, low parity, and late menopause-relate to the hormonal (largely oestrogen) milieu to which the breast is exposed from menarche to the cessation of ovulation at menopause (Pike *et al.,* 1983; Bray *et al.,* 2004).

2.3.2 Exposure to Exogenous Hormones: Exposure to exogenous hormones including oral contraceptives and hormone replacement therapy could result in an increase in the risk of breast cancer (CGHFBC, 1996; Beral, 2003). The risk conferred by oral contraceptive use could persist for up to 10 years after cessation. There is much evidence that the rate of exposure to endogenous and exogenous oestrogen is on the increase. This is consistent with upward trends in incidence of breast cancer (Bray *et al*., 2004).

2.3.3 Socio-Economic Status: The association between socio-economic status and risk of breast cancer has been suggested. Certain studies observed that women in higher socio-economic groupings were at higher risk. Conversely, women with low socio-economic status were reportedly at higher risk of breast cancer, owing to ignorance which made them report late in the hospital (Heck and Pamuk, 1997; Adams *et al*., 2004).

2.4 Normal Mammary Gland Development

The development of mammary gland occurs throughout the female life time (Ronnov-Jessen *et al*., 1996; Russo and Russo 1998). At puberty, the female mammary gland responds to the production of the ovarian steroid hormone, oestrogen, which makes the breast epithelium branch into numerous ducts with terminal end buds or alveoli, collectively referred to as the terminal ductal lobular unit (TDLU). In humans, the TDLU is composed of clusters of 6 to 11 ductules per lobule referred to as lobule type 1 (or Lob 1) (Russo and Russo, 1998). Lob 1 progresses to lobule type 2 (Lob 2) in the post pubertal virgin gland, with only modest alveolar proliferation producing a higher number of ductular structures per lobule during the menstrual cycle. Once pregnancy occurs, Lobs 1 and 2 are stimulated by the elevated levels of oestrogen and progesterone, thus resulting in Lob 3. Lob 3 is formed by epithelial expansion of existing pubertal alveoli to 80 small lobules per alveoli. These changes prime the mammary gland for milk secretion from the alveoli, now called secretory lobules type 4 (Lob 4). After parturition, the lactating mammary gland becomes insensitive to oestrogen-dependent regulation of growth, during the post-weaning involution phase, responsiveness to oestrogen is restored. Finally, with the cessation of lactation, the alveoli collapse and the mammary gland regresses apoptotically to its resting, pre-pregnancy state, reverting to Lob 3 and Lob 2, retaining a more extensive framework of branching than Lob 1. Thus, the adult female mammary gland experiences recurrent cycles of regulated growth, differentiation and apoptosis, while oestrogen and progesterone play important roles in this process (Ronnov-Jessen *et al*., 1996; Russo and Russo, 1998).

2.4.1 The Composition of the Mammary Gland

The mammary epithelium is a bilayered structure consisting of an inner continuous layer of luminal epithelial cells and an outer layer of myoepithelial cells. The epithelial bilayer is polarized; the apical layer (luminal epithelial cells) faces the lumen of the ducts and the alveoli, and the basal layer (myoepithelial cells) is in close contact with a laminin-rich basement membrane (BM). The epithelium is embedded in the mammary stroma, which makes up more than 80% of the breast volume (Ronnov-Jessen *et al*., 1996). The breast stroma includes fat tissue, interstitial/interlobular dense connective tissue, intra lobular loose connective tissue and blood vessels. The stromal cells are also surrounded by extracellular matrix (ECM) that is

sometimes referred to as stromal ECM. Extracellular matrix refers to the insoluble proteinaceous components that exist in the mammary tissue. In the normal female breast, approximately 20% of the luminal epithelial cells are in direct contact with the basement membrane (BM), the remaining cells are adjacent to the myoepithelial cells (Gusterson *et al*., 1982; Petersen & van Deurs, 1988). The precise relationship between the luminal epithelial cells, myoepithelial cells and the origin of these cells is largely unknown, making this an important problem for developmental biology of the mammary gland. It was recently reported that a portion of luminal epithelial cells, cultivated in culture to maintain correct functional characteristics gave rise to myoepithelial cells in an appropriate medium, but myoepithelial cells do not produce luminal epithelial cells (Pechoux *et al.,* 1999). This observation suggests a linear relationship between these two epithelial cell types and may be important to tumour biology because most breast cancers are luminal rather than myoepithelial in origin (Wellings *et al*., 1975; Rudland, 1993). Myoepithelial cells have been hypothesized to play a 'tumour suppressive' role by maintaining the differentiated state of luminal epithelial cells (Bani *et al*., 1994; Liu *et al*., 1996). Moreover, it is believed that luminal epithelial cell transformation may prevent conversion to myoepithelial cells. This may explain why in premalignant lesions, there are fewer myoepithelial cells. In invasive breast cancer, myoepithelial cells are either missing or less differentiated (Gusterson *et al*., 1982; Guelstein *et al*., 1988; Rudland *et al*., 1995). In more than 90% of cases, tumour cells are restricted to a luminal-like phenotype (Altmannsberger *et al.,* 1986; Nagle *et al*., 1986; Dairkee *et al*., 1988; Guelstein *et al.,* 1988; Bocker *et al*., 1992), and only a small proportion of these cells are in contact with myoepithelial cells (Gusterson *et al.,* 1982; Petersen & van Deurs, 1988). Although, breast cancer cells originate mainly in the epithelium, evidence suggests that the stroma is an active participant in cancer progression (and possibly even induction) and constitutes the majority of the tumour mass (Dvorak, 1986; Thomasset *et al.,* 1998). The tumour stroma contains changes in the cellular composition and in the amounts of certain protein constituents, often referred to as reactive stroma or desmoplasia when compared with normal mammary gland stroma. For example, the most prominent cellular change in tumour stroma is the appearance of myofibroblasts which are found in close proximity to tumour cell nests (Ronnov-Jessen *et al.,* 1996). Myofibroblasts produce proteases such as urokinase plasminogen activator and stromelysin-3 which degrade ECM and also contribute to tumour cell invasion (Wolf *et al.,* 1993; Unden *et al.,* 1996).

2.4.2 Mammary Gland Morphology and Breast Cancer Origin

The development of breast cancer is characterized by the acquisition or loss of discrete cellular functions. This results in altered tissue organization which has long been recognized by pathologists and used to classify breast tumours as specific morphological types (Beckmann *et al*., 1997). Observations have been made that specific morphological types of breast cancer are associated with specific breast structures or developmental stages of the mammary gland (Russo and Russo, 1998). For example, the common breast malignancy, ductal carcinoma which is thought to originate within the fairly undifferentiated epithelial cells of the terminal ductal lobular unit (TDLU), corresponds to Lob 1. Similarly, lobular carcinomas in situ are found in Lob 2, benign breast lesions originate in Lob 3, and lactating adenomas arise in Lob 4. It was however concluded from these observations that less functionally differentiated breast cells (Lob 1) are more susceptible to giving rise to the most undifferentiated and aggressive neoplasms (Russo and Russo, 1998). Thus, the developmental stage of the breast appears to affect neoplastic transformation. Supporting this hypothesis are studies demonstrating the higher risk of malignancy in nulliparous and late parous women (Lambe *et al.,* 1996). In spite of this evidence, it is yet to be understood how the morphological and developmental stages of the mammary gland are associated with breast cancer. It is generally accepted that the development of invasive breast cancer occurs through the multistep transformation of epithelial cells via steps of hyperplasia, premalignant change, in situ carcinoma, and invasive carcinoma (Wellings *et al.*, 1975; Gould, 1993; Beckmann *et al*., 1997). However, there is no evidence that each step is a necessary precursor of the next stage. This is because it has been difficult to develop model systems with cells representing various types of breast lesions from benign tumours to invasive carcinoma. Markers of malignant cells have been partially defined, however, the characteristics of the precursor cells are less well known, making identification difficult. Evidence does suggest, however, that certain regions of the mammary gland may be predisposed to tumour formation (Deng *et al*., 1996). Studies have indicated that whole regions of the breast may originate from the same cells, i.e. that they are clonal. If these cells are ‘primed’ for tumour formation by harbouring genetic mutations, one might expect to find normal-appearing cells with genetic abnormalities in the region surrounding tumours (Tsai *et al*., 1996). It is now known that morphologically normal breast epithelia could contain many genetic mutations which may give rise to cancer (Deng *et al.,* 1996).

2.5 Anthropometric Measurements, Adiposity and Breast Cancer Risk

The World Health Organization (WHO) defines obesity as an abnormal or excessive fat accumulation in the adipose tissue to the extent that health is impaired. The classification of obesity for epidemiological purposes defines overweight as Body Mass Index (BMI) greater than 25kg/m^2 and obesity as BMI greater than 30 kg/m^2(Gill *et al*., 2003).

Adipose tissue is principally deposited in two compartments; subcutaneously and centrally. It is thought that centrally deposited or visceral fat is more metabolically active than peripheral subcutaneous fat (Kershaw and Flier, 2004; Vohl *et al*., 2004; Galic *et al*., 2010). Visceral adipose tissue largely comprises of omental adipose tissue but also includes other intra-abdominal fat sources such as mesenteric fat. Visceral fat has been reported to be more strongly associated with an adverse metabolic risk profile even after accounting for the contribution of other standard anthropometric indices (Pot and Simmins, 1994; Despress and Lemieux, 2006; Snijder *et al*., 2006; Charles-Davies *et al*., 2012). These systemic effects exerted by visceral adiposity are putatively involved in cancer biology (van Kruijsdijk *et al*., 2009; Amadou *et al*., 2013) and are the focus of much research (Donohoe *et al*., 2011).

Studies have shown that obesity is marked by alteration in the production of adipocytokines; leptin and adiponectin. Increased leptin levels and decreased adiponectin levels promote breast carcinogenesis (Tworoger *et al*., 2007; Mantovani *et al*., 2009). Leptin is strongly angiogenic and may increase tumour angiogenesis by directly acting on the endothelium or by increasing local vascular endothelial growth factor (VEGF) secretion (Hanahan and Weinberg, 2000; Rutkowski *et al*., 2009). Studies in Ibadan, Nigeria showed elevated leptin levels in apparently healthy premenopausal women with metabolic syndrome compared with those without metabolic syndrome. Leptin levels were similar in both pre and postmenopausal women with metabolic syndrome (Fabian *et al*., 2015). Our earlier study showed that elevated levels of leptin in individuals with metabolic syndrome might reflect adiposity and could be a compensatory mechanism for maintaining weight/fat loss and blood pressure (Fabian *et al*., 2015).

Body mass index (BMI) has been reported as a measure of overall adiposity. Its commonly used cut off values to diagnose obesity has been reported to have a high specificity (Okorodudu *et al*., 2010). High BMI has been associated with an increased incidence of many types of cancer

(Renehan *et al*., 2008). There are reports that overweight or obesity is associated with poorer prognosis in most studies that have examined body mass and breast cancer risk (Ryu *et al*., 2001; Berclaz *et al*., 2004; McTiernan, 2005; Dignam *et al*., 2005; Whiteman *et al*., 2005; Kroenke *et al*., 2005; Loi *et al*., 2005). This is because, the obese state may be thought of as a pro-tumourigenic environment which can act to facilitate tumour development by promotion of the acquisition of some of the hallmark properties that characterize cancerous lesions (Hanahan and Weinberg, 2000; Mantovani, 2009).

Women with a BMI of ≥25 had about 58% increased risk of breast cancer in a reported study (Hirose *et al*., 2007). Other studies reported an increased BMI or body weight to be a significant risk factor for recurrent breast cancer, breast cancer survival, or both (Ryu *et al*., 2001; Berclaz *et al*., 2004; McTiernan, 2005; Dignam *et al*., 2005; Whiteman *et al*., 2005; Kroenke *et al*., 2005; Loi *et al*., 2005). In postmenopausal women, epidemiologic evidence suggest a positive association between body mass, body weight and breast cancer (Key *et al*., 2001; Carpenter *et al*., 2003; Feigelson *et al*., 2004; Sweeney *et al*., 2004; Ursin *et al*., 1995; van den Brandt *et al*., 2000; Friedenreich, 2001; Lahmann *et al*., 2004).

Moreover, Height and BMI were reportedly associated with postmenopausal breast cancer in another study (Trentham-Dietz *et al*., 1997; Shu *et al*., 2001; Iwasaki *et al*., 2007). This effect was most pronounced in women with oestrogen receptor positive (ER+) tumours. Ogundiran *et al*. (2010) demonstrated that height was a significant risk factor for female breast cancer in both premenopausal and postmenopausal women. The underlying mechanism could be that childhood energy balance is associated with mammary gland mass and increased insulin-like growth factors (Adami *et al*., 1998; Lovegrove, 2002). Attained height is determined by genetic makeup and environmental factors, including energy intake during childhood and adolescence. In societies with an insufficient food supply, caloric intake plays a more important role in determining height than in societies with an abundant food supply. Thus, energy intake in earlier life may play an important role in breast carcinogenesis.

Waist Circumference has been reported to be an accurate predictor of visceral adiposity, either alone or in combination with BMI or waist to hip ratio (Zhu *et al*., 2004). Its accuracy compared to waist to hip ratio has also been reported (Donohoe *et al*., 2011). This is because it directly reflects total abdominal fat mass (Lemieux *et al*., 1996; Bose and Mascie-Taylor, 1998;

Kopelman, 2000; Kashihara *et al*., 2009; Chakraborty and Bose, 2009). These measures of adiposity have been widely recommended for epidemiological surveys because of their independent association with major non-communicable metabolic diseases including breast cancer (Chakraborty and Bose, 2009).

Waist Height Ratio (WHtR) is an index of assessing central fat distribution. Several studies have demonstrated that waist to height ratio (WHtR) is a better predictor of metabolic risk in oriental people (Ho *et al*., 2003; Hsieh *et al*., 2003; Tseng, 2005). Although, the mechanisms that explain the health risk predicted by WHtR are not firmly established, it is often suggested that the risk is explained by its association with elevations in abdominal obesity (Ashwell *et al*., 1996). WHtR has an added advantage over isolated waist circumference measurement, because its adjustment for height allows establishment of a single, population-wide cut off point that remains applicable regardless of gender, age, and ethnicity (Ashwell and Hsieh, 2005).

Cancer mortality associated with obesity has been reported. A prospective study of 900,000 adults in the United States reported that obesity could account for 20% of all deaths from cancer in women. Women with a BMI greater than 40kg/m^2 had a death rate of about 62% in when compared with those with normal weight (Calle *et al*., 2003).

2.6 Steroid Hormones Biosynthesis

Cholesterol is the building block of steroid hormones. *De novo* synthesis of all steroid hormones starts with the conversion of cholesterol to pregnenolone by CYP11A, one of the cytochrome P450 enzymes (Miller, 1988; Parker and Schimmer, 1995). CYP11A is bound to the inner membrane of the mitochondrion and is found in all steroidogenic tissues (Miller, 1988; Reincke *et al.*, 1998). Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD), one of the several non-CYP450 enzymes that are involved in steroidogenesis which is found in both mitochondria and smooth endoplasmic reticulum. 3β-HSD is widely distributed in steroidogenic and non steroidogenic tissues and consists of two isoenzymes (types 1 and 2, 3 β-HSD), which are regulated in a tissue-specific manner (Leers-Sucheta *et al.*, 1997; Mason *et al.*, 1997; Gingras *et al.*, 2001; Simard *et al.*, 2005). The type 2 3β-HSD is predominantly expressed in steroidogenic tissues including the adrenal gland and ovary, whereas type 1 is found in

placenta and in non steroidogenic tissues such as liver, kidney and skin. Pregnenolone and progesterone form the precursors for all other steroid hormones.

2.6.1 Steroidogenesis in the Ovaries

The main role of the ovary is to produce eggs for fertilization and steroid hormones for sexual and reproductive function. The ovum inside the developing follicle is directly surrounded by layers of granulosa cells followed by thecal cells, which is where steroidogenesis predominantly takes place. The *theca interna* is highly vascularized and produces large amounts of progesterone and androgens, which act as precursor for oestrogen synthesis in the granulosa cells. Androstenedione and testosterone diffuse into the neighbouring poorly vascularized granulosa cells where they are converted to predominantly oestradiol via the concerted action of aromatase and 17β-HSD types 1 and 7, which favour the conversion of oestrone to oestradiol (Luu-The, 2001; Mindnich *et al.*, 2004). In the pre-ovulatory follicular stage during which the follicle matures, oestrogen synthesis increases gradually due to up regulation of aromatase by LH and FSH. During this critical phase, oestrogen appears to be responsible for the up regulation of LH receptors and the initiation of the positive feedback loop responsible for the LH and FSH surge which triggers ovulation (Greenwald and Roy, 1994). Interference with the synthesis of oestrogens during this critical window of time would prevent ovulation. After the LH surge, the follicle enters the luteal phase and becomes a corpus luteum which predominantly synthesizes progesterone. Decreased LH concentration and subsequently decreased aromatase expression result in declining oestrogen production (Fitzpatrick *et al.*, 1997), while a concurrent increase in CYP11A and 3β-HSD activity promotes the synthesis of progesterone which via its receptor initiates the process of follicle rupture.

2.7 Oestrogens

The oestrogens are a family of steroid hormones synthesized in a variety of tissues including ovaries, placenta and adrenal cortex (Tsang *et al*., 1980; Rotestein, 2011). They are responsible for the development and maintenance of the female sex organs and secondary sexual characteristics. More than 97% of circulating oestradiol is bound to plasma proteins. It is bound specifically and with high affinity to sex hormone binding globulin (SHBG) and non-specifically to albumin. Only a tiny fraction circulates as free (unbound) hormone (Martin *et al.*, 1981; Siiteri

et al., 1982; Rotstein, 2011). Both the free and albumin-bound fractions of oestradiol are thought to be available, but measurement of this (protein-bound) fraction has not been shown to be clinically important. In conjunction with progesterone, oestrogens also participate in the regulation of the menstrual cycle, breast and uterine growth as well as the maintenance of pregnancy (Carl and Edward, 2001). Oestrogenic activity is effected via oestrogenic-receptor complexes which trigger the appropriate response at the nuclear level in the target sites. These include ovarian follicles, uterus, breast, vagina, urethra, hypothalamus, pituitary and to a lesser extent the liver and skin (Carl and Edward, 2001). The principal function of the oestrogens is to cause the cellular proliferation and growth of tissues of the sex organs and other tissues related to reproduction (Tsang *et al*., 1980; Guyton and Hall, 2000). In the female, oestrogens cause (1) the development of the stromal tissues of the breast (2) growth of an extensive ductile system and (3) deposition of fat in the breast, subcutaneous tissues, the buttocks and thighs. More than 20 oestrogens have been identified, but only 17β –oestradiol (E_2), oestrone (E_1) and oestriol (E_3) are known to have clinical importance (Heldring *et al*., 2007). The most potent natural oestrogen secreted by the ovaries is 17β –oestradiol (Rotstein, 2011). It is a C18 steroid hormone with a phenolic ring and a molecular weight of 272.4 kDa (Tsang *et al*., 1980). In pregnancy, relatively more oestriol is produced and this comes from the placenta.

2.7.1 Metabolism of Oestrogens

Glandular synthesis of oestrogen occurs in the granulosa and theca cells of the ovaries, as well as the corpus luteum, while extraglandular synthesis is by aromatization of androgens in non gonadal sites. This is a complex process that involves three hydroxylation steps, each of which requires O_2 and NADPH (Mark and Paul, 2001). Oestradiol is formed if the substrate of this enzyme complex is testosterone, whereas, oestrone results from the aromatization of androstenedione. The conversion of androstenedione to oestrone is the major source of oestrogens in postmenopausal women from the aromatization in extragonadal tissues such as the liver, muscle and adipose tissues (Saten *et al*., 1986; Rotstein, 2011). Increase activity of the enzyme aromatase may contribute to excess oestrogen that characterizes such diseases as breast cancer among other chronic diseases. Oestrogens are catabolized mainly by hydroxylation reactions (Mark and Paul, 2001) resulting in the formation of; 2-hydroxyestrone and 2-hydroxyestradiol, 4-hydroxyestrone and 4-hydroxyestradiol and 16ά-hydroxyestradiol and 16ά-

hydroxyestrone. 4-hydroxyestrone and 16α-hydroxyestradiol of these metabolites are known to be oestrogenic and are thought to be carcinogenic (Mark and Paul, 2001).

2.8 Progesterone

Progesterone is a 21 carbon steroid that is primarily produced by the granulosa-lutein cells of the corpus luteum during the luteal phase and also by the syncytiotrophoblast of the placenta during pregnancy (Al-Asmakh, 2007). It is transported in the blood by transcortin and albumin with approximately 2% present in the free, unbound state. The half life of progesterone is approximately 5 minutes in the blood and its principal degradation product, pregnanediol, is formed in the liver. The plasma progesterone concentration is usually below 5 nmol/L (1.5 ng/ml) during the follicular phase of the menstrual cycle. However, it rises to the peak value of 40-50nmol/L (12-16ng/mL) in the luteal phase (Laycock and Wise, 1996; Pfeifer and Strauss, 1996; Al-Asmakh, 2007). Progesterone is essential for the regulation of normal female reproductive functions. Its major physiological actions are: facilitation of implantation and maintenance of early pregnancy in the uterus, lobular-alveolar development in preparation for milk secretion in the breast, neurobehavioral expression associated with sexual responsiveness in the brain and prevention of bone loss (Clark and Sutherland, 1990; Graham and Clarke, 1997; Genazzani *et al.,* 2000; Balasch, 2003).

2.8.1 The Function of Progesterone during the Menstrual Cycle

Progesterone is essential for the implantation and maintenance of early pregnancy. The follicular phase of the menstrual cycle is oestrogen dominated, while the luteal phase is progesterone dominated (Cameron *et al.,* 1996). The secretion of progesterone converts an oestrogen primed proliferative endometrium into a secretory one, which is receptive to the blastocyst. The granulosa cells in the follicle biosynthesize and secrete oestrogen before ovulation takes place. Upon follicle rupture and release of the ovum, these granulosa cells mature to form the corpus luteum, which is responsible for secretion of progesterone and oestrogen in the latter part of the cycle (Al-Asmakh, 2007). In humans, if fertilization does not occur within 1 to 2 days, the corpus luteum continues to enlarge for 10–12 days, this is followed by regression of the gland and concomitant cessation of oestrogen and progesterone release. The corpus luteum then continues to grow and function for the first 2 to 3 months of pregnancy if fertilization occurs.

After this time, it slowly regresses as the placenta assumes the role of hormonal biosynthesis for the maintenance of pregnancy (Graham and Clarke, 1997; Al-Asmakh, 2007).

2.8.2 The Effects of Progesterone on Ovulation and Luteinization

In primates, luteinization and follicular rupture occur 36–38 hours after the onset of mid-cycle gonadotropin surge. During this pre-ovulatory phase, granulosa cells undergo changes in response to the ovulatory stimulus that result in terminally differentiated luteal cells. These differentiating (luteinizing) granulosa cells secrete large amounts of progesterone (Suzuki *et al.,* 1994). Acute administration of 3ß-hydroxysteroid dehydrogenase (3ß-HSD) inhibitors or progesterone receptor antagonists prevented ovulations in monkeys (Hibbert *et al*., 1996) and mice (Loutradis *et al.,* 1991). Moreover, follicles from progesterone-depleted monkeys and progesterone receptor knockout mice (PRKO) do not luteinize (Lydon *et al.,* 1995). The increase in progesterone levels and in progesterone receptor expression within 12 hours of the ovulatory stimulus in the macaque (monkey) follicle supports a critical early role for progesterone in ovulation and luteinization (Chaffin *et al.,* 1999). The pre-ovulatory surge of gonadotropins activates a cascade of proteolytic enzymes resulting in the rupture of the follicular wall and the release of a fertilizable ovum during ovulation. Several lines of evidence support a role for progesterone in the induction of proteolytic activity in the pre-ovulatory follicle of primate and non-primate species. The levels of mRNAs for matrix metalloproteinases-1 (MMP-1) and tissue inhibitor matrix metalloproteinases-1 (TIMP-1) increased dramatically within 12 hours of gonadotropin stimulus and were up-regulated by progesterone (Chaffin and Stouffer, 1999). Moreover, inhibition of progesterone synthesis or blocking progesterone action with RU486 decreased MMP activity in the rat and ewe in a reported study (Curry and Osteen, 2003). A regulatory role for progesterone in the activation of other ovulation-associated proteases, such as plasminogen-activator (PA), has been suggested as well, because administration of a selective progesterone receptor antagonist, Org 31710 to gonadotropin-treated rats resulted in lower PA activity levels (Pall *et al*., 2000).

2.8.3 The Effects of Progesterone on Cellular Proliferation in the Uterus during the Menstrual Cycle

The changes in proliferative activities of the glandular epithelium and stromal elements of the human endometrium correlate with the circulating levels of oestrogens and progesterone. During oestrogen-dominated follicular phase, cellular proliferations occur in both epithelial and stromal cells. This is followed by a decline in proliferation in the first half of the secretory, progesterone-dominated phase of the cycle. In the late luteal phase, while proliferative activity remains low in the epithelium, a second peak of proliferation, consistent with decidual changes, is seen in the stromal elements (Al-Asmakh, 2007). Oestrogen stimulates epithelial cell proliferation, while progesterone opposes the mitotic effects of oestrogen and inhibits proliferation (Graham and Clarke, 1997; Conneely *et al*., 2002). In progesterone receptor knockout (PRKO) mice, ablation of both progesterone receptor-α (PR-A) and progesterone receptor-β (PR-B) isoforms resulted in a marked hyperplasia in the endometrial epithelium due to unopposed proliferative oestrogen action (Lydon *et al.,* 1995). However, in a PR-A knockout mice (PRAKO), in which the expression of the PR-A isoform is selectively ablated, the PR-B isoform functions to mediate rather than inhibit cellular proliferations. This gain of PR-B-dependent proliferative activity upon removal of PR-A indicates that PR-A is necessary not only to oppose oestrogen-induced proliferations, but also required to inhibit proliferations induced by progesterone acting through the PR-B proteins (Conneely *et al.,* 2002; Mulac-Jericevic and Conneely, 2004; Al-Asmakh, 2007).

2.8.4 Progesterone's Effects on Cellular Differentiation

As a result of the inhibitory effects of progesterone on cellular proliferation, progesterone induces secretory differentiation in the glandular epithelium and stromal fibroblast. The differentiating action of progesterone is terminal: if implantation does not occur, the tissue is shed and endometrial renewal from the basal portion of the endometrium takes place. Progesterone's effect on the stromal decidualization is described as the progesterone mediated differentiation of small stromal fibroblast into large epitheloid decidual cells. This process occurs around day 23 of the menstrual cycle and is accompanied in fertile cycles by the implantation event (Mulac-Jericevic and Conneely, 2004). The decidual reaction is inhibited in PRAKO mice, but not PRBKO mice, suggesting a critical significance of PR-A in this process (Conneely *et al.,*

2002; Mulac-Jericevic and Conneely, 2004; Brosens *et al.,* 2004). In humans, decidual transformation occurs in stromal cells surrounding the spiral arteries approximately 10 days after the postovulatory rise in ovarian progesterone level, indicating that the expression of the deciduas-specific gene is unlikely to be under the direct control of activated PR. Evidence has emerged to suggest that the initiation of decidual transformation requires elevated intracellular cAMP levels and sustained activation of protein kinase A (PKA) pathway (Gellersena and Brosens, 2003; Al-Asmakh, 2007).

2.8.5 The Roles of Progesterone on Menstruation and Regenerative Phase

Menstruation is defined as the shedding of the superficial layer of the endometrium due to withdrawal of progesterone following luteolysis (Cameron *et al.,* 1996). It is the result of enzymatic autodigestion and ischaemic necrosis. During the first part of the secretory phase, acid phosphatase and lytic enzymes are restricted to the lysosomes. Progesterone plays a role in stabilizing the lysosomal membranes. In the second part of the secretory phase, these lysosomal membranes are degraded resulting in the release of lytic enzymes into the cytoplasm and intracellular membrane. The lytic enzymes digest cell elements, including intracellular bridges and desmosomes (Bergeron, 2000). Matrix metalloproteinases have an important role causing degradation of many components of the uterine extracellular matrix, including proteoglycan, glycoproteins and basement membrane collagen (Curry and Osteen, 2003). There is substantial evidence that MMPs are produced in the endometrium and that expression of their mRNAs is closely correlated with the process of normal menstruation (Hampton and Salamonsen, 1994). Studies showed that production of endometrial MMPs is modulated by progesterone withdrawal in vitro and in vivo (Salamonsen *et al*., 1997; Zhang and Salamonsen, 2002). Progesterone has a role in maintaining coagulation and as a result any fall in serum progesterone level will engender fibrinolysis and initiate menstrual bleeding (Bergeron, 2000). Vasoconstriction of the spiral arterioles also plays a role in the breakthrough of the menstrual bleeding (Al-Asmakh, 2007). Prostaglandin F 2- alpha (PGF2α) causes vasoconstriction. It is negatively controlled by progesterone and causes reduction in blood flow to the corpus luteum, thus, it may cause luteolysis by depriving the gland of nutrients and substrates needed for steroidogenesis (Bergeron, 2000; Niswender *et al.,* 2000). Finally, apoptosis takes place. Apoptosis is a phenomenon regulated by the gene bcl-2 (B cell lymphoma/leukemia-2) causing gland cell death

and shedding of the menstrual blood (Bergeron, 2000). The protooncogene bcl-2 functions to prolong the survival of healthy and pathological cells by blocking apoptosis. Several studies showed a decrease in the expression of bcl-2 during menstruation and following the withdrawal of progesterone (Dahmoun *et al.*, 1999; Mertens *et al.*, 2002). Angiogenesis (new blood vessel formation) is rare in adult tissue, however, the female reproductive tract is an exception, with blood vessel formation taking place during regeneration, development of spiral arterioles in the late secretory phase and at the time of implantation. Three peaks of regeneration have been indicated in endometrial tissue. Two peaks of endometrial regeneration under the control of oestrogen occur immediately postmenstrually and during the mid proliferative phase of the cycle. The third peak is progesterone related and occurs during the secretory phase of the cycle. This peak involves the growth of spiral arterioles. The persistence of stromal progesterone receptors provides evidence that progesterone influence the development of spiral arterioles (Critchley and Heal.y, 1998).

2.9 Hormone Receptors and Breast Cancer

Oestrogen receptor (ER) and progesterone receptor (PR) play important roles in the growth and differentiation of breast cancers making them important prognostic markers (Patel *et al*., 2013; Mohamed *et al*., 2015; Deepti *et al*., 2015). Two isoforms of ER are known to exist; oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ) (Green *et al.*, 1986, Greene *et al.*, 1986, Kuiper *et al.,* 1996). A strong expression of ERα is reportedly observed in tissues related to female reproduction; ovary, womb, mammary gland (Kerdivel *et al*., 2013). Recent studies have reported a mild expression of ERβ in the mammary gland (Dotzlaw *et al*., 1997, Saji *et al*.2000). There is paucity of information on its role in mammary gland (Cowley *et al.*, 1997; Kuiper *et al*., 1997; Pace *et al*., 1997; Pettersson *et al*., 1997; Hansen and Bissell, 2000; Saji *et al*., 2000).

Oestrogen receptor, PR and HER 2 are determined by immunohistochemistry (Recareanu *et al*., 2011; Qiao *et al*., 2013). The biologic, prognostic and predictive importance of assessment of ER expression in breast cancer is well established. However, the added value of PR assessment appears controversial in some climes (Olivotto *et al*., 2004; Colozza *et al*., 2005; Fuqua *et al*., 2005; Hefti *et al*., 2013; Qiao *et al*., 2013). In spite of this, the American Society of Clinical Oncology and the College of American Pathologists recommend testing for both ER and PR on

all newly diagnosed cases of invasive breast cancer (Hammond *et al*., 2010). Since the 1970s, it has been hypothesized that PR expression will be associated with response to hormonal therapies in ER+ breast cancer, as it is thought that ER and PR co-expression demonstrates a functionally intact oestrogen response pathway (Horwitz *et al*., 1978; Horwitz and McGuire, 1978; Horwitz and McGuire, 1975; Horwitz and McGuire, 1979). Analyses from observational studies showed that loss of PR expression was associated with worse overall prognosis among ER+ breast cancers (Bardou *et al*., 2003; Grann *et al*., 2005; Dunnwald *et al*., 2007; Cancello *et al*., 2013; Prat *et al*., 2013). These results suggested that evaluation of PR status in ER+ breast cancer might be used to help guide clinical management, as high levels of PR expression may identify a subset of ER+ patients most likely to benefit from hormonal therapy (Davies *et al*., 2011; Hefti *et al*., 2013).

The biological and clinical significance of the ER-/PR+ breast cancer subtype has been reported to be controversial, with some reports claiming it represents a distinct, clinically useful biologic entity (Thor et al., 1998; Scawn and Shousha, 2002; Rakha *et al*., 2007; Rhodes and Jasani, 2009; Suvarchala and Negesrwararao, 2011; Al-Khafaji *et al*., 2014) while others are of the view that ER-/PR+ classification is primarily a technical artifact (De Maeyer *et al*., 2008; Nadji *et al*., 2005) and too rare to be of clinical use (Chariyalerstak *et al*., 1996; Olivotto *et al*., 2004). In large published series, the percentage of ER-/PR+ cases has been in the range of zero (Nadji *et al*., 2005) to four percent (Bardou *et al*., 2003; Colditz *et al*., 2004). In the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis, PR expression was not significantly predictive of tamoxifen treatment response in ER-negative breast cancer, although, there was a slight trend, which failed to reach statistical significance (Davies *et al*., 2011). In the EBCTCG analysis, the investigators noted that as methods for assessment of hormone receptor status have improved. The proportion of cases reported as ER-/PR+ has decreased from approximately 4% in the early 1990s to only 1% in recent SEER (Surveillance, Epidemiology, and End Results) cancer registry data. This suggests that as methods of ER testing and interpretations have improved, the rates of false negative ER results have decreased (Davies *et al*., 2011). However, There are reports that breast cancer patients with tumours that are ER+ and/or PR+ have lower risks of mortality after their diagnosis compared to women with ER- negative and/or PR-negative disease (Fisher *et al*., 1988; Parl *et al*., 1984; Crowe *et al*., 1991; Aaltomaa *et al*., 1991;

Lethaby *et al*., 1996; Anderson *et al*., 2001). Clinical trials have also shown that the survival advantage for women with hormone receptor-positive tumours is enhanced by treatment with adjuvant hormonal and/or chemotherapeutic regimens (Smith and Good, 2003; Goldhirsch, *et al*., 2003; Fisher *et al*., 2004).

Human epithelial receptor 2 (HER 2), a proto-oncogene also known as ErbB2-neu, located on chromosome 17q21 is also considered to be closely associated with occurrence and development of breast cancer (Gown, 2008). Under normal physiological conditions HER 2 is inactive; however, once activated it may enhance tumour invasion and metastases and increase the degree of malignancy (Revillion *et al*., 1998; Guo and Bai, 2008), which may explain HER 2 association with intermediate to high grade tumours and large tumour sizes (Makanjuola *et al*., 2014). Status of HER 2 is important when considering treatment choice especially for patients with metastatic tumours, who respond better to additional medication such as Herceptin (Cobleigh *et al*., 1999; Shak, 1999; Khokher *et al*., 2013).

Different expression patterns of ER, PR and HER2 have been identified, making the knowledge of the receptor content of breast carcinoma essential in planning the management of the disease (Low *et al*., 1992; Sacks and Baum, 1993). ER over-expression has been predominantly observed in lower grade, smaller size-tumours, more likely to be node negative, and shows better survival outcome than ER-negative cancers (Fisher *et al*., 1988; Low *et al*., 1992; Grann *et al*., 2005). PR over-expression is also associated with well differentiated tumours with good overall survival (Reiner *et al*., 1990). The over-expression of ER is reported to occur in approximately 70-80% of invasive breast carcinoma at the point of diagnosis (SjÃgren *et al*., 1998). Over-expression of HER2 is associated with higher grade (SjÃgren *et al*., 1998) and ER-negative tumours (Gago *et al*., 2006) which demonstrate poor overall survival (Yamauchi *et al*., 2001). The HER2 over-expression is reported to occur in 10-30% of invasive breast cancers (Ciocca *et al*., 2006). Another subtype usually identified in breast cancer classification is the triple-negative. Triple-negative breast cancers are tumours characterized by their lack of hormone receptors (ER and PR) and HER2. They are the most aggressive form and account for 10-17% of all breast cancers (Nwachukwu *et al*., 2009). This subtype is reportedly more prevalent in African-Americans than in their white counterparts (Carey *et al*., 2006; Bauer *et al*., 2007; Ihemelandu *et al*., 2007; Yang *et al*., 2007).

2.10 Mechanisms of Action of Sex Hormones

Oestrogen and progesterone promote proliferation and differentiation in the normal breast epithelium. They function via binding to their corresponding intracellular receptors, ER and PR, which are members of the nuclear hormone receptor super-family (Evans, 1988). The process by which oestrogen and progesterone interact with their receptors is similar for all members of the nuclear hormone receptor family (White and Parker, 1998). In the absence of hormones, the receptors are inactive. When hormones pass through the cell membrane and bind the receptors, the inactive oligomeric complex dissociates and the receptors are transformed into an active state that regulates gene expression either directly as a transcription factor by binding DNA at a specific response-element (Beato and Sanchez-Pacheco, 1996; Glass *et al.,* 1996; Horwitz *et al.,* 1996), or indirectly by cooperative interactions with other transcription factors e.g. activator protein 1 (AP-1) (Gaub *et al.,* 1990; Philips *et al*., 1993; Umayahara *et al*., 1994). As DNA-binding transcription factors, steroid hormone receptors do not function alone but interact with general transcription factors and receptor interacting proteins. In addition to this complexity, members of the nuclear hormone receptor super family are expressed in multiple forms.

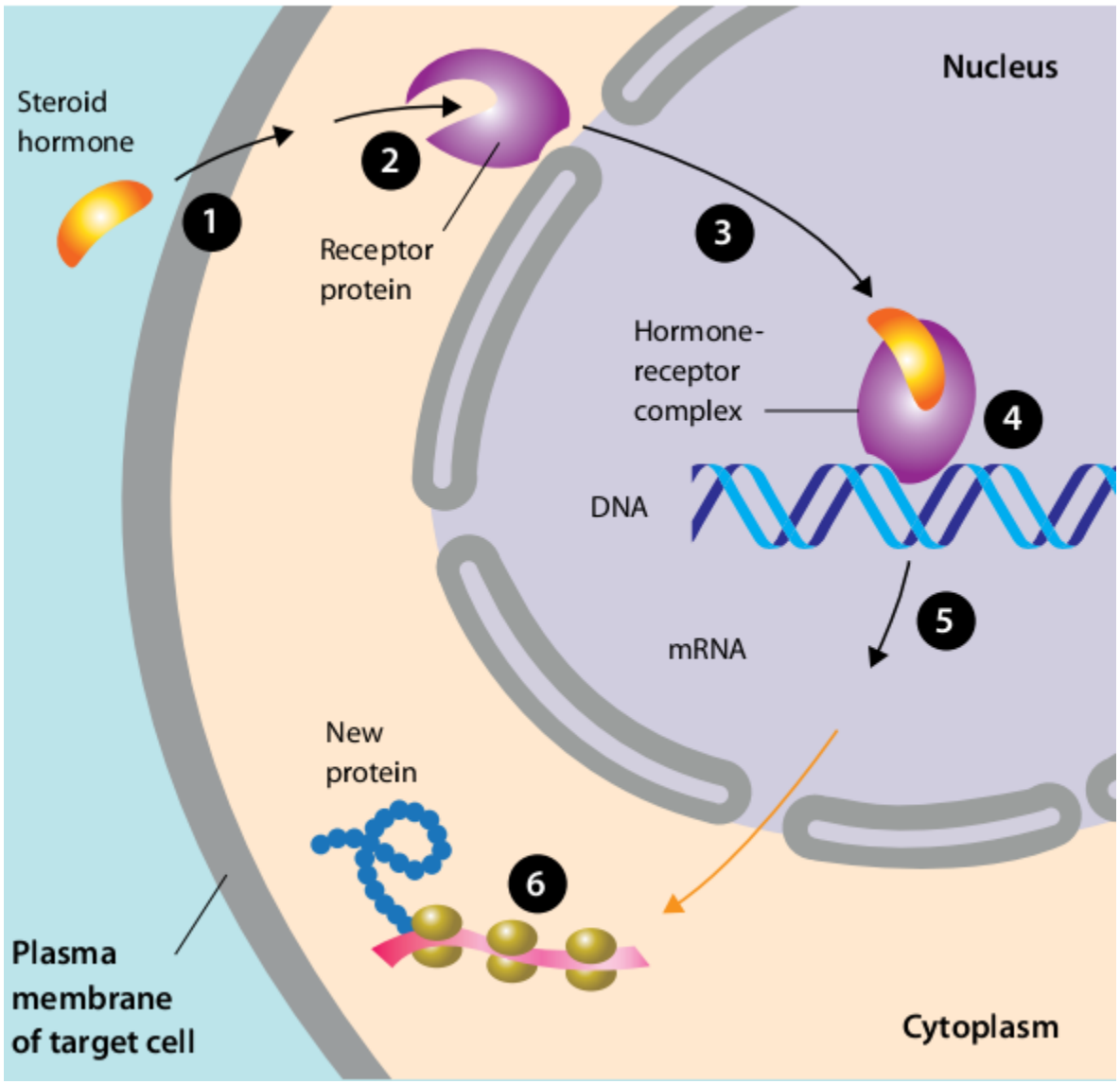

Figure 2.1: Mechanisms of Action of Sex Hormones (Bergman *et al*., 2013).

2.11 Oestrogens and Breast Cancer

Oestrogens play a role in breast cancer. It is thought that in promoting the growth of breast's end buds, oestrogens may also contribute to an increase in cells that later in life become prone to cancerous growth (Russo and Russo, 1998). During the periods when the duct structures grow, especially during puberty, the breast is particularly vulnerable to cancer-causing influences (Russo and Russo, 1998). The cyclical secretion of oestrogen during a woman's life is now recognized as a key determinant of breast cancer risk. The more oestrogens reach the sensitive structures in the breast during her lifetime, the higher the overall risk. Thus, every year of delay in the onset of regular ovulations corresponds to 5% reduction in breast cancer risk. Conversely, every year of delay in menopause increases the risk by 3% (Travis and Key, 2003). On the other hand, pregnancies have a protective influence (Hinkula *et al*., 2001). Each child birth is thought to decrease the risk of breast cancer by 7% and this effect is even more pronounced before the age of 20 years (Travis and Key, 2003). The very high levels of oestrogen and other hormones that are secreted during pregnancy stimulate the full maturation of the duct system of the breast. It is thought that this leads to a reduction in the number of cells in the buds that are vulnerable to cancer-causing factors and thus to a decrease in cancer risk. Moreover, E_2 not only trigger cell proliferation/division but alter breast micro-environment. They change intercellular communication and have systemic effects with secondary consequences for breast tissue. All these changes are important for the formation of new milk ducts during normal breast development and may promote progression of breast cancer (Brisken, 2008).

2.12 Progesterone and Breast Cancer

Progesterone's role in breast cancer is controversial (Ho *et al*., 2009). It has been hypothesised that its activity of opposing oestrogenic stimulation of the breast decreases breast cancer risk (Kelsey, 1979; Foidart *et al.*, 1998; Ho *et al*., 2009). On the other hand, some believe that the risk of breast cancer is increased because breast mitotic rates are highest in the luteal phase of the menstrual cycle (Harris *et al*., 1992; Foidart *et al*., 1998). In recent times, the findings of studies of serum progesterone levels in premenopausal women have been conflicting (Bernstein *et al*., 1990; Ho *et al*., 2009). A number of case-control studies have observed lower levels of serum progesterone in premenopausal cases (Key and Pike, 1988; Bernstein *et al*., 1990). Currently,

there is paucity of information on the serum progesterone levels in Nigerian women with breast cancer.

2.13 Follicle Stimulating Hormone (FSH)

Follicle stimulating hormone is a 35.5kD glycoprotein dimer. Its structure is similar to those of luteinizing hormone (LH), thyroid stimulating hormone (TSH) and human chorionic gonadotropin (hCG). The protein dimer contains 2 polypeptide units, labelled alpha and beta subunits. The alpha subunits of LH, FSH, TSH and hCG are identical and contain 92 amino acids. The beta subunits vary. Follicle stimulating hormone has a beta subunit of 111 amino acids (FSH β), which confers its specific biologic action and is responsible for interaction with the FSH receptor (Jiang *et al.,* 2012). The sugar part of the hormone is composed of fucose, galactose, mannose, galactosamine, glucosamine, and sialic acid, the latter being critical for its biologic half-life. The half-life of FSH is 3-4 hours.

2.13.1 The Physiological Roles of FSH in Females

Follicle stimulating hormone stimulates the growth and recruitment of immature ovarian follicles in the ovary. FSH is the major survival factor that rescues the small antral follicles (2–5 mm in diameter for humans) from apoptosis. In the luteal-follicular phase transition period, the serum levels of progesterone and oestrogen (primarily oestradiol) decrease and no longer suppress the release of FSH, consequently FSH peaks at about day three (day one is the first day of menstrual flow). The cohort of small antral follicles is normally sufficient in number to produce enough Inhibin B to lower FSH serum levels. In addition, there is evidence that gonadotropin surge-attenuating factor produced by small follicles during the first half of the follicular phase also exerts a negative feedback on LH secretion amplitude, thus allowing a more favourable environment for follicle growth and preventing premature luteinization (Fowler *et al.,* 2003). When the follicle matures and reaches 8–10 mm in diameter it starts to secrete significant amounts of oestradiol. Normally in humans, only one follicle becomes dominant and survives to grow to 18–30 mm in size and ovulate, the remaining follicles undergo atresia. The sharp increase in oestradiol production by the dominant follicle (possibly along with a decrease in gonadotropin surge-attenuating factor) cause a positive effect on the hypothalamus and pituitary gland,thus, rapid gonadotropin-releasing hormone (GnRH) pulses occur and an LH surge results.

The increase in serum oestradiol level causes a decrease in FSH production by inhibiting GnRH production in the hypothalamus (Dickerson *et al.*, 2008).

The decrease in serum FSH level causes the smaller follicles in the current cohort to undergo atresia as they lack sufficient sensitivity to FSH to survive. Occasionally two follicles reach the 10 mm stage at the same time by chance and as both are equally sensitive to FSH, both survive and grow in the low FSH environment and thus two ovulations can occur in one cycle possibly leading to non identical (dizygotic) twins. As a woman nears perimenopause, the number of small antral follicles recruited in each cycle diminishes and consequently insufficient Inhibin B is produced to fully lower FSH and the serum level of FSH begins to rise. Eventually, the FSH level becomes so high that down regulation of FSH receptors occurs and by menopause any remaining small secondary follicles no longer have FSH receptors (Radu *et al*., 2010). FSH binding is thought to upregulate neo-vascularization via at least two mechanisms – one is the Vascular Endothelial Growth Factor (VEGF) pathway and the other VEGF independent - related to the development of umbilical vasculature when physiological. This presents possible use of FSH and FSH-receptor antagonists as an anti tumour angiogenesis therapy (Radu *et al*., 2010).

2.14 Luteinizing Hormone (LH)

Luteinizing hormone, also known as lutropin or lutrophin is a hormone produced by gonadotroph cells in the anterior pituitary gland. In females, an acute rise of LH ("LH surge") triggers ovulation and development of the corpus luteum. (Louvet *et al*., 1975). It acts synergistically with FSH in females. LH supports theca cells in the ovaries that provide androgens and hormonal precursors for E_2 production. At the time of menstruation, FSH initiates follicular growth, specifically affecting granulosa cells (Mahesh, 2011). With the rise in oestrogens, LH receptors are also expressed on the maturing follicle, which causes it to produce more E_2. Eventually, when the follicle is fully mature, a spike in 17-hydroxyprogesterone production by the follicle inhibits the production of oestrogen, leading to a decrease in oestrogen-mediated negative feedback of GnRH in the hypothalamus, which then stimulates the release of LH from the anterior pituitary (Carr, 1998). This increase in LH production only lasts for 24 to 48 hours. This "LH surge" triggers ovulation, thereby not only releasing the egg from the follicle, but also initiating the conversion of the residual follicle into a corpus luteum that, in turn, produces

progesterone to prepare the endometrium for a possible implantation (Yeh and Adashi, 1999). If pregnancy occurs, LH levels will decrease, and luteal function will instead be maintained by the action of hCG, a hormone very similar to LH but secreted from the new placenta). The release of LH from the pituitary gland, and is controlled by pulses of GnRH. When the levels are low, GnRH is released by the hypothalamus, stimulating the pituitary gland to release LH (Carr, 1998; Yeh and Adashi, 1999; Yen, 1999).

2.15 FSH, LH and Breast Cancer

FSH stimulates follicle growth and development in the ovaries (Zhou *et al.,* 2013). FSH has been reported to be associated with certain cancers including prostate, endometrial and ovarian cancers (Ben-josef *et al.,* 1999; Bax *et al.,* 2000; Chen *et al*., 2009; Huhtaniemi, 2010). There are reports that FSH induces cancer cell proliferation, differentiation and metastasis by activating adenylyl cyclase, thereby resulting in increased cAMP levels (Tunizicker-Dunn and Maizels, 2006; Fan *et al.,* 2007). High FSH levels have been associated with a significantly poor prognosis in patients with premenopausal breast cancer (Pujol *et al.,* 2001). FSH has also been linked to breast cancer cell proliferation and an increased risk of breast cancer development in females who have undergone infertility treatments (Zreik *et al.,* 2010). While the oestrogen signal pathway on tumourigenesis and tumour progression in breast cancer has been widely discussed, there is paucity of information on the FSH and LH pathway(s) in breast cancer. Moreover, the specific functions of FSH and LH have not been fully elucidated with regards to the progression of breast cancer (Zhou *et al*., 2013). There is paucity of information on the role of gonadotropin and breast cancer based on menopausal status.

2.16 Thyroid Physiology and Pathophysiology

Thyrotropin Releasing Hormone (TRH) acts on the pituitary thyrotropes to stimulate both the synthesis and release of TSH (Krassas *et al*., 2010). TSH in turn controls the thyroid gland and the synthesis and release of thyroid hormones. TSH also controls the size and number of thyroid follicular cells. Thyroid hormones are the only iodine-containing substances of physiologic significance in vertebrates (Bello and Bakari, 2012). Thyroid cells actively extract and concentrate iodide from plasma. A tightly controlled feedback system exists between the thyroid gland, the hypothalamus and pituitary gland (Bello and Bakari, 2012). These three glands

function closely thereby ensuring that thyroid hormone concentration in the blood are maintained within certain limits in the face of large changes in basal metabolic and physiological need for thyroid hormone (Surks *et al.,* 2004). A rise in the serum thyroid hormone concentration elicits an inhibitory effect on the pituitary response to TRH (negative feedback). Thyroxine (T_4), a prohormone, is converted to triiodothyronine (T_3), the active form of thyroid hormone, in the peripheral tissues by 5'-deiodination. Normal thyroid gland produces all of the circulating T_4 and about 20% of the circulating T_3 (Surks *et al.,* 2004). Most of the biologic activity of thyroid hormones is due to the cellular effects of T_3, which has a greater affinity for the thyroid hormone receptor and is approximately 4 to 10 times more potent than T_4 (Surks *et al*., 1973; Sawin *et al*., 1977). 80% of serum T_3 is derived from the de-iodination of T_4 in tissues such as the liver and kidney. Once T_4 and T_3 are released into the circulation, they are bound by Thyroxine Binding Globulin (TBG), transthyretin (thyroxine-binding pre-albumin), and albumin. Thyroxine Binding Globulin has the highest affinity for T_4 and T_3 and the lowest capacity, whereas albumin has the lowest affinity and the highest capacity. Only the free (unbound) fraction of T_4 and T_3 is able to bind to specific thyroid hormone receptors in peripheral tissues and possesses biologic activity. Normally, approximately 0.03% of T_4 and 0.5% of T_3 is free (Oppenheimer *et al*., 1972; TNACB, 1996). Changes in the binding capacity of thyroid hormone transport proteins may significantly affect the measurement of total thyroid hormone concentration and thereby complicate the diagnosis of hypothyroidism. The accurate diagnosis of thyroid disease is more difficult in patients with multiple abnormalities in thyroid hormone-binding proteins (Robbins, 1992).

Localized disease of the thyroid gland that results in decreased thyroid hormone production is the most common cause of hypothyroidism. Under normal circumstances, the thyroid releases 100 to 125 nmol of T_4 daily and only small amounts of T_3. Decreased production of T_4 causes an increase in the secretion of TSH by the pituitary gland. TSH stimulates hypertrophy and hyperplasia of the thyroid gland and thyroid T_4-5'-deiodinase activity. This in turn causes the thyroid to release more T_3. Deficiency of the hormone has a wide range of effects, because all metabolically active cells require thyroid hormone. The systemic effects are due to either derangements in metabolic processes or direct effects by myxedematous infiltration (that is, accumulation of glucosaminoglycans in the tissues).

2.16.1 The Metabolism of Thyroid Hormones

One of the earliest recognized physiologic actions of thyroid hormones was its effect on the basal metabolic rate (Dickerman and De Vries, 1997). In general, thyroid hormone deficiency results in a reduction in the metabolic rate. This is manifested as the intolerance to cold temperatures experienced by many hypothyroid patients. Thyroid hormone is also an important modulator of intermediary metabolism. Thyroid hormone replacement therapy may slow the progression of coronary artery disease, because of its beneficial effects on lipids (Sundaram *et al.,* 1997). Glucose homeostasis may be altered due to the slower rate of glucose absorption from the gastrointestinal tract. Insulin secretion in response to glucose load varies in hypothyroid individuals, but there is evidence of insulin resistance and reduced glucose utilization (Pedersen *et al.*, 1988; Fowler *et al.,* 1996). Hypothyroid patients generally exhibit decreased appetite (Bello and Bakari, 2012). Some studies have found an association between thyroid hormones and adiposity. Leptin regulates the hypothalamic-pituitary-thyroid axis by regulating TRH gene expression in the paraventricular nucleus in the hypothalamus thus, prompting TSH to stimulate leptin secretion (Feldt-Rasmussen, 2007; Menendez *et al*., 2003; Oge *et al*., 2005; Santini *et al*., 2010; Mehran *et al*., 2014).

2.16.2 Thyroid Hormones and the Reproductive System

A possible relationship between the thyroid hormones and ovarian function has been well documented in the literatures based on in vivo studies. The effects of hypothyroidism on fertility are mediated by a disruption of gonadotropin secretion and steroidogenesis. Serum levels of FSH and LH may be increased, normal, or decreased, and the preovulatory LH surge may be absent (Ottesen *et al.,* 1995). Delayed LH response to GnRH has been reported in some hypothyroid women (Valenti *et al*., 1984; Marino *et al.,* 2006).

In females, hypothyroidism is associated with menstrual irregularities, i.e. changes in cycle length and amount of bleeding (Joshi *et al*., 1993). The latter is probably due to oestrogen breakthrough bleeding secondary to anovulation (Krassas *et al.,* 1999). Defects in haemostasis factors (such as decreased levels of factors VII, VIII, IX, and XI) that occur in hypothyroidism may also contribute to polymenorrhea and menorrhagia (Ansell, 1996). Menstrual disturbances

specifically, amenorrhea, clinical metropathia haemorrhagica (haemorrhage during the menstrual cycle), and menorrhagia was reported in patients with primary myxedema (Krassas *et al*., 2010).

Anovulation and infertility have also been reported in hypothyroid females (Stradtman, 1993). Hypothyroid women have decreased rates of metabolic clearance of oestrone and exhibit an increase in peripheral aromatization (Longscope *et al.,* 1990; Redmond, 2004). An increase in excretion of 2-oxygenated oestrogens has been reported in hypothyroid women (Gallergher *et al.,* 1966). Plasma binding activity of SHBG is decreased, which results in decreased plasma concentrations of both total testosterone and E_2 but their unbound fractions are increased. Alterations in steroid metabolism disappear when a euthyroid state is restored (Gordon and Southren, 1977).

2.16.3 Gonadotropins (LH, FSH) in Hyperthyroid Women

Gonadotropin dysfunction has been reported in women with hyperthyroidism. It was reported that the mean LH levels in both the follicular and luteal phases of the menstrual cycle are significantly higher in hyperthyroid women than in normal women (Akande and Hockaday, 1972). Similar results were obtained in women at the middle of the luteal phase of the menstrual cycle (Pontikides *et al.* 1990). Some authors found that LH secretion was increased, whereas, the pulsatile characteristics of LH and FSH secretion did not differ in patients when compared with controls in the early follicular phase of the menstrual cycle. However, LH peaks may be absent in patients with amenorrhea (Zähringer *et al.,* 2000). Serum LH levels decrease to normal after a few weeks of treatment with antithyroid drugs (ATD) (Akande, 1974). Baseline FSH levels may be increased, although, data on this are limited (Tanaka *et al*., 1981; Pontikides *et al.*, 1990). However, some reports claim that FSH levels remain normal in thyrotoxic women (Distiller *et al.,* 1975; Zähringer *et al.*, 2000). The mechanism for the increase in serum LH and FSH in hyperthyroid women is unclear (Krassas *et al.,* 2010). It has been reported that hyperthyroxinemia resulted in an augmented gonadotropin response to GnRH (Tanaka *et al.*, 1981). Other studies, however, have been unable to confirm these findings (Distiller *et al.*, 1975).

The biochemical and hormonal abnormalities, nutritional disturbances and emotional upheavals that are commonly associated with hyperthyroidism may individually or in combination be the cause of the menstrual disturbances (Krassas, 2005). A study in India showed menstrual

irregularities in 65% of hyperthyroid women, compared with 17% among healthy controls (Joshi *et al.*, 1993). These irregularities sometimes preceded the identification of thyroid dysfunction (Krassas *et al*., 2010). Similar results were observed in other studies (Krassas *et al.,* 1994). Although, these findings indicate that menstrual disturbances are 2.5-fold more frequent in thyrotoxicosis than in the normal population (Krassas *et al*., 2010).

2.17 Thyroid Hormones and Breast Cancer

The growing and developing breasts require the coordinated action of several hormones such as oestrogen (E_2), progesterone, and thyroid hormones (Lai, 2002; Neville *et al*., 2002). While oestradiol has been reported to be a potent mitogen for normal mammary gland, thyroid hormones appear to stimulate lobular development, contributing to the differentiation of normal breast tissue (Neville *et al.*, 2002). However, the relationship between breast cancer and thyroid hormone is controversial (Saraiva *et al*., 2005). Even though, many studies have shown that thyroid diseases are common in women with breast cancer, other reports have not confirmed this association (Gogas *et al.,* 2001; Turken *et al.,* 2003; Smyth *et al*., 1996; Smyth *et al*., 1998; Cengiz *et al*., 2004; Giustarini *et al*., 2006; Conde *et al*., 2006; Tosovic *et al*., 2010; Tosovic *et al*., 2012). Almost every form of thyroid disease including hyperthyroidism has been identified in association with breast cancer (Takatani *et al.,* 1989; Goldman. 1990; Cengiz *et al.,* 2004; Rasmusson *et al*., 1987; Lemaire and Baugnet-Mahieu, 1989; Takatani *et al*., 1989). For instance, it was speculated that subclinical hyperthyroidism in postmenopausal patients contributes to breast tumour growth **(**Saraiva *et al.,* 2005). It has also been suggested that free triiodothyronine (FT_3) plays an important role in the physiology of fibrocystic breast disease (Martinez *et al.,* 1995). There is currently paucity of information on the link of thyroid hormones with breast cancer in Nigeria, However, physiological concentrations of T_3, the more active form of thyroid hormone is reported to significantly enhance oestradiol growth stimulation of a number of human breast carcinoma cell lines (Shao *et al*., 1995). In T47D breast cancer cells, E_2 and T_3 similarly regulate cell cycle progression and proliferation raising the p53 level and causing hyperphosphorylation of pRb (Dinda *et al.,* 2002). Moreover, it was demonstrated that in breast cancer cell lines, T_3 at supra-physiologic concentrations and in the absence of oestradiol mimics the effects of oestradiol, possibly through the ER (Nogueira and Brentani, 1996).

2.18 Contraceptives Use and Breast Cancer Risk

More than 100 million women worldwide use Oral Contraceptives (OCs), which are the most commonly used contraceptive method for US women (Bensyl *et al.*, 2005). They are prescribed because they are reportedly safe, effective, well tolerated and convenient (Bensyl *et al.*, 2005). The effectiveness of OCs and of the other combination hormonal contraceptives including the patch and ring is 99.7% if used exactly as directed and only slightly lower at 92% if the dose is occasionally taken late or not taken. The effectiveness of other contraceptives ranges from approximately 85% for barrier methods such as condom, sponge, and diaphragm, to upward of 99% for intrauterine devices, subdermal implants, progesterone injection and sterilization in both men and women (Trussell, 1998; Implanon, 2006). There have been suspicions for many years that the use of hormonal contraception is linked to an increased risk of breast cancer. These suspicions have been fuelled by the fact that widespread use of hormonal contraceptives, particularly OCs has paralleled an increased incidence of breast cancer in many countries. Increasing evidence that breast cancer is hormonally mediated has heightened concern about a possible link. Yet the numerous investigations of possible OC/breast cancer associations that have been carried out around the world have not provided conclusive answers (Trusell, 1998; Casey *et al.*, 2008). In general, these studies have been characterized by weak, sometimes conflicting associations (PATH, 1997). It has been reported that 5 years of combined Hormone Replacement Therapy (HRT) of oestrogen and progesterone was associated with a 26% increased risk of invasive breast cancer in postmenopausal women (WGWHII, 2002). Moreover, the carcinogenic effect of oestrogen-progestagen contraceptives and replacement hormones has been reported (Cogliano *et al.*, 2005). This has been confirmed and acknowledged by the World Health Organization (IARC, 2005). However, progestin-only pills were associated with a relative risk of breast cancer of 1.17 within 5 years of use, while the relative risk for use within 10 years is 0.99 (Casey *et al.*, 2008).

Two major potential mechanisms have been postulated by which oestrogens (both endogenous and exogenous) increase the risk of breast cancer. The first mechanism is the stimulation of oestrogen receptor–mediated transcription that results in cell proliferation. The second mechanism is direct carcinogenesis via metabolic activation and direct binding of DNA. One

hypothesis is that these 2 mechanisms act in an additive or even synergistic fashion to induce carcinogenesis (Yager, 2000; Santen *et al*., 2004).

2.19 Induced Abortion (IA) and Breast Cancer

Childbearing has been consistently shown to reduce the risk of breast cancer in the long term (CGHFBC, 1996). Until recently, incomplete pregnancies were thought to have no effect, or perhaps slightly reduce the risk of breast cancer (Vessey *et al*., 1982). However, the outcome of a study involving literature review suggested that induced abortion might increase the risk of breast cancer (Remennick, 1990). This was further supported by similar findings (Brind *et al.*, 1996). However, others who reviewed the evidence made by Remennick (1990) and Bind et al (1996) arrived at different conclusions (Michels and Willett, 1996; Wingo *et al*., 1997; Batholomew and Grimes, 1998). Induced abortion (IA) was reportedly significantly associated with an increased risk of breast cancer among Chinese females, and the risk of breast cancer increased as the number of IA increased (Huang *et al*., 2014). As of 2004, 41 studies had been published in the worldwide medical literature (including 16 American studies) reporting data on the risk of breast cancer among women with a history of induced abortion (AAPLOG, 2008). Twenty nine (70%) of these studies, reported increased risk. Thirteen of the 16 (81%) American studies reported increased risk, 8 (50%) with statistical significance (at least 95% probability that the result was not due to chance) irrespective of age at first full-term pregnancy. The relative risk increase of the 41 studies combined was 30% (AAPLOG, 2008). In the current American abortion experience, this would result in approximately 5,000 additional cases of breast cancer per year in the U.S. (There are about 190,000 new cases of breast cancer diagnosed in the US each year) (AAPLOG, 2008). Moreover, a 50% breast cancer risk increase by age 45 in United States' women who have had an induced abortion has been reported (Daling *et al*., 1994). However, a 12% lifetime chance of developing breast cancer was equally reported. Among women with a family history of breast cancer (mother, grandmother, sister or aunt), the increase in risk was 80% (Daling *et al.*, 1994). Few studies have focussed on the association of induced abortion with breast cancer risk in indigenous African women.

2.19.1 The Proposed Mechanism of Induced Abortion in Breast Cancer

The hypothesized mechanism by which induced abortion influence the development of breast cancer has been described (Russo *et al*., 2001). Prior to puberty, a woman's breast contains immature lobules, called type 1 lobules. After puberty, with increasing oestrogen levels, these lobules begin to increase in number and in maturity, and are called type 2 lobules. Pregnancy produces a huge increase in oestrogen levels (about 20 times non-pregnant levels). This causes an immense increase in the number of type 1 and 2 (relatively immature, in accelerated growth phase) lobules. More vulnerable lobules make more places where cancer can start. In the 3rd trimester and with lactation, the lobules complete their maturation into type 3 and 4 lobules, which have been reported to be more resistant to cancer influences/genetic mutations than are the less mature type 1 and type 2 lobules. The post abortive woman is left with a huge increase in the more vulnerable type 1 & 2 lobules. Thus, the process of lobular maturation in a full term pregnancy could account for "the protective effect" that is observed. Abortion abruptly interrupts this process before the 3rd trimester maturation of lobules happens by causing an immediate and marked drop in the oestrogen levels. This leaves the type 1 and 2 lobules, now greatly increased in number, in non-mature (only partially differentiated) growth phase. This could make them more susceptible to malignant change with exposure to carcinogens at a future time. This could be a major factor in the increased risk between induced abortion and subsequent breast cancer that many studies show (Russo *et al.*, 2001; Beiler *et al*., 2003; Butt *et al*., 2012).

2.20 Diet and Breast Cancer Risk

2.20.1 Fibre-Based Diet

A plant based diet is naturally high in fibre. A diet rich in natural fiber obtained from fruits, vegetables, legumes (lentils, split peas, black beans, pinto beans etc.), and whole-grains may reduce cancer risk and/or reduce risk of cancer progression (Harris *et al*., 1993). Certain case-control studies have reported that the greater the fibre intake, the lower the incidence of breast cancer (Howe *et al*., 1990; Freudenheim *et al*., 1996; De Stefani *et al*., 1997; La Vecchia *et al*., 1997; Challier *et al*., 1998). A high fibre diet is also associated with less obesity (Stoll, 1996). However, data from prospective studies is mixed, reporting protective effects (Rohan *et al*., 1993; Mattisson *et al*., 2004) or no effect observed (Terry *et al*., 2002; Cho *et al*., 2003). Total

dietary fibre intake, particularly from cereals and fruit was found to reduce the risk of breast cancer in premenopausal but not postmenopausal women (Cade *et al*., 2007). Moreover, a cohort study reported that high fibre intake was associated with a 42% lower risk of postmenopausal breast cancer (Mattisson *et al*., 2004). Indigenous African women who ate beans and lentils at least twice a week had a 24% lower risk of developing breast cancer than women who ate them less than once a month (Adebamowo *et al*., 2005).

Various mechanisms have been proposed for the protective effects of dietary fibre against cancer. These include: Increased faecal bulk and decreased intestinal transit time, which allow less opportunity for faecal mutagens to interact with the intestinal epithelium (Slavin, 2000), binding to bile acids, which are thought to promote cell proliferation (Slavin, 2003). Fermentation in the gut produces short-chain fatty acids (SCFA) which improves the gut environment and may provide immune protection beyond the gut (Slavin, 2000; Slavin, 2003). Additionally, whole grains are rich in antioxidants, including trace minerals and phenolic compounds, which have been linked to disease prevention (Slavin, 2003). Furthermore, there are reports that a high fibre diet works to reduce hormone levels that may be involved in the progression of breast cancer (Bagga *et al*., 1995; Stoll, 1996; Slavin, 2000; Rock *et al*., 2004; Wayne *et al*., 2007). In a high-fibre, low-fat diet intervention study, fibre reduced serum E_2 concentration in women diagnosed with breast cancer, the majority of whom did not exhibit weight loss. Thus, increased fiber intake was independently related to the reduction in serum oestradiol concentration (Rock *et al*., 2004). This decrease in oestrogen levels in the blood thereby may potentially reduce the risk of hormone-related cancers, such as breast cancer (Slavin, 2000; Rock *et al*., 2004). Reduced levels of serum oestrone and oestradiol were observed in premenopausal women with a greater intake of dietary fibre (Bagga *et al*., 1995). Similarly, a high intake of dietary fibre was significantly associated with low serum levels of oestradiol in postmenopausal breast cancer survivors (Wayne *et al*., 2007). Dietary fibre intake increases the amount of oestrogen excreted in the stool (Goldin *et al*., 1982).

.2.20.2 Fruits and Vegetables

Fruits and vegetables contain vitamins, minerals, fibre, and various cancer-fighting phytonutrients (i.e. carotenoids, lycopene, indoles, isoflavones, flavonols). Vibrant, intense colour is one indicator of phytonutrient content in fruits and vegetables. There is extensive and

consistent evidence that diets high in fruits and vegetables are associated with decreased risks of many cancers, and while results for breast cancer risk are not yet conclusive, they are promising (Riboli and Norat, 2003; Gaudet *et al*., 2004; Hirose *et al*., 2005; World Cancer Research Fund, 2007; de Lima *et al*., 2008). In a study of about 3000 postmenopausal women, a protective effect for vegetables was observed (Gaudet *et al*., 2004). Women who consumed 25 or more servings of vegetables weekly had a 37% lower risk of breast cancer compared with women who consumed fewer than 9 vegetable servings weekly. An epidemiological study reported a significant protective effect of vegetables against breast cancer when case-control and cohort studies were considered together (Riboli and Norat, 2003). A recent case-control study reported women who consumed more than 3.8 servings of fruits and vegetables daily had a lower risk of breast cancer when compared with women who consumed fewer than 2.3 daily servings (Shannon *et al*., 2005). Japanese women following a prudent dietary pattern (high in fruits and vegetables, low in fat) had a 27% decreased risk of breast cancer (Hirose *et al*., 2007). A Korean case-control study reported that a high intake of certain fruits and vegetables resulted in a significantly lower risk of breast cancer in premenopausal and postmenopausal women (Do *et al*., 2007). These observations indicate that regular consumption of fruits and vegetables could reduce the risk of breast cancer.

2.20.3 Refined Carbohydrates

When carbohydrates are refined, nearly all of the vitamins, minerals and fibres are removed leaving only calories. Certain products like white flour and sugars are refined and then enriched meaning that only certain nutrients removed in the refining process are added back into the product. In white flour, the kernel of the grain is processed to remove the germ portion. This removes about 33 nutrients. Enriching adds 4-6 nutrients back into the product. This creates the nutritive deficit. White flour is literally sugar in itself, and where it is mixed with fats in processed foods, the fats are commonly hydrogenated, increasing consumer's susceptibility to a number of disease processes (Sieri *et al*., 2007). A case-control study reported that carbohydrate intake significantly increased the risk of breast cancer; sucrose (table sugar, a refined carbohydrate) imparted the greatest risk (Romieu *et al*., 2004). This risk was lessened considerably with a higher fibre intake. Adding credence to the idea that blood sugar levels may affect disease progression. Women who consumed a high glycemic index (GI) and glycemic load

(GL) diet had a high risk of breast cancer. This effect was reportedly most pronounced in premenopausal women and those women of a healthy body weight (Sieri *et al*., 2007). Similarly, GI and GL were both associated with an increased risk of breast cancer among postmenopausal overweight women; this effect was most pronounced for women with ER (negative) breast cancer (Lajous *et al*., 2008). A meta-analysis showed that GI to modestly increased the risk of breast cancer (Barclay *et al*., 2008).

2.20.4. Meat

Reports have associated the consumption of red meat with the risk of breast cancer (Zheng *et al*., 1998; Taylor *et al*., 2007). Meat consumption increased the risk of breast cancer risk by 56% for each additional 100 g (3.5 oz) daily of meat consumption in a French case-control study (Wakai *et al*., 2005). Regular consumption of fatty red meat and pork fat greatly increased the risk of breast cancer in a Brazilian study (Di Pietro *et al*., 2007). In a study of over 35,000 women, meat consumption significantly increased the risk of breast cancer in both premenopausal and postmenopausal women (Taylor *et al*., 2007). Women who eat 1.75 ounces of processed meat daily, increased the risk of breast cancer by 64% in postmenopausal women compared to women who did not eat meat (de Lima *et al*., 2008). Consumption of red and fried meat quadrupled the risk of breast cancer in a case-control study in Brazil (de Lima *et al*., 2008). A large case-control study found that women who consumed meat for hamburger, bacon, and steak had a 54%, 64%, and 221% increased risk for breast cancer, respectively (Zheng *et al*., 1998).

2.21 Alcohol Consumption and the Risk of Breast Cancer

Alcohol consumption has been considered a plausible risk factor of breast cancer (Qian *et al*., 2014). Certain studies found a positive relationship between alcohol consumption and breast cancer (Nasca *et al.,* 1990; Bowlin *et al*., 1997; Thun *et al*., 1997; Bagnardi *et al*., 2001; Hamajima *et al.,* 2002; Key *et al*., 2006; Suzuki *et al.,* 2008) However, other studies did not (Kinney *et al*., 2000; Zhang and Holman 2011; Llanos *et al*., 2012; Chandran *et al*., 2013). Studies of the relationship between alcohol consumption and breast cancer risk among African-Americans have found inconclusive results (Hiatt and Bawol 1984; Hiatt *et al.,* 1988; Brinton *et al.,* 1997; Kinney *et al*., 2000; Zhu *et al*., 2003). The extent of alcohol drinking's effect on breast cancer risk may vary across races, possibly due to different drinking habits, metabolism and

genetic factors (Dumitrescu and Shields, 2005). In general, alcohol drinking is less common among African women than their counterparts in North America and Europe (Martinez *et al*., 2011; Peer *et al*., 2014). This could be due to racial differences in the distributions of genetic polymorphisms related to ethanol metabolism (McCarver *et al*., 1998; Dumitrescu and Shields, 2005). As women in Africa are increasingly influenced by western cultures and begin to change their lifestyle and as the populations in African countries are becoming more affluent, more and more women may be exposed to alcohol (Martinez *et al.,* 2011; Francis *et al*., 2014; Peer *et al*., 2014). There is currently paucity of information on the association of alcohol consumption with the risk of breast cancer in indigenous sub-Saharan African women.

2.22 Endocrine Disruptors (EDs)

An endocrine disruptor is defined as an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub) populations **(**Sprangler, 1996; IPCS, 2002). There is increasing evidence that various chemicals introduced into the environment have the potential to adversely interfere with the endocrine system in humans and wildlife (IOMC, 2013). EDs are widespread in food chains and in the environment. Certain studies have found that potential EDs at very low levels in the environment may result in harmful effects especially when several different compounds act on one target. The homeostasis of sex steroids and the thyroid appears to be the main targets of endocrine disrupting substances (Caserta *et al*., 2008).

Many EDs have been reported to act as agonists of oestrogen receptors (ER), e.g. bisphenol-A, or to antagonize androgen receptor (AR). Progesterone receptors are also a potential target for many chlorinated endocrine disruptor (Scippo *et al*., 2004). Some of these endocrine disruptors could also inhibit hormone synthesis, transport or metabolism. Moreover, some could inhibit the conversion of androgens to oestrogens (Matsui *et al*., 2005). Despite several studies done on endocrine disruptors, relatively few studies have addressed the roles of known carcinogens, such as metals in the initiation, promotion and progression of breast cancer (Adachi and Tainosho, 2004).

2.22.1 Metabolism of Toxic Metals

Humans have found an increasing number of uses for various metals in industry, agriculture, and medicine since the industrial revolution (Juracek and Ziegler, 2006). These activities have increased exposure not only to metal-related occupational workers, but also to consumers of the various products (Adachi and Tainosho, 2004). Metals like lead, cadmium and arsenic can be harmful pollutants when they enter the soil and water. Once in the environment, metals are almost impossible to eliminate because they do not decompose. Metals get into the body through air, food, water, or dermal exposure. They cross the plasma membrane to enter the cell in order to exert toxicity. Lipophilic metals like the arsenic and cadmium readily penetrate the plasma membrane (Lakowicz and Anderson, 1980). Cadmium can also bind to a protein, metallothionein to form cadmium-metallothionein, which allows cadmium to be actively taken into the cell by endocytosis (Antila *et al.* 1996). Other metals, like lead may be absorbed by passive diffusion (Karmakar and Jayaraman 1988).

These metals among other toxic metals have been reported as a major source of oxidative stress (Ragab *et al*., 2014). Oxidative stress describes the steady state level of oxidative damage in a cell, tissue, or organ caused by Reactive Oxygen Species (ROS). Oxidative stress occurs when the generation of ROS in a system exceeds that system's ability to neutralize and eliminate them. The imbalance can result from a disturbance in production or the distribution of antioxidants, as well as an overabundance of ROS from an environmental or behavioral stressor (Danilova 2006). Oxidative stress induces a cellular redox imbalance which has been found to be present in various cancer cells compared with normal cells; the redox imbalance thus may be related to oncogenic stimulation (Valko *et al*., 2007). The permanent modification of genetic material resulting from oxidative damage incidents represents the first step involved in mutagenesis and carcinogenesis. Elevated levels of oxidative DNA lesions have been noted in various tumours, strongly implicating such damage in the aetiology of cancer (Valko *et al*., 2007).

Moreover, these metals could also act through direct binding to DNA (De Bont and van Larebeke, 2004). They have been shown to directly modify and/or damage DNA by forming DNA adducts that induce chromosomal breaks (Chakrabarti *et al*., 2001). DNA damage can result in the arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Valko

et al., 2006). DNA damage, mutations and altered gene expression are thus key players in the process of carcinogenesis (Hartwig *et al*., 2002; Valko *et al*., 2007).

2.22.2 Toxic Metals and Breast Cancer

Heavy metals are reported to play critical roles in cancer biology (Kirkwood, 2002; Ragab *et al*., 2014). A large number of epidemiological studies indicate a close association between heavy metals such as lead (Pb), arsenic (As), cadmium (Cd) and development of breast cancer (Ragab *et al*., 2014). There are reports that these metals are a major source of oxidative stress (Leonard *et al.* 2004; Wang *et al.*, 2004; Hei and Filipic, 2004). Substantial data suggest that oxidative stress is involved in the development of breast cancer (Gammon *et al*., 2002; Wu, 2004; Rossner, 2006). Certain studies suggested that these toxic substances are agonists or antagonists for the oestrogen receptor in various *in vitro* systems. Although, usually with very low affinities relative to endogenous hormones such as 17β-oestradiol and oestrone (Stoica *et al*., 2000a; Johnson *et al*., 2003).

2.23 Cadmium (Cd)

Cadmium is a toxic, bio-accumulating, non-essential and highly persistent heavy metal with a variety of known adverse health effects (McElroy *et al.*, 2006). It occurs naturally in the soil, rocks and water. It has been classified among the most important carcinogens (EPA, 1987; Garcia-Morales *et al.*, 1994; Stoica *et al.*, 2000b; Johnson *et al.*, 2003). For non-occupationally exposed women who do not smoke, food is the largest source of Cd intake (Amzal *et al*., 2009). Particularly, root vegetables, potatoes, and grain, including rice and wheat, grown on Cd rich soils, and shellfish (Vahter *et al*., 1996; Mueller *et al.*, 1996; McLaughlin *et al*., 1997; Olsson *et al.*, 2005; Perez and Anderson, 2009; EFSA, 2009; Reuben, 2010). Inhalation of tobacco smoke is the predominant source of exposure (CDC, 2005) for smokers. The estimated daily intake of Cd in food in a non-hazardous environment for heavy metals is between 8 and 25 μg/day whereas one pack of cigarettes is estimated to add 1 μg/day (Satarug *et al*., 2010). Only a small fraction of inhaled or ingested Cd is excreted, resulting in increased body burden over time (Klaassen, 1981; Fujishiro *et al*., 2012; Tekin *et al*., 2012). Women tend to have higher Cd levels than men presumably because of lower iron stores, which increase Cd absorption (Olsson *et al*.,

2002; Reeves and Chaney, 2008). Thus, comparable environmental exposures to Cd may disproportionately affect women compared to men (Reeves and Chaney, 2008).

Cadmium-containing products are rarely recycled. Instead, they are frequently dumped together with household waste, thereby contaminating the environment, especially if the waste is incinerated. Cadmium is a known cumulative toxicant with a biological half-life of more than 10 years in humans. Thus, chronic low level exposure will eventually result in accumulation to toxic levels. Cadmium has the potential to disrupt endocrine function by behaving like sex hormones (Stoica *et al.,* 2000b). At low concentrations the metal mimics the effects of oestradiol and binds with high affinity to the hormone-binding domain of ER-alpha. This binding involves several amino acids, suggesting that Cd activates the receptor through the formation of a complex with specific residues in the hormone-binding domain (Stoica *et al.,* 2000b; Johnson *et al*., 2003). Cadmium affects cell proliferation, differentiation, apoptosis and signal transduction by enhancement of protein phosphorylation and activation of transcription and translation factors (Siewt *et al.,* 2010). Early puberty has been associated with breast cancer (Colditz and Frazier, 1995; Hamilton and Mack, 2003; Johnson *et al*., 2003).

2.24 Lead (Pb)

The heavy metals of greatest concern for health with regard to drinking water exposure are Pb and arsenic (ATSDR, 2005). Lead in gasoline was removed during the early 1990s. Lead solder in food cans was banned in the 1980s and Pb in paint was severely restricted in 1978 in the U.S. Both the nervous and reproductive systems are susceptible targets for Pb toxicity. Results of epidemiologic studies investigating the association of Pb exposure with cancer are inconsistent and vary according to the type of cancers reported (Steenland *et al.,* 1992; Wong and Harris, 2000). The ability of Pb to function as potent oestrogens suggests that it may be an important class of endocrine disruptors (Martin *et al.,* 2003). There are reports from New Delhi, India and Cairo, Egypt that support an association between environmental exposure to Pb and the risk of breast cancer (Siddiqui *et al*., 2003; Ragab *et al.,* 2014). There is currently a paucity of information on the role of Pb in breast cancer aetiology in Nigeria. It is however reasonable to examine the possible association between environmental exposure to Pb and risk of breast cancer, given the known impact of Pb on human health. The mechanisms of Pb carcinogenicity

involve direct DNA damage as a result of oxidative stress, clastogenicity, inhibition of DNA synthesis or repair (Martin *et al.,* 2003; Ragab *et al.,* 2014).

2.25 Arsenic (As)

The major source of human exposure to As is through food. Microorganisms convert As to dimethylarsenate, which can accumulate in fish, providing a source for human exposure (ATSDR 2005). Arsenic compounds are lipid soluble and within 24 hours of absorption distribute throughout the body where they can bind to sulfhydryl (SH) groups on proteins. Arsenic may also replace phosphorus in bone tissue and be stored for years (Bartolome *et al*., 1999). Methylation efficiency in humans appears to decrease at high As doses and studies show that aging is associated with a diminishing capacity to methylate inorganic As, resulting in its increased retention in soft tissues (Tseng *et al*., 2005). The oestrogenic-like activities of As have been studied in human ER-positive breast cancer cell line MCF-7 (Stoica *et al.,* 2000a; MartÃnez-Campa *et al*., 2006).

Arsenite (AsO_3^{3-}) blocked the binding of oestradiol to ER-alpha, acted as a ligand for ER activating it in the absence of hormone, suggesting that the metal interacts with the hormone binding domain of the receptor. It increased cell growth and mimicked the effects of oestradiol, decreased the amount of ER-alpha and increased the expression of the progesterone receptor (Stoica *et al.,* 2000a). Kaltreider *et al*. (1999) in a recent study examined the effect of single low-dose As, potentially directly relevant to human exposures, on binding of transcription factors in human MDA-MB-435 breast cancer and rat H4IIE hepatoma cells. These transcription factors were sensitive to the toxic metal at low doses. The specific effects were dependent on the transcription factor, time, dose, and cell line. This study showed that alteration in gene expression may play a role in long term effects of low dose environmental exposures, such as in metal induced carcinogenesis. Regulation and activation of transcription factors is an important part of mediating cellular response to target genes by metals. However, the pathways remain to be known.

2.26 Bisphenol-A (BPA)

Bisphenol-A is formed by the condensation of phenol with acetone. It has a low vapour pressure, high melting point and moderate solubility (Howard, 1989; Cousins *et al*., 2002; Shareef *et al*.,

2006). It is thus expected to have low volatility. Less than 1% of environmental BPA is thought to occur in the atmosphere, where it is believed to photooxidize and breakdown rapidly (Cousins *et al*., 2002; Howard, 1989). It is estimated that the largest environmental compartments of BPA are abiotic and are associated with water and suspended solids, soil, or sediments (Staples *et al*., 1998; Cousins *et al*., 2002; Environment Canada, 2008). Bisphenol-A has become ubiquitous in the environment within the past 80 years. This is because of its presence in a multitude of products including food and beverage packaging, flame retardants, adhesives, building materials, electronic components, and paper coatings (Staples *et al*., 1998; Flint *et al*., 2012). This has resulted in a widespread human exposure (Brody *et al*, 2007; Betancourt *et al*, 2012). As demand for these products has increased, so has BPA production. In 1964, 42 metric tons of BPA were produced in the United States (Dermer, 1977). As at 2003, global production of BPA was 3.2 million metric tons (Tsai, 2006), approximately one-third of which was manufactured in the United States (NIH, 2008). Global consumption of BPA in 2011 was predicted to exceed 5.5 million metric tons (Greiner *et al*., 2007). The oestrogenic effects of BPA were first reported in 1936 (Dodds and Lawson, 1936) but its use as a synthetic oestrogen was not pursued (Dodds *et al*., 1938). A study indicates that BPA may be as effective as oestradiol in triggering some receptor responses (Stahlhut *et al*., 2009) and it may act as an androgen receptor antagonist (Roy *et al*., 2004; Zoeller *et al*., 2005; Urbatzka *et al*., 2007). The safety of BPA is currently controversial. High levels of these endocrine disruptors have been suggested in the serum of breast cancer patients (Brisken, 2008; Calafat *et al*, 2013).

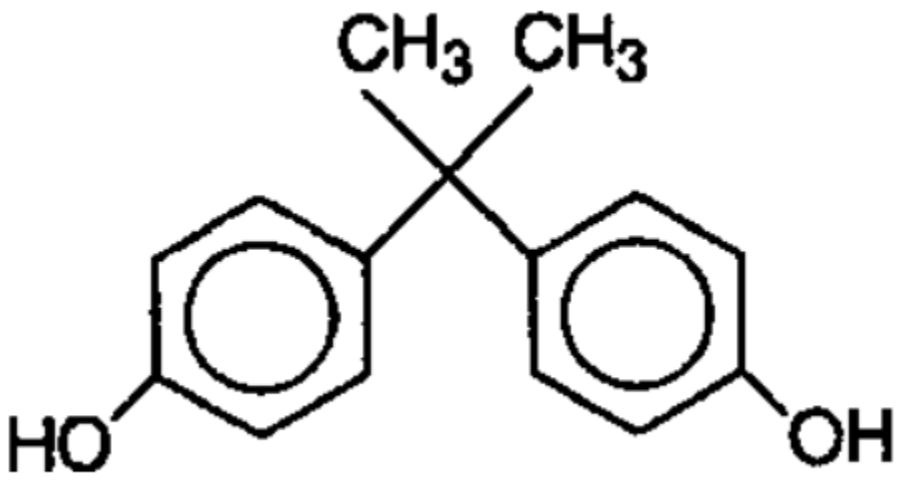

Figure 2.2: The chemical structure of bisphenol-A (Nieminen, 2002).

2.27 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls are members of a chemical family that were widely used in the past in industry as lubricants, coatings and insulation materials for dielectric equipment like transformers and capacitors (Iyengar, 2005; Gray *et al*, 2009). Human exposure to PCBs is through inhalation of contaminated air (outdoor or indoor), ingestion of contaminated food or non-food items, and dermal contact of contaminated surfaces. The primary route of exposure to PCBs is through consumption of contaminated lipid-enriched foods (e.g. fish and cooking oils) as PCBs can accumulate in these and other foodstuffs (Van-Emon *et al*., 2013). Polychlorinated biphenyls were classified as probable human carcinogens (2A group) (Van-Emon *et al*., 2013). The semi-volatile chemically stable nature of these compounds, combined with their resistance to bio-degradation and photolysis, has resulted in "global distillation" and redistribution via the atmosphere (Atlas *et al*., 1986; Atlas and Giam, 1998; Van-Emon *et al*., 2013). Concern over the harmful ecological and human effects and the persistence of PCBs in the environment led the United States Congress to ban their domestic production in 1977. Polychlorinated biphenyls are still detected in various micro-environments (e.g., air, soil, dust, sediment, food, tissue) either as Aroclors or as individual congeners (Wilson *et al*., 2003; Kim *et al*., 2004; Sapozhnikova *et al*., 2004; Martinez *et al*., 2010). There is paucity of information on the serum level of PCBs in non-occupationally exposed women in Nigeria.

MATERIALS AND METHODS

3.1 Study Design

The study was a prospective case-control study conducted in the Surgical Oncology Clinic of the Department of Surgery, University College Hospital, Ibadan. The study protocol was approved by the University of Ibadan and University College Hospital Health Review Committee (UI/EC/10/0193, **Appendix 1**). Informed consent was obtained from the participants before recruitment into the study. Women with breast cancer were recruited between April, 2011 and March, 2012.

3.2 Study Participants

One hundred and seventy women aged 28-80 years were consecutively recruited for this study. Eighty-five were histologically confirmed breast cancer patients who had not commenced treatment (Cases). They were recruited from the Surgical Oncology Clinic of the Department of Surgery, University College Hospital, Ibadan, by a Consultant Surgical Oncologist. Eighty-five non-pregnant, apparently healthy women aged 28-80 years were recruited as controls. The controls were recruited at three Primary Health Clinics (PHC) in Ibadan North Local Government Area of Oyo state (PHC, Idi Odundun, Agodi, PHC, Agbowo and Elderly Women/Widows Clinic, Agodi-gate). Their breasts were examined by trained nurses for the presence of any breast lump. They were asked if they felt any pain or had any discomfort in their breasts. Those that complained of pain, discomfort and/or had lump in their breasts were excluded from the study. One of the controls was excluded from the study due to incomplete data on questionnaire and insufficient blood sample.

Each of the cases was matched for age and menstrual phases (follicular, luteal and postmenopausal) with the controls. Participants were reported as postmenopausal if they had stopped menstruating over the last twelve months (Wang *et al*., 2009). Participants that had bilateral oophorectomy were also considered postmenopausal.

3.2.1 Inclusion Criteria

Non pregnant, non hypertensive participants with histologically confirmed breast cancer who had not commenced treatment and gave informed consent.

3.2.2 Exclusion Criteria

Pregnant women and those who reported being on hormonal drugs (i.e. contraceptives), had other types of cancers and/or chronic diseases were excluded from the study. Postmenopausal women on hormone replacement therapy were also excluded.

3.3 Demographic, Social, Dietary and Reproductive History

Semi-structured pre-test questionnaire was administered to each participant to obtain data on demography, social, diet and reproductive history (**Appendix 3**).

3.4 Anthropometric Indices

Anthropometric indices were weight, height, BMI, waist circumference, hip circumference, waist hip ratio, waist height ratio.

3.4.1 Weight

This was taken with a bathroom weighing scale placed on a flat surface. The participants while wearing light clothing and without shoes were made to stand on the scale with the indicator at zero. The reading was recorded to the nearest 0.5kg.

3.4.2 Height

This was measured against a pre-graduated flat, vertical surface with the participants standing bare footed in an upright position without any head gear on, without raising the heels from the ground and the feet kept together. Measurements were taken with a sliding headpiece brought to the vertex of the participant's head. The reading at this level was taken to the nearest 0.1 cm.

3.4.3 Body Mass Index

This was calculated from the body weight and height of the participants using the formula stated below.

BMI (kg/m^2) =weight (kg)/height (m^2).

3.4.4 Waist and Hip Circumferences

Waist circumference (in cm) was measured using a measuring tape placed at the navel level, while hip circumference (in cm) was measured at the widest circumference of the hip over light clothing using a non-stretchable measuring tape without any pressure on the body surface. Both indices were recorded to the nearest 0.1cm.

3.4.5 Waist Hip Ratio (WHR)

This was calculated as the ratio of the waist circumference to the hip circumference

WHR=Waist Circumference (cm)/ Hip Circumference (cm)

3.4.6 Waist Height Ratio (WHtR)

This was calculated as the ratio of waist circumference to height measurements using the formula

WHtR= Waist Circumference (cm)/ Height (cm)

3.5 Blood Pressure (BP) Measurement

Blood pressure was determined using a mercury sphygmomanometer and recorded to the nearest mmHg. Each of the participants was allowed to rest for about ten minutes and in a sitting position before the BP was taken. The rotocuff was tied around the forearm and was inflated to obstruct the brachial artery. A stethoscope was placed at the cubital fossa and the pressure released. As the blood flowed through the arm, the first and the second sound produced were systolic blood pressure and diastolic blood pressure respectively.

3.6 Sample Collection

Ten millilitres of venous blood samples were drawn into plain bottle from participants after diagnosis and histological confirmation of invasive ductal carcinoma. This was done by applying a tourniquet 10-15 cm above the intended puncture site to obstruct the return of venous blood to the heart and to distend the vein. The site of puncture, the medial cubital vein in the antecubital fossa was cleansed with alcohol swab.

For premenopausal participants, blood samples were drawn between days 5 and 9 of their menstrual cycle in follicular phase (forward dating) and 5 to 9 days before the anticipated start of their next menstrual cycle in the luteal phase (backward dating) i.e. days 19-23 (Wang *et al.*,

2009). The blood was allowed to retract and centrifuged at 3500 rpm for 5 minutes. The resulting serum was aliquoted and stored at -20^{o}C until analysis. Breast biopsy samples of the affected breast were obtained from women with breast cancer for the determination of oestrogen receptor (ER), progesterone receptor (PR) and HER 2.

3.7 Biochemical Investigations

The biochemical indices assayed in serum were hormones (oestradiol, progesterone, LH, FSH, TSH, FT_3 and FT_4), and endocrine disruptors (lead, cadmium, arsenic, bisphenol-A and polychlorinated biphenyls). The expression of oestrogen receptor, progesterone receptor and HER 2 were determined by immunohistochemistry.

3.7.1 Determination of Progesterone

Serum progesterone was analysed by Enzyme Immuno Assay (EIA) on TOSOH AIA System Analyzers (Tosoh Corporation, Tokyo 105-8623, Japan).

The Principle of Test

The ST AIA-PACK Progesterone is a competitive enzyme immunoassay which was performed within the AIA-PACK test cups. Progesterone present in the test sample competed with enzyme-labelled progesterone for a limited number of binding sites on a progesterone-specific antibody immobilized on magnetic beads. The beads were washed to remove the unbound enzyme-labelled progesterone and were then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labelled progesterone bound to the beads is inversely proportional to the progesterone concentration in the test sample. A standard curve using a range of known standard concentration was constructed and unknown progesterone concentrations were calculated using the curve.

Material and Reagents

Plastic test cups containing:

(1) Lyophilized twelve magnetic beads with anti-progesterone rabbit polyclonal antibody.

(2) 75µL of progesterone conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

Assay Procedure

The substrate solution, wash solution and diluents were poured into their respective containers provided by the manufacturer and placed in their respective position on the analyzer.

75µL of serum was pipetted into the analyzer's test cups and loaded into the analyzer for the determination of progesterone.

The controls were pipetted into their respective cups, thereafter, the analyzer was instructed to commence analysis by pressing the START icon on the operational menu on the analyzer's screen. The TOSOH AIA System Analyzers performed all reagent and sample handling operations automatically (i.e. immunoextraction of hormone, washing, labelled hormone-antibody reaction and colour development).

Calculation

The system analyzer read the rate of fluorescence produced by the reaction and automatically converts the rate to progesterone concentration.

3.7.2 Determination of Oestradiol (E_2)

Serum oestradiol was analysed by Enzyme Immuno Assay (EIA) on TOSOH AIA System Analyzers. (Tosoh Corporation, Tokyo 105-8623, Japan).

The Principle of Test

The ST AIA-PACK E_2 is a competitive enzyme immunoassay which was performed within the AIA-PACK test cups. Oestradiol present in the test sample competed with enzyme-labelled E_2 for a limited number of binding sites on an anti-E_2 monoclonal antibody immobilized on magnetic beads. The magnetic beads were washed to remove the unbound enzyme-labelled E_2 and were then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labelled E_2 that was bound to the beads was inversely proportional to the

E_2 concentration in the test sample. A standard curve using a range of known standard concentration was constructed and unknown E_2 concentrations were calculated using the curve.

Material and Reagents

Plastic test cups containing:

(1) Lyophilized twelve magnetic beads with anti- E_2 rabbit polyclonal antibody.
(2) 50µL of E_2 conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

Assay Procedure

The substrate solution, wash solution and diluents were poured into their respective containers provided by the manufacturer and placed in their respective position on the analyzer.

75µl of serum was pipetted into the analyzer's test cups and loaded into the analyzer for the determination of E_2.

The controls were pipetted into their respective cups, thereafter, the analyzer was instructed to commence analysis by pressing the START icon on the operational menu on the analyzer's screen. The TOSOH AIA System Analyzers performed all reagent and sample handling operations automatically (i.e. immunoextraction of hormone, washing, labelled hormone-antibody reaction and colour development).

Calculation

The system analyzer read the rate of fluorescence produced by the reaction and automatically converts the rate to E_2 concentration.

3.7.3 Determination of FSH

Serum (FSH) was analysed by Enzyme Immuno Assay (EIA) on TOSOH AIA System Analyzers. (Tosoh Corporation, Tokyo 105-8623, Japan).

The Principle of Test

The ST AIA-PACK FSH is a two site immunoenzymometric assay which was performed within the AIA-PACK test cups. FSH present in the test sample was bound with monoclonal antibody immobilized on a magnetic solid phase and enzyme-labelled monoclonal antibody in the AIA-PACK test cups. The magnetic beads were washed to remove unbound enzyme-labelled monoclonal antibody and were then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labelled monoclonal antibody that was bound to the beads was directly proportional to the FSH concentration in the test sample. A standard curve using a range of known standard concentration was constructed and unknown sample concentrations were calculated using the curve.

Material and Reagents

Plastic test cups containing:

(1) Lyophilized twelve magnetic beads with anti-FSH mouse monoclonal antibody.
(2) 100µL of anti-FSH mouse monoclonal antibody (to human FSH) conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

Assay Procedure

The substrate solution, wash solution and diluents were poured into their respective containers provided by the manufacturer and placed in their respective position on the analyzer.

50µL of serum was pipetted into the analyzer's test cups and loaded into the analyzer for the determination of FSH.

The controls were pipetted into their respective cups, thereafter, the analyzer was instructed to commence analysis by pressing the START icon on the operational menu on the analyzer's screen. The TOSOH AIA System Analyzers performed all reagent and sample handling operations automatically (i.e. immunoextraction of hormone, washing, labelled hormone-antibody reaction and colour development).

Calculation

The system analyzer read the rate of fluorescence produced by the reaction and automatically converts the rate to FSH concentration.

3.7.4 Determination of LH

Serum LH was analysed by Enzyme Immuno Assay (EIA) on TOSOH AIA System Analyzers. (Tosoh Corporation, Tokyo 105-8623, Japan).

The Principle of Test

The ST AIA-PACK Progesterone is a two-site immunoenzymometric assay which was performed within the AIA-PACK test cups. LH present in the test sample was bound with monoclonal antibody immobilized on a magnetic solid phase and enzyme-labelled monoclonal antibody in the AIA PACK CUPS. The magnetic beads were washed to remove unbound enzyme-labelled monoclonal antibody and were then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labelled monoclonal antibody that was bound to the beads was directly proportional to the LH concentration in the test sample. A standard curve using a range of known standard concentration was constructed and unknown sample concentrations were calculated using the curve.

Material and Reagents

Plastic test cups containing:

(1) Lyophilized twelve magnetic beads coated with mouse anti-LH monoclonal antibody

(2)100µL of mouse anti-LH monoclonal antibody (to human LH) conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

Assay Procedure

The substrate solution, wash solution and diluents were poured into their respective containers provided by the manufacturer and placed in their respective position on the analyzer.

40µL of serum was pipetted into the analyzer's test cups and loaded into the analyzer for the determination of LH.

The controls were pipetted into their respective cups, thereafter, the analyzer was instructed to commence analysis by pressing the START icon on the operational menu on the analyzer's screen. The TOSOH AIA System Analyzers performed all reagent and sample handling operations automatically (i.e. immunoextraction of hormone, washing, labelled hormone-antibody reaction and colour development).

Calculation

The system analyzer read the rate of fluorescence produced by the reaction and automatically converts the rate to LH concentration.

3.7.5 Determination of Free Thyroxine (FT_4)

Serum FT_4 was analysed by Enzyme Immuno Assay (EIA) on TOSOH AIA System Analyzer. (Tosoh Corporation, Tokyo 105-8623, Japan).

The Principle of Test

The ST AIA-PACK FT_4 is a competitive enzyme immunoassay which was performed within the AIA-PACK test cups. The thyroxine not bound to serum protein (free T_4) competed with enzyme-labelled T_4 for a limited number of binding sites on a T_4-specific antibody immobilized on magnetic beads. After incubation, the beads were washed to remove the unbound enzyme-labelled FT_4 and were then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labelled T_4 that was bound to the beads was inversely proportional to the FT_4 concentration in the test sample. A standard curve using a range of known standard concentration was constructed and unknown FT_4 concentrations were calculated using the curve.

Material and Reagents

Plastic test cups containing:

(1) lyophilized twelve magnetic beads with anti-thyroxine rabbit polyclonal antibody.
(2) 140µL of thyroxine conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

Assay Procedure

The substrate solution, wash solution and diluents were poured into their respective containers provided by the manufacturer and placed in their respective position on the analyzer.

10µl of serum was pipetted into the analyzer's test cups and loaded into the analyzer for the determination of FT_4

The controls were pipetted into their respective cups, thereafter, the analyzer was instructed to commence analysis by pressing the START icon on the operational menu on the analyzer's screen. The TOSOH AIA System Analyzers performed all reagent and sample handling operations automatically (i.e. immunoextraction of hormone, washing, labelled hormone-antibody reaction and colour development).

Calculation

The system analyzer read the rate of fluorescence produced by the reaction and automatically converts the rate to FT_4 concentration

3.7.6 Determination of Free Triiodothyronine (FT_3)

Serum FT_3 was analysed by Enzyme Immuno Assay (EIA) on TOSOH AIA System Analyzer. (Tosoh Corporation, Tokyo 105-8623, Japan).

The Principle of Test

The ST AIA-PACK FT_3 is a competitive enzyme immunoassay which was performed within the AIA-PACK FT_3 test cups. Free triiodothyronine present in the test sample competed with enzyme-labelled progesterone for a limited number of binding sites on a T_3-specific antibody immobilized on magnetic beads. The beads were washed to remove the unbound enzyme-labelled T_3 and were then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labelled T_3 that was bound to the beads was inversely proportional to the T_3 concentration in the test sample. A standard curve using a range of known standard concentration was constructed and unknown T_3 concentrations were calculated using the curve.

Material and Reagents

Plastic test cups containing:

(1) Lyophilized twelve magnetic beads with anti-T_3 rabbit polyclonal antibody.

(2) 100µL of FT_3 conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

Assay Procedure

The substrate solution, wash solution and diluents were poured into their respective containers provided by the manufacturer and placed in their respective position on the analyzer.

50µL of serum was pipetted into the analyzer's test cups and loaded into the analyzer for the determination of FT_3.

The controls were pipetted into their respective cups, thereafter, the analyzer was instructed to commence analysis by pressing the START icon on the operational menu on the analyzer's screen. The TOSOH AIA System Analyzers performed all reagent and sample handling operations automatically (i.e. immunoextraction of hormone, washing, labelled hormone-antibody reaction and colour development).

Calculation

The system analyzer read the rate of fluorescence produced by the reaction and automatically converts the rate to FT_3 concentration

3.7.7 Determination of Thyroid Stimulating Hormone (TSH)

Serum TSH was analysed by Enzyme Immuno Assay (EIA) on TOSOH AIA System analyzer. (Tosoh Corporation, Tokyo 105-8623, Japan).

The Principle of Test

The ST AIA-PACK TSH was a two-site immunoenzymometric assay which was performed within the AIA-PACK test cups. TSH present in the test sample was bound with monoclonal antibody immobilized on magnetic beads and monoclonal antibody conjugated with bovine

alkaline phosphate in the AIA-PACK test cups. The beads were washed to remove the unbound enzyme-labelled monoclonal antibody and were then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme conjugated with monoclonal antibody that binds to the beads was directly proportional to the TSH concentration in the test sample. A standard curve was constructed and unknown sample concentrations were calculated using this curve.

Material and Reagents

Plastic test cups containing:

(1) Lyophilized twelve magnetic beads coated with anti-TSH mouse monoclonal antibody

(2) 50µL of anti-TSH mouse monoclonal antibody (to human TSH) conjugated to bovine alkaline phosphatase with sodium azide as a preservative.

Assay Procedure

The substrate solution, wash solution and diluents were poured into their respective containers provided by the manufacturer and placed in their respective position on the analyzer.

100µL of serum was pipetted into the analyzer's test cups and loaded into the analyzer for the determination of TSH.

The controls were pipetted into their respective cups, thereafter, the analyzer was instructed to commence analysis by pressing the START icon on the operational menu on the analyzer's screen. The TOSOH AIA System Analyzers performed all reagent and sample handling operations automatically (i.e. immunoextraction of hormone, washing, labelled hormone-antibody reaction and colour development).

Calculation

The system analyzer read the rate of fluorescence produced by the reaction and automatically converts the rate to TSH concentration.

3.8 Determination of Serum Toxic Metals (Pb, Cd and As) using Flame Atomic Absorption Spectrophotometry (Buck Scientific, 210 / 211VGP. Atomic absorption spectrophotometer. Connecticut, USA).

Serum lead, cadmium and arsenic were determined with atomic absorption spectrophotometer (AAS) based on the direct method described by Kaneko (1999).

Principle

Atomic absorption spectrophotometry is an accurate and sensitive analytical method for the determination of trace metals. The elements were not appreciably excited in the flame but merely dissociated from its bonds and placed in an unexcited or ground state. The atoms were at a low level in which they were capable of absorbing radiation at a very narrow band width corresponding to their own line spectrum. Hollow cathode lamps made of the materials analysed were used to produce a wavelength of specific for the kind of metal in the cathode.

3.8.1 Determination of Lead

Serum lead was determined using atomic absorption spectrophotometry (AAS). Samples were treated with Triton X-100. A beam of light from a hollow cathode lamp (coated with lead) was passed through a flame containing the vapourized metal to be determined. The amount of light absorbed by the metal was proportional to the concentration of Lead in the solution and was determined at 283.3nm (wavelength).

Reagents

Triton X-100 (TX) (an alkyl phenoxy polyethoxy)

Ethanol (BDH Chemicals Ltd., Poole, England)

Sample Preparation and Procedure of Analysis

A 1:2 dilution of the serum was made by mixing 1.0 ml each of triton X-100 solution

1. A two-fold dilution of the sample was made with 0.1% triton X-100 and was mixed thoroughly. 0.2ml of the digested sample was aspirated into AAS.

2. The burner was lit under flow conditions of air and acetylene. The acetylene flow was then reduced until the flame was blue.
3. The air flow was then adjusted to remove all traces of yellow from the flame.
4. The machine was then properly calibrated with the appropriate standard solutions before analyzing the test samples.
5. Serum lead was detected at a wavelength of 283.3nm.
6. The assay results were displayed on the instrument reader's screen in µg/dL.
7. Analytical quality control was performed by analyzing an aliquot of pooled serum several times during the assay.

3.8.2 Determination of Cadmium

Serum cadmium was determined by the methods of (Kaneko, 1999) using atomic absorption spectrophotometry.

Reagents

Triton X-100 (TX) (an alkyl phenoxy polyethoxy)

Ethanol (BDH Chemicals Ltd., Poole, England)

Sample Preparation and Procedure of Analysis

1. A two-fold dilution of the sample was made with 0.1% triton X-100 and was mixed thoroughly. 0.2ml of the digested sample was aspirated into AAS for analysis at a wavelength of 228.9nm
2. The prepared samples were analysed in Buck 210/211 VGP atomic absorption spectrophotometer (Buck Scientific, Inc. 58 Fort Point St. East Norwalk, Ct. 06855)
3. The burner was lit under flow conditions of air and acetylene. The acetylene flow was then reduced until the flame was blue
4. The air flow was then adjusted to remove all traces of yellow from the flame
5. The machine was then properly calibrated with the appropriate standard solutions before analyzing the test samples.
6. Serum cadmium was detected at a wavelength of 228.9 nm.
7. The assay results were displayed on the instrument reader's screen in µg/dL

8. Analytical quality control was performed by analyzing an aliquot of pooled serum several times during the assay.

3.8.3 Determination of Arsenic

Serum arsenic was determined by the methods of (Kaneko, 1999) using atomic absorption spectrophotometry.

Reagents

Triton X-100 (TX) (an alkyl phenoxy polyethoxy)

Ethanol (BDH Chemicals Ltd., Poole, England).

Reagents

Triton X-100 (TX) (an alkyl phenoxy polyethoxy)

Ethanol (BDH Chemicals Ltd., Poole, England)

Sample Preparation and procedure of Analysis

1. A two-fold dilution of the sample was made with 0.1% triton X-100 and was mixed thoroughly. 0.2ml aspirated into AAS for analysis at a wavelength of 193.7nm
2. The prepared samples were analysed in Buck 210/211 atomic absorption spectrophotometer (Buck Scientific, Inc. 58 Fort Point St. East Norwalk, Ct. 06855).
3. The burner was lit under flow conditions of air and acetylene. The acetylene flow was then reduced until the flame was blue.
4. The air flow was then adjusted to remove all traces of yellow from the flame.
5. The machine was then properly calibrated with the appropriate standard solutions before analyzing the test samples.
6. The assay results were displayed on the instrument reader's screen in µg/dL.
7. Analytical quality control was performed by analyzing an aliquot of pooled serum several times during the assay.

3.9 Determination of Bisphenol-A

Serum BPA was estimated by high performance liquid chromatography (ALLIANCE, e2695 Waters, USA).

Principle

This based on the separation of the solutes of a sample mixture by their differential distribution between stationary and mobile phases.

Reagents

Labelled bisphenol-A (50ng)

4-methylumbelliferone glucuronide (250 ng)

Ammonium acetate buffer (pH 6.5) (300µL)

β-glucuronidase (10µL) (Escherichia coli K12, Roche Biomedical).

Assay Procedure

Serum samples were fortified with 12.5 nanograms of isotopically labelled phthalate metabolites, 50 nanograms of labelled bisphenol-A, 250 nanograms of 4-methylumbelliferone glucuronide, 300 microlitres of ammonium acetate buffer (pH 6.5) and 10 microliters of β-glucuronidase (Escherichia coli K12, Roche Biomedical). The samples were mixed and incubated at 37^{o}C overnight to allow for the deglucuronidation. Following enzymatic hydrolysis, a 20µL aliquot of the sample was added to 70µL of HPLC- grade water and 10ng of labelled 4-methylumbelliferone to determine deglucuronidation efficiency. The remaining sample was loaded on to Zymark rapid trace solution for automated solid phase extraction (SPE). The 60 milligram/3mL Oasis-HLB cartridges were conditioned with HPLC-grade methanol (2ml) and 0.1 M formic acid (2mL). The samples were diluted with 5 mL of 0.1 M formic acid and loaded on the SPE cartridge at a rate of 1.0mL/min. The cartridge was washed with water (1mL) and 10% methanol in water (2mL) at a flow rate of 1mL/min. The samples were eluted with 1.0mL of acetonitrile at a flow rate of 0.5 mL/min. The eluate was evaporated to dryness under a stream of dry nitrogen and the residue was re-suspended in 85% methanol in water (200 microliters) and

transferred to glass autosampler vials. Quality control of the analysis was maintained by analyzing a method blank (calf serum) and two spiked calf serum samples (20ng/mL). The detection limit (0.2ng/mL) was based upon a lower calibration standard (0.5ng/ml) which gave an instrument signal to noise response of 3:1

3.10 Determination of Polychlorinated Biphenyls

Serum polychlorinated biphenyl was determined by gas chromatography-electron capture detector (GC-ECD).

Principle

This is based on the separation of the solutes of a sample mixture by their differential distribution between stationary and mobile phases.

Reagents

Methanol

N-hexane-diethyl-ether

Concentrated sulphuric acid

Assay Procedure

The determination of serum PCBs consisted of three steps (1) extraction of PCBs from the serum by organic solvent (2) Clean up of PCBs from impurities on chromatographic columns (3) Quantitation by Gas chromatography with a suitable detector (electron capture detector).

Serum samples were mixed with methanol and a mixture of internal standards were added to correct for recovery and ensure quality control. The samples were then extracted three times with n-hexane-diethyl-ether (1:1 v/v). After evaporation of the solvents the fat content was determined gravimetrically. The fat was re-dissolved in n-hexane and treated with concentrated sulphuric acid. The PCBs were separated from the bulk of the chlorinated compounds by elution through a silica gel column (4.5g of 3% water-deactivated silica-gel). The first fraction, containing the PCBs was eluted with 30ml of n-hexane. The columns were of different polarity to ease identification of analytes which was based on retention times relative to internal

standards. Quantification was performed using multilevel calibration curves obtained by injection of standard solutions of at least three different concentrations. The limit of determination (LOD) was determined as three standard deviations (SD) above the value of the blank and varied between 1 and 7 pg/g serum (not lipid adjusted). Samples with concentrations of LODs three SD above the blank have a 99% probability of being non-zero. To increase this probability, the quantification limits (LOQ) were set at higher levels than the LODs. In this case the lowest standard concentration was used; 10pg/g serum. The reproducibility of the method was demonstrated by 21 replicate determinations using an in-house control serum sample included in the analytical batches during the course of the study.

3.11 Determination of the Expression of ER, PR and HER 2

Immunohistochemistry was performed on 79 breast tissue biopsy samples obtained from the breast cancer participants in this study. The tissue samples were collected in bottles containing 10% buffered formalin. This was followed by embedding of tissue in paraffin wax pending when analysis will be done.

Principle

Immunohistochemistry combines histological, immunological and biochemical techniques for the identification and localization of specific tissue components (localization of antigens in tissue section). This is by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold. It is among the most sensitive and specific histochemical techniques.

Reagents

1. 70%, 90% and 100% (2 jars) ethanol solutions.
2. Epitope retrieval buffer (citrate buffer 6.0 or EDTA buffer 9.0).
3. Xylene (2 jars).
4. Wash Buffer (2 jars).
5. Mounting solution.
6. Hematoxylin stain.
7. Diaminobenzidine tetrahydrochloride (DAB) substrate/ Chromogen.

8. Antibody/ Antibody diluents.
9. Secondary antibody.

Materials

1. Humidified chamber.
2. Oven.
3. Water Bath.
4. Cover slip (22x32, 22x40, 22x22).

Method

(1) Sample Preparation (Deparaffination and Rehydration)

Paraffin specimens were cut into 4-µm sections using a microtome and mounted on positively charged slides. Slides containing the breast tissue section were incubated for 10 minutes at 70^{o}C. This was followed by incubation for 5 minutes in xylene jar #1. The slides were incubated for 5 minutes in xylene jar #2. Slides were incubated in graded alcohols i.e. Slides were incubated in 100% ethanol jar #1 for 2 minutes. This was followed by incubation in 100% ethanol jar #2 for another 2 minutes. The slides were further incubated in 95% ethanol jar #1 for 2 minutes. This was followed by incubation in 95% ethanol jar #2 for another 2 minutes. The slides were then incubated in 70% ethanol for 2 minutes. The slides were thereafter transferred into wash buffer for 2 minutes. The above step was aimed at deparaffinising and rehydrating the tissue samples.

(2) Epitope Retrieval (Antigen Unmasking)

Slides were transferred into pre-heated retrieval solution at 95^{o}C and were incubated for 20-30 minutes. The slides were immediately transferred into wash buffer for 2 minutes and were drained off.

(3) Blocking for Endogenous Enzymes

The tissue area on the slide was marked with a hydrophobic pen. Peroxidase block solution was applied drop-wise to cover the tissue. This was followed by incubation for 15 minutes at room temperature in a humidity chamber. The slides were thereafter transferred to and immersed in wash buffer jar for 5 minutes.

(4) Immunoperoxidase Staining

Excess liquid was wiped off the slide while care was taken not to clean off the tissue from the slide. The slides were placed in the humidity chamber, diluted primary antibody was applied. This was followed by incubation for 1 hour. It was thereafter washed in TBSt (wash solution) 3x2 minutes. Excess liquid was wiped off and secondary antibody was applied. This was followed by incubation in humidity chamber for 30 minutes. It was washed in TBSt (wash solution) 3x2 minutes.

(5) Detection

3, 3 diaminobenzidine tetrahydrochloride (DAB) solution was prepared (1ml + a drop DAB). The DAB was applied to the tissue section and incubated for 7 minutes. The slides were thereafter washed with running tap water.

(6) Counterstaining

The slides were dipped in Gill's hematoxylin for 10 seconds.

(7) Dehydration

The slides were immersed in 70% ethanol for 1 minute and thereafter were immersed in 95% ethanol for 1 minute. The slides were immersed in 100% ethanol for 1 minute. The slides were immersed in 50% ethanol for 5 minutes.

(8) Clearing and Mounting

The slides were immersed in xylene for 5 minutes and thereafter in a mounting solution (DPX). The samples were covered immediately with a cover slip using mounting solution and air dried for approximately 30 minutes before being examined under a light microscope by a pathologist.

3.12 Statistical Analysis

Data obtained from the research participants were collated and analyzed using the statistical package for social scientists (SPSS 18.0) SPP, Inc., Richmond, CA.

For quantitative variables;

(a) Student t-test was used to test the significance of difference between mean values. Data were expressed in mean±SEM (standard error of mean).
(b) Multiple regression analysis was employed to determine interrelationships between variables.

For qualitative variables;

(a) Chi-square test was used for association of qualitative variables

A two sided probability value at $p<0.05$ was considered statistically significant

CHAPTER FOUR

4.0 RESULTS

Table 4.1 shows the association of breast cancer stage, affected breast and hormone receptors in pre and postmenopausal women with breast cancer. There was significant difference in the hormone receptors ($p<0.05$).

Table 4.2 shows the association of age and demographic indices in women with and without breast cancer. There was an association in the occupation ($p=0.006$). No association was observed in age, marital status, educational status and ethnic group ($p>0.05$).

Table 4.3 shows the association of contraceptive use and history of breast cancer and tumour in women with and without breast cancer. No association was observed ($p>0.05$).

Table 4.4 shows the association of diet history in women with breast cancer and non breast cancer women. An association was observed in vegetable, fruit, red meat and diary product intake ($p<0.05$). There was no association in beans and beans product, refined carbohydrates and refined carbohydrate type intake ($p>0.05$).

Table 4.5 shows the reproductive history in women with breast cancer and non breast cancer women. Age at menarche was significantly higher in women with breast cancer when compared with non breast cancer women ($p=0.033$). Menstrual cycle was significantly lower in women with breast cancer when compared with non breast cancer women ($p=0.003$). Number of previous pregnancies was significantly higher in women with breast cancer ($p=0.009$). Number of induced abortion was significantly higher in women with breast cancer ($p<0.001$).

Table 4.1 Breast Cancer Stages, Sites of Affected Breast and Hormone Receptors in Premenopausal and Postmenopausal Women with Breast Cancer

Variable	Premeno-HCBCa (n=54)	Postmeno-HCBCa (n=31)	Total 85 (100%)	χ^2	p
Breast Cancer Stage				3.394	0.335
1	5(9.3%)	1(3.2%)			
2	3(5.6%)	5(16.1%)			
3	24(44.4%)	14(45.2%)			
4	22(40.7%)	11(35.5%)			
Breast Site	**(n=54)**	**(n=31)**		2.236	0.135
Right Breast	30 (55.6%)	12 (38.7%)			
Left Breast	24 (44.4%)	19 (61.3%)			
Receptors	**n=52**	**n=27**	**n=79(100%)**		
ER				22.050	<0.001*
Positive	0	10(37.0%)			
Negative	52(100%)	17(63.0%)			
PR				17.143	<0.001*
Positive	0	8(29.6%)			
Negative	52(100%)	19(70.4%)			
HER2				5.488	0.019*
Positive	6(11.5%)	9(33.3%)			
Negative	46(88.5%)	18(66.7%)			

n=number of participants, χ^2 =Chi-Squared test, Fishers=Fishers Exact ratio, p=Probability value, * significant at p<0.05. ER=Oestradiol Receptor, PR=Progesterone Receptor, HER2=Human epithelial receptor 2, Premeno-HCBCa=premenopausal women with histologically confirmed breast cancer, Postmeno-HCBCa= postmenopausal women with histologically confirmed breast cancer,

Table 4.2 Demographic Indices of Women with and without Breast Cancer.

Variable	HCBCa (n=85) %	AHWB (n=84)%	χ^2	p
Age	48.3±1.3	48.45±1.27	t=-0.07	0.941
Marital Status			7.795	0.050
Married	63(74.1%)	55(65.5%)		
Single	2(2.4%)	11(13.1%)		
Widow	19(22.4%)	18(21.4%)		
Divorced/separated	1(1.2%)	0		
Educational Status			4.400	0.221
None	16(18.8%)	19(22.6%)		
Primary	21(24.7%)	13(15.5%)		
Secondary	24(28.2%)	20(23.8%)		
Tertiary	24(28.2%)	32(38.1%)		
Occupation			12.432	0.006*
Trading	60(70.6%)	41(48.8%)		
Civil Servants	14(16.5%)	26(31.0%)		
Unemployed (House wife)	5(5.9%)	14(16.7%)		
Others (farmers, clergy)	6(7.1%)	3(3.6%)		
Ethnic Group			14.704	0.070
Igbo	14(16.5%)	2(2.4%)		
Ebira	2(2.4%)	0		
Hausa	1(1.2%)	2(2.4%)		
Isan	2(2.4%)	2(2.4%)		
Isoko	2(2.4%)	1(1.2%)		
Tiv	1(1.2%)	0		
Urhobo	2(2.4%)	1(1.2%)		
Yoruba	61(71.8%)	76(90.5%)		

n=number of participants, χ^2 =Chi-Squared test, p=probability value, *= significant at $p<0.05$, t=Student's t-test, HCBCa=Women with histologically confirmed breast cancer.

AHWB=Apparently healthy women without breast cancer

Table 4.3 Contraceptive use and History of Breast Cancer andTumour in Women with and without Breast Cancer.

Variable	HCBCa(n=85) %	AHWB (n=84) %	χ^2	p
Contraceptive Use			1.201	0.273
Yes	31(36.5%)	24(28.6%)		
No	54(63.5%)	60(71.4%)		
Contraceptive Type			6.658	0.471
Pills	11(12.9%)	6(7.1%)		
Injectibles	5(5.9%)	3(3.6%)		
Barrier	11(12.9%)	12(14.3%)		
Pills and injectible	2(2.4%)	1(1.2%)		
Pills, injectible and barrier	0	1(1.2%)		
Barrier and injectible	0	1(1.2%)		
Implant	2(2.4%)	0		
Nil	54(63.5%)	60(71.4%)		
Family History of Breast Cancer			0.137	0.711
Yes	4(4.7%)	3(3.6%)		
No	81(95.3%)	81(96.4%)		
Relative with B. Cancer			4.994	0.288
Mother	1(1.2%)	1(1.2%)		
Aunty	0	2(2.4%)		
Half Sister	1(1.2%)	0		
Sister	2(2.4%)	0		
Nil	81(95.3%)	81(96.4%)		
Personal History of Breast Tumour			2.983	0.084
Yes	3(3.5%)	0		
No	82(96.5%)	84(100%)		
Breast Tumour Mgt.			3.018	0.080
Surgery	3(3.5%)	0		
Nil	82(96.5%)	84(100%)		

n=number of participants, χ^2 =Chi-Squared test, p=probability value, *=significant at $p<0.05$. [a]= Student's t-test value LMC=Length of menstrual cycle, DMM=Duration of monthly menstruation. NPP=Number of previous pregnancies, NIA=Number of induced abortion. HCBCa=Women with histologically confirmed breast cancer. AHWB=Apparently healthy women without breast cancer

Table 4.4 Diet History in Women with and without Breast Cancer.

Variable	HCBCa (n=85)	AHWB (n=84)	χ^2	p
Beans/Beans Product Intake			0.491	0.782
Daily	10(11.8%)	13(15.5%)		
Weekly	20(23.5%)	18(21.4%)		
Occasionally	5362.4%)	53(63.1%)		
Nil	2(2.4%)	0		
Vegetable Intake			6.933	0.031*
Daily	23(27.1%)	23(27.4%)		
Weekly	43(50.6%)	28(33.3%)		
Occasionally	19(22.4%)	33(39.3%)		
Fruit Intake			6.824	0.033*
Daily	17(20.0%)	20(23.8%)		
Weekly	32(37.6%)	45(53.6%)		
Occasionally	36(42.4%)	19(22.6%)		
Red Meat Intake			56.869	<0.001*
Daily	70(82.4%)	22(26.2%)		
Weekly	4(4.7%)	26(31.0%)		
Occasionally	10(11.8%)	36(42.9%)		
Nil	1(1.2%)	0		
Dairy Product Intake			11.438	0.010*
Daily	2(2.4%)	2(2.4%)		
Weekly	7(8.2%)	23(27.4%)		
Occasionally	75(88.2%)	59(70.3%)		
Nil	1(1.2%)	0		
Refined Carbohydrate Intake			0.080	0.778
Yes	58(68.2%)	59(70.2%)		
No	27(31.8%)	25(29.8%)		
Refined Carbohydrate Type			4.272	0.370
Any	54(63.5%)	59(70.2%)		
Indomie	2(2.4%)	0		
Indomie/spaghetti	1(1.25)	0		
Spaghetti	1(1.2%)	0		
Nil	27(31.8%)	25(29.8%)		

n=number of participants, χ^2 =Chi-Squared test, p=Probability value, * significant at $p<0.05$. HCBCa=Women with histologically confirmed breast cancer. AHWB=Apparently healthy women without breast cancer

Table 4.5 Reproductive History in women with Breast Cancer and without Breast Cancer

Variable	HCBCa (n=85)%	AHWB (n=84)%	t	p
Menstrual History				
Age at Menarche (Years)	15.4±0.2	14.7±0.2	2.154	0.033*
LM C (days)	27.8±0.1	28.3±0.1	-2.988	0.003*
DMM(days)	4.5±0.1	4.6±0.0	-1.026	0.306
NPP	5.1±0.3	4.0±0.3	2.632	0.009*
Number of Live births	3.6±0.2	3.1±0.2	1.664	0.098
NIA	0.9±0.1	0.3±0.1	3.816	<0.001*
Number of Miscarriage(s)	0.1±0.1	0.3±0.1	-1.635	0.104

Student's t-test value LMC=Length of menstrual cycle, DMM=Duration of monthly menstruation. NPP=Number of previous pregnancies, NIA=Number of induced abortion. HCBCa=Women with histologically confirmed breast cancer. AHWB=Apparently healthy women without breast cancer

Table 4.6 shows the mean blood pressure and anthropometric indices (WC, HC, weight, height, BMI, WHR, WHtR) and blood pressure of women with breast cancer compared with controls. WC, HC, weight, height, WHR, WHtR and SBP were significantly higher in women with breast cancer compared with controls ($p<0.05$). No significant differences were observed in the mean body mass index and diastolic blood pressure ($p>0.05$).

Table 4.7 shows the serum levels of hormones (progesterone, oestradiol, LH, FSH, FT_4, FT_3 and TSH) and endocrine disruptors (Pb, Cd, As, BPA and PCBs). FT_4, Pb, Cd, As, BPA and PCBs were significantly higher in women with breast cancer compared with controls ($p<0.05$). No significant differences were observed in other indices ($p>0.05$).

Table 4.6 Blood Pressure and Anthropometric Indices in Women with and without Breast Cancer.

Variable	HCBCa (n=85)	AHWB (n=84)	t	p
Blood Pressure				
Systolic BP(mmHg)	122.7±1.1	119.4±1.0	2.215	0.028*
Diastolic BP (mmHg)	81.7±0.9	80.7±0.8	0.811	0.418
Anthropometric Indices				
WC(cm)	89.9±1.1	82.6±1.2	4.535	<0.001*
HC (cm)	101.8±1.1	98.5±1.0	2.212	0.028*
Body Weight (kg)	69.2±1.4	62.1±1.1	3.975	<0.001*
Height (m)	1.63±0.0	1.58±0.0	4.882	<0.001*
Body Mass Index	26.1±0.5	24.9±0.4	1.826	0.070
WHR	0.9±0.0	0.8±0.0	4.856	<0.001*
WHtR	55.3±0.7	52.4±0.7	2.809	0.006*

Values are in mean±SEM (Standard error of mean), n=number of participants, t=Student's t-test, p=Probability value, *=significant at $p<0.05$, WC=Waist circumference, HC=Hip circumference, WHR=Waist hip ratio, WHtR=Waist height ratio, BP=Blood pressure. HCBCa=Women with histologically confirmed breast cancer, AHWB=Apparently healthy women without breast cancer.

Table 4.7 Serum Hormones and Endocrine Disruptors in Women with and without Breast Cancer.

Variable	HCBCa (n=85)	AHWB (n=84)	t	p
Hormones				
Progesterone (nmol/L)	8.6±1.8	5.9±1.4	1.167	0.245
Oestradiol (pmol/L)	344.8±31.9	307.8±34.7	0.786	0.433
LH (IU/L)	14.5±1.5	14.6±1.4	-0.038	0.970
FSH (IU/L)	26.8±3.7	32.9±4.2	-1.101	0.273
FT_3(pmol/L)	3.4±0.3	3.4±0.1	-0.045	0.964
FT_4 (pmol/L)	17.8±0.4	14.7±0.3	6.373	<0.001*
TSH (mIU/L)	1.7±0.1	1.4±0.1	1.880	0.062
Endocrine Disruptors				
Lead (µg/dL)	5.5±0.2	1.8±0.0	24.167	<0.001*
Cadmium (µg/dL)	0.04±0.0	0.01±0.0	24.602	<0.001*
Arsenic (µg/dL)	0.3±0.0	0.04±0.0	23.209	<0.001*
BPA(mg/dL)	0.8±0.7	0.4±0.0	6.81	<0.001*
PCBs(µg/dL)	0.8±0.5	0.3±0.0	10.12	<0.001*

Values are mean±SEM (Standard error of mean), n=number of participants, t=Student's t-test, p=Probability value, *=significant at $p<0.05$, LH=Luteinizing hormone, FSH=Follicle-stimulating hormone, FT_3=Free triiodothyronine, FT_4=Thyroxine, TSH=Thyroid-stimulating hormone, Pb=Lead, Cd=Cadmium, As=Arsenic, BPA=Bisphenol-A, PCBs=Polychlorinated biphenyls. nmol/L=nanomole per liter, IU/L=mili international units per mililitre, pmol/L=picomol per litre, mIU/L=milliinternational unit per litre, µg/dL=micrograms per decilitre. HCBCa=Women with histologically confirmed breast cancer, AHWB=Apparently healthy women without breast cancer.

Table 4.8 shows the mean age, age at menarche, age at menopause (postmenopausal women only), blood pressure and anthropometric indices (WC, HC, weight, height, BMI, WHR, WHtR) of pre and postmenopausal women with breast cancer compared with their respective controls.

Premenopausal women

Age at menarche, WC, HC, weight, height, WHR, WHtR and SBP were significantly higher in premenopausal women with breast cancer ($p<0.05$). No significant differences were observed in the mean BMI and diastolic blood pressure in both premenopausal cases and control ($p>0.05$)

Postmenopausal women

Weight, height were significantly higher in postmenopausal women with breast cancer compared with controls ($p<0.05$). No significant differences were observed in the mean age, age at menarche, age at menopause other anthropometric indices and blood pressure measurements ($p>0.05$)

Table 4.9 shows the serum levels of sex hormones (progesterone and oestradiol, LH, FSH, FT_4, FT_3 and TSH) and endocrine disruptors (Pb, Cd, As, BPA and PCBs) in pre and postmenopausal women with breast cancer and their respective controls.

Premenopausal women:

LH, FSH, FT_4, Pb, Cd, As, BPA and PCBs were significantly higher in premenopausal women with breast cancer when compared with controls ($p<0.05$). No significant differences were observed in levels of progesterone, oestradiol, FT_3 and TSH ($p>0.05$)

Postmenopausal women:

Progetsreone, oestradiol, FT_4, Pb, Cd, As, BPA and PCBs were significantly higher while FSH was significantly lower in postmenopausal women with breast cancer than controls ($p<0.05$). No significant difference was observed in LH, FT_3 and TSH ($p>0.05$)

Table 4.8 Age, Reproductive History, Blood Pressure and Anthropometric Indices in Women with and Without Breast Cancer in Pre and Postmenopausal Groups.

Variable	Premeno-HCBCa (n=54)	Premeno-AHWB (n=53)	t	p	Postmeno-HCBCa (n=31)	Postmeno-AHWB (n=31)	t	p
Age (years)	40.9±0.7	40.7±0.6	0.187	0.852	61.2±1.5	61.6±1.5	-0.199	0.843
AM.1 (years)	15.3±0.3	14.5±0.3	2.081	0.040*	15.6±0.39	15.1±0.38	0.840	0.404
AM.2 (years)	n/a	n/a	-	-	51±0.7	50±0.9	0.255	0.799
BP								
SBP(mmHg)	123.0±1.4	119.0±1.2	2.062	0.042*	122.3±1.8	120.0±1.6	0.925	0.360
DBP (mmHg)	82.4±1.1	80.9±1.0	0.967	0.336	80.3±1.3	80.3±1.2	0.000	1.000
AI								
WC (cm)	88.5±1.4	78.3±1.3	5.321	<0.001*	92.2±1.7	89.8±1.5	0.968	0.337
HC (cm)	100.5±1.5	95.9±1.0	2.512	0.014*	103.9±1.7	102.7±1.7	0.500	0.619
Wt (Kg)	68.0±1.9	60.1±1.3	3.435	0.001*	71.4±2.2	65.6±1.7	2.103	0.010*
Ht (m)	1.63±0.0	1.57±0.0	4.345	<0.001*	1.63±0.0	1.59±0.0	2.340	0.023*
BMI(Kg/m^2)	25.7±0.7	24.5±0.5	1.401	0.164	26.8±0.7	25.7±0.7	1.048	0.217
WHR	0.9±0.0	0.8±0.0	6.073	<0.001*	0.89±0.0	0.88±0.0	0.716	0.480
WHtR	54.6±1.0	49.9±0.9	3.516	0.001*	56.6±1.2	56.5±0.9	0.093	0.930

Values are mean±SEM (Standard error of mean), n=number of participants, t=Student's t-test, n/a=not applicable, p=Probability value, *=significant at $p<0.05$, Premeno-HCBCa=Premenopausal women with histologically confirmed breast cancer, Postmeno-HCBCa=Postmenopausal women with histologically confirmed breast cancer, SBP=Systolic blood pressure, DBP=Diastolic blood pressure. AM.1=Age at menarche, AM.2=Age at menopause. WC=Waist circumference, HC=Hip circumference, Wt=Body weight, Ht=Height, BMI=Body mass index, WHR=Waist hip ratio, WHtR=Waist height ratio, Systolic=Systolic blood pressure, Diastolic=Diastolic blood pressure. AI=Anthropometric Indices.

Table 4.9 Hormones and Endocrine Disruptors in Women with and without Breast Cancer in Pre and Postmenopausal Groups

Variable	Premeno-HCBCa (n=54)	Premeno-AHWB (n=53)	t	p	Postmeno-HCBCa (n=31)	Postmeno-AHWB (n=31)	t	p
Hormones								
Progest (nmol/L)	12.3±2.6	8.8±2.2	1.023	0.309	2.1±0.4	1.0±0.1	2.919	0.005*
Oestradiol (pmol/L)	452.8±43.3	430.8±46.5	0.347	0.729	156.5±12.4	90.4±3.6	5.036	<0.001*
LH (IU/L)	7.7±0.7	5.8±0.5	2.298	0.024*	26.4±2.9	29.7±1.1	-1.061	0.290
FSH (IU/L)	7.2±0.6	5.6±0.4	2.183	0.031*	60.9±6.4	79.6±4.1	-2.455	0.020*
FT_3(pmol/L)	3.6±0.4	3.5±0.1	0.249	0.804	3.1±0.1	3.3±0.1	-1.372	0.175
FT_4 (pmol/L)	17.8±0.6	14.9±0.3	4.507	<0.001*	17.7±0.6	14.3±0.4	4.785	<0.001*
TSH (mIU/L)	1.8±0.17	1.5±0.1	1.360	0.178	1.6±0.2	1.3±0.1	1.355	0.181
Endocrine Disruptors								
Lead (µg/dL)	5.4±0.2	1.8±0.1	18.349	<0.001*	5.8±0.2	1.8±0.1	15.975	<0.001*
Cadmium (µg/dL)	0.04±0.0	0.01±0.0	18.788	<0.001*	0.05±0.0	0.01±0.0	15.993	<0.001*
Arsenic (µg/dL)	0.3±0.0	0.04±0.0	17.413	<0.001*	0.3±0.0	0.04±0.0	15.219	<0.001*
PCBs (µg/dL)	0.8±0.1ǂ	0.4±0.0ǂ	4.515	<0.001*	0.8±0.1ǂ	0.3±0.0ǂ	5.178	<0.001*
BPA (mg/dL)	0.8±0.1ǂ	0.3±0.0ǂ	6.910	<0.001*	0.8±0.1ǂ	0.3±0.0ǂ	7.338	<0.001*

Values are mean±SEM (Standard error of mean), n=number of participants, t=Student's t-test, p=Probability value, *=significant at p<0.05. Premeno=Premenopausal, Postmeno=Postmenopausal, Progest=Progesterone. LH=Luteinizing hormone, FSH=Follicle stimulating hormone, FT_3=Free triiodothyronine, FT_4=Thyroxine, TSH=Thyroid-stimulating hormone, Pb=Lead, Cd=Cadmium, As=Arsenic, BPA=bisphenol-A, PCBs=polychlorinated biphenyls. nmol/L=nanomole per liter, IU/L=mili international units per mililitre, pmol/L=picomol per litre, mIU/L=milliinternational unit per litre, µg/dL=micrograms per decilitre. ǂ =n for PCBs and BPA in premenopausal cases and controls are 22 and 23 respectively, while n for PCBs and BPA in postmenopausal cases and controls are 18 and 17. Premeno-HCBCa=Premenopausal women with histologically confirmed breast cancer, Postmeno-HCBCa=Postmenopausal women with histologically confirmed breast cancer. Premeno-AHWB=controls, Postmeno-AHWB=Postmenopausal controls

Table 4.10 shows the mean age, anthropometric indices (WC, HC, weight, height, BMI, WHR, WHtR) and blood pressure of pre and postmenopausal women with breast cancer. Age was significantly lower in premenopausal women with breast cancer than postmenopausal women with breast cancer ($p<0.05$). No significant differences were observed in the mean anthropometric indices and blood pressure measurements ($p>0.05$).

Table 4.11 shows the serum levels of sex hormones (progesterone, oestradiol, LH, FSH, FT_4, FT_3, TSH) and endocrine disruptors (Pb, Cd, As, BPA and PCBs) in pre and postmenopausal women with breast cancer. Progetsreone, oestardiol were significantly higher in premenopausal women with breast cancer than postmenopausal women with breast cancer ($p<0.05$). LH and FSH were significantly lower in premenopausal women with breast cancer than postmenopausal women with breast cancer ($p<0.05$). No significant differences were observed in the levels of FT_3, FT_4 TSH, Pb, Cd, As, BPA and PCBs ($p>0.05$).

Table 4.10 Age, Blood Pressure and Anthropometric Indices in Pre and Postmenopausal Women with Breast Cancer

Variable	Premeno-HCBCa (n=54)	Postmeno-HCBCa (n=31)	t	p
Age (years)	40.9±0.7	61.2±1.5	-14.223	<0.001*
BP				
Systolic BP (mmHg)	123.0±1.4	122.3±1.8	0.299	0.765
Diastolic BP (mmHg)	82.4±1.1	80.32±1.3	1.182	0.241
Anthropometric Indices				
WC (cm)	88.5±1.4	92.2±1.7	-1.581	0.118
HC (cm)	100.5±1.5	103.9±1.7	-1.467	0.146
Wt (Kg)	70.0±1.9	71.4±2.2	-1.147	0.255
Ht (m)	1.62±0.0	1.63±0.0	-0.275	0.784
BMI (Kg/m^2)	25.7±0.7	26.8±0.7	-1.081	0.283
WHR	0.88±0.0	0.89±0.0	-0.510	0.611
WHtR	54.6±1.0	56.6±1.2	-1.348	0.181

Values are Mean±SEM (Standard error of mean), n=Number of subjects, t=Student's t-test, p=Probability value, *=significant at $p<0.05$, WC=Waist circumference, HC=Hip circumference, Wt=Body weight, Ht=Height, BMI=Body mass index, WHR=Waist hip ratio, WHtR=Waist height ratio, Systolic BP=Systolic blood pressure, Diastolic BP=Diastolic blood pressure. Premeno-HCBCa=Premenopausal women with histologically confirmed breast cancer, Postmeno-HCBCa=Postmenopausal women with histologically confirmed breast cancer,

Table 4.11 Serum Hormones and Endocrine Disruptors in Pre and Postmenopausal Women with Breast Cancer

Variable	Premeno-HCBCa (n=54)	Postmeno-HCBCa (n=31)	t	p
Hormones				
Prog (nmol/L)	12.3±2.6	2.1±0.4	2.907	0.005*
E_2 (pmol/L)	452.8±43.3	156.5±12.4	5.100	<0.001*
LH (IU/L)	7.7±0.7	26.4±2.9	-7.711	<0.001*
FSH (IU/L)	7.2±0.6	60.9±6.4	-11.034	<0.001*
FT_3(pmol/L)	3.6±0.4	3.1±0.1	0.868	0.388
FT_4 (pmol/L)	17.8±0.7	17.7±0.6	0.211	0.833
TSH (mIU/L)	1.8±0.2	1.6±0.2	0.492	0.624
Endocrine Disruptors				
Lead (µg/dL)	5.4±0.2	5.8±0.2	-1.293	0.200
Cadmium (µg/dL)	0.04±0.0	0.05±0.0	-1.049	0.297
Arsenic (µg/dL)	0.3±0.0	0.3±0.0	-0.294	0.769
PCB (µg/dL)	0.79±0.1ǂ	0.8±0.1ǂ	-0.206	0.838
BPA (mg/dL)	0.76±0.1 ǂ	0.8±0.1 ǂ	-0.529	0.600

Values are Mean±SEM (Standard error of mean), n=number of subjects, t=Student's t-test, p=Probability, *=significant at p<0.05, Prog=Progesterone, E_2=Oestradiol, LH=Luteinizing hormone, FSH=Follicle stimulating hormone, FT_3=Free triiodothyronine, FT_4=Thyroxine, TSH=Thyroid stimulating hormone, Pb=Lead, Cd=Cadmium, As=Arsenic. nmol/L=nanomole per liter, IU/L=mili international units per mililitre, pmol/L=picomol per litre, mIU/L=milliinternational unit per litre, µg/dL=micrograms per decilitre. ǂ =n for PCBs and BPA in premenopausal and postmenopausal women with breast cancer are 22 and 18 respectively. Premeno-HCBCa=Premenopausal women with histologically confirmed breast cancer, Postmeno-HCBCa=Postmenopausal women with histologically confirmed breast cancer.

Table 4.12 shows the mean age, anthropometric indices (WC, HC, weight, height, BMI, WHR and WHtR) and blood pressure of pre and postmenopausal women without breast cancer. Age, waist circumference, hip circumference, weight, waist-hip ratio and waist-height ratio were significantly lower in premenopausal women without breast cancer than postmenopausal women without breast cancer ($p<0.05$). No significant differences were observed in height, weight, systolic and diastolic blood pressure ($p>0.05$)

Table 4.13 shows the serum levels of sex hormones (progesterone, oestradiol, LH, FSH, FT_3, FT_4 and TSH) and endocrine disruptors (Pb, Cd, As, BPA and PCBs) in pre and postmenopausal women without breast cancer. Progesterone, oestradiol were significantly higher in premenopausal women than postmenopausal women ($p<0.05$). Luteinizing hormone and FSH were significantly lower in premenopausal women compared with postmenopausal women. No significant differences were observed in the levels of the endocrine disruptors ($p>0.05$).

Table 4.12 Age, Blood Pressure and Anthropometric indices in Pre and Postmenopausal Women without Breast Cancer.

Variable	Premeno-AHWB (n=53)	Postmeno-AHWB (n=31)	t	p
Age (years)	40.7±0.6	61.7±1.5	-14.846	<0.001*
Blood Pressure				
Systolic BP (mmHg)	119.0±1.2	120.0±1.6	-0.475	0.636
Diastolic BP (mmHg)	80.9±1.0	80.3±1.2	0.386	0.701
Anthropometric Indices				
WC (cm)	78.3±1.3	89.9±1.5	-5.741	<0.001*
HC (cm)	95.9±1.0	102.7±1.7	-3.619	0.001*
Wt (Kg)	60.1±1.3	65.6±1.7	-2.566	0.012*
Ht (m)	1.57±0.0	1.59±0.0	-1.777	0.079
BMI (Kg/m^2)	24.5±0.5	25.9±0.7	-1.612	0.111
WHR	0.8±0.0	0.9±0.0	-5.154	<0.001*
WHtR	49.9±0.9	56.5±0.9	-4.840	<0.001*

Values are Mean±SEM (Standard error of mean), n=Number of subjects, t=Student's t-test, p=Probability value, *=significant at $p<0.05$, WC=Waist circumference, HC=Hip circumference, Wt=Body weight, Ht=Height, BMI=Body mass index, WHR=Waist hip ratio, WHtR=Waist height ratio, Systolic=Systolic blood pressure, Diastolic=Diastolic blood pressure. Premeno-AHWB=apparently healthy premenopausal women. Postmeno-AHWB=apparently healthy postmenopausal women.

Table 4.13 Hormones and Endocrine Disruptors in Pre and Postmenopausal women without Breast Cancer.

Variable	Premeno-AHWB (n=53)	Postmeno-AHWB (n=31)	t	p
Hormones				
Prog (nmol/L)	8.8±2.2	1.0±0.1	2.756	0.007*
E_2 (pmol/L)	430.8±46.5	90.4±3.6	5.491	<0.001*
LH (IU/L)	5.8±0.5	29.8±1.1	-22.862	<0.001*
FSH (IU/L)	5.6±0.4	79.6±4.1	-23.147	<0.001*
FT_3(pmol/L)	3.5±0.1	3.3±0.1	1.218	0.227
FT_4 (pmol/L)	14.9±0.3	14.3±0.4	1.088	0.280
TSH (mIU/L)	1.5±0.1	1.3±0.1	0.833	0.407
Endocrine Disruptors				
Lead (µg/dL)	1.8±0.1	1.8±0.1	0.042	0.967
Cadmium (µg/dL)	0.01±0.0	0.01±0.0	-0.230	0.818
Arsenic (µg/dL)	0.39±0.0	0.04±0.0	-1.579	0.118
PCBs(µg/dL)	0.4±0.0ǂ	0.3±0.0ǂ	0.899	0.374
BPA (mg/dL)	0.3±0.0 ǂ	0.3±0.0 ǂ	1.397	0.171

Values are Mean±SEM (Standard error of mean), n=Number of subjects, t=Student's t-test, p=Probability value, *=significant at $p<0.05$, Prog=Progesterone, E_2=Oestradiol, LH=Luteinizing hormone, FSH=Follicle stimulating hormone, FT_3=Free triiodothyronine, FT_4=Thyroxine, TSH=Thyroid-stimulating hormone.nmol/L=nanomole per liter, IU/L=mili international units per mililitre, pmol/L=picomol per litre, mIU/L=milliinternational unit per litre, µg/dL=micrograms per decilitre. ǂ =n for PCBs and BPA in premenopausal and postmenopausal women without breast cancer are 23 and 17 respectively. Premeno-AHWB=apparently healthy premenopausal women. Postmeno-AHWB=apparently healthy postmenopausal women.

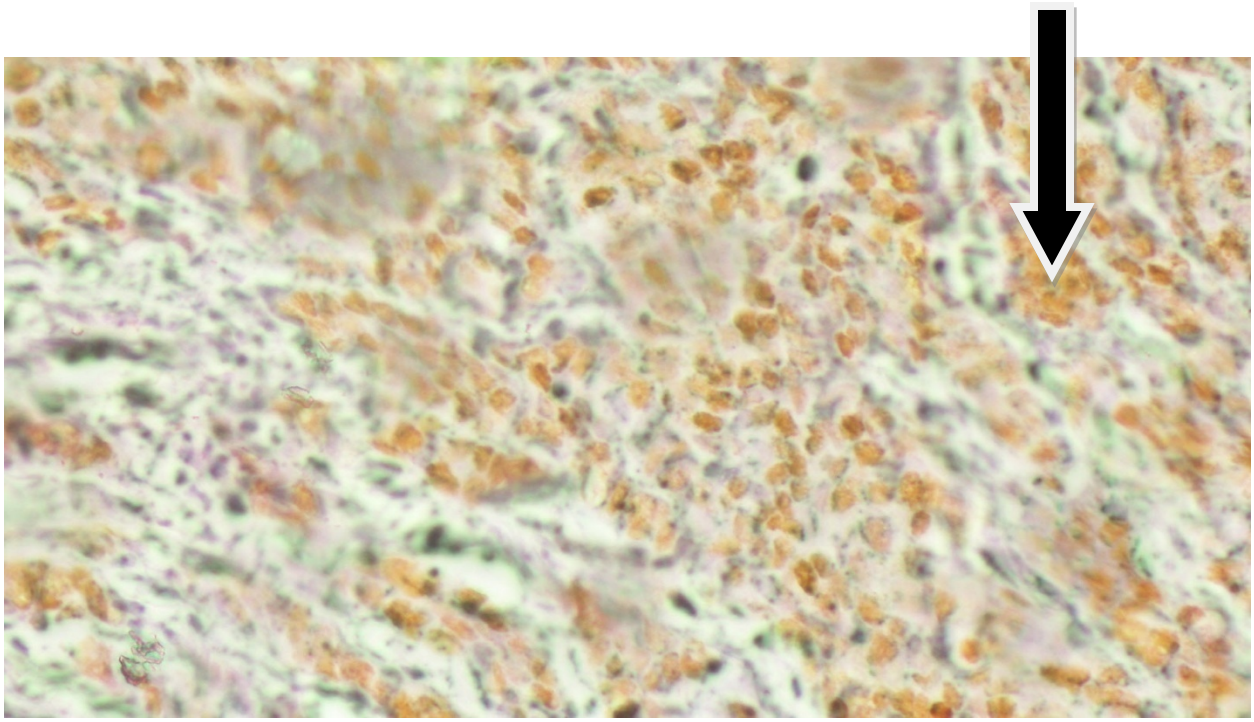

Figure 4.1: Photomicrograph of ER Positive (X400). Marked area indicates stained cells

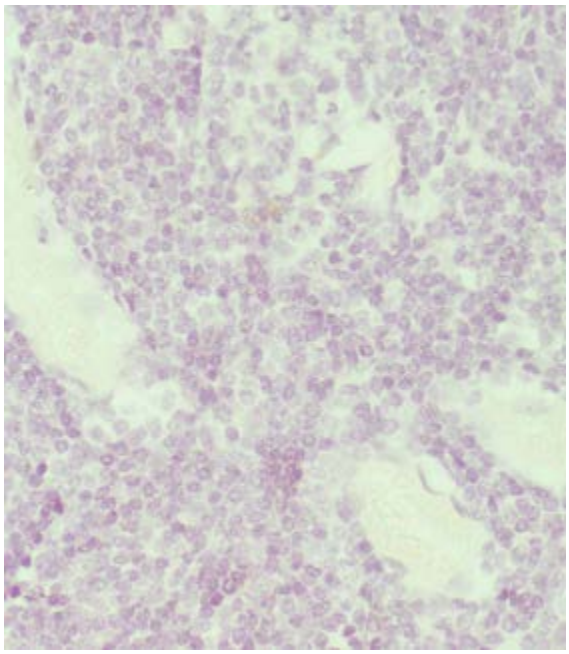

Figure 4.2: Photomicrograph of ER Negative (X400)

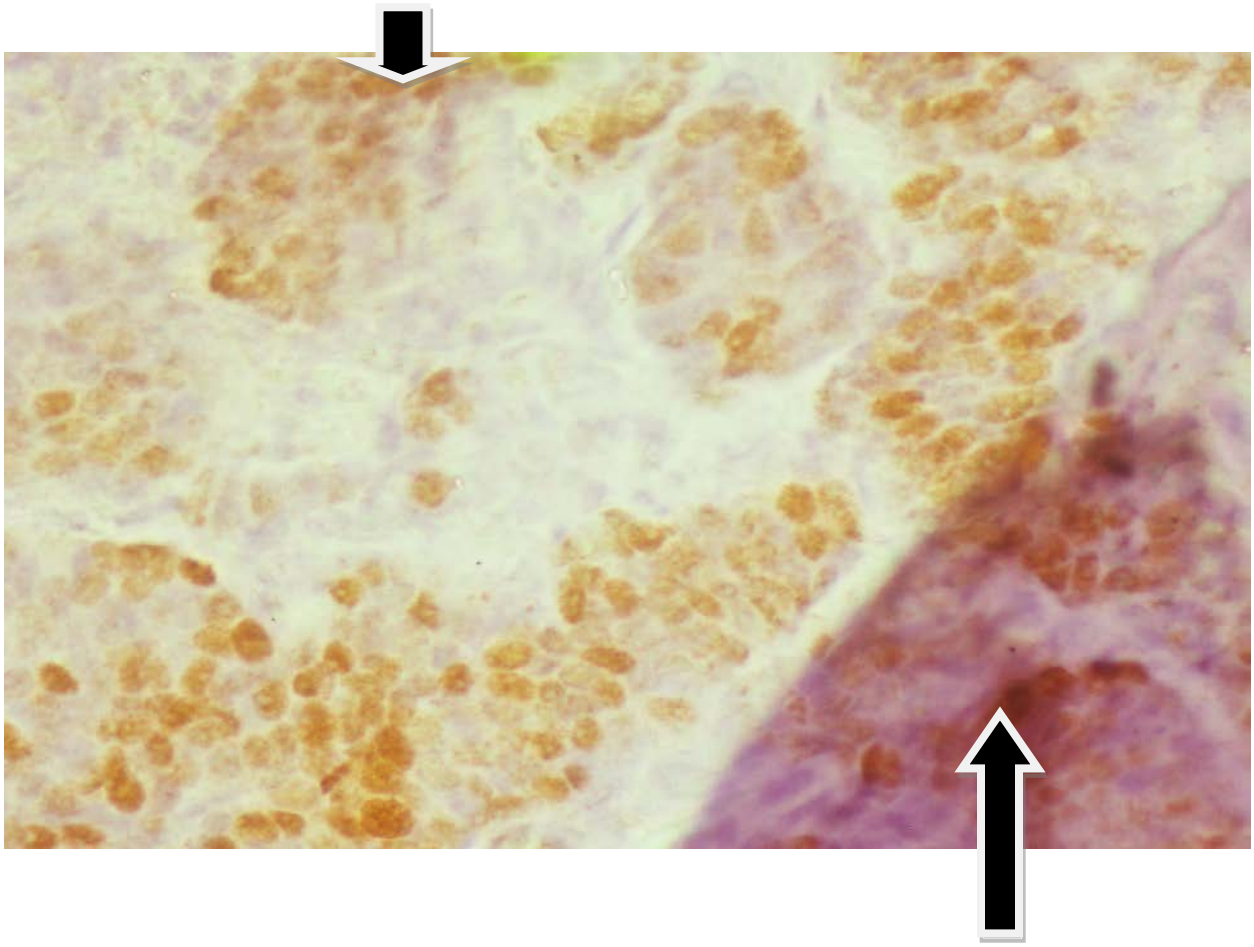

Figure 4.3: Photomicrograph of PR Positive (X400). Marked area represents stained cells

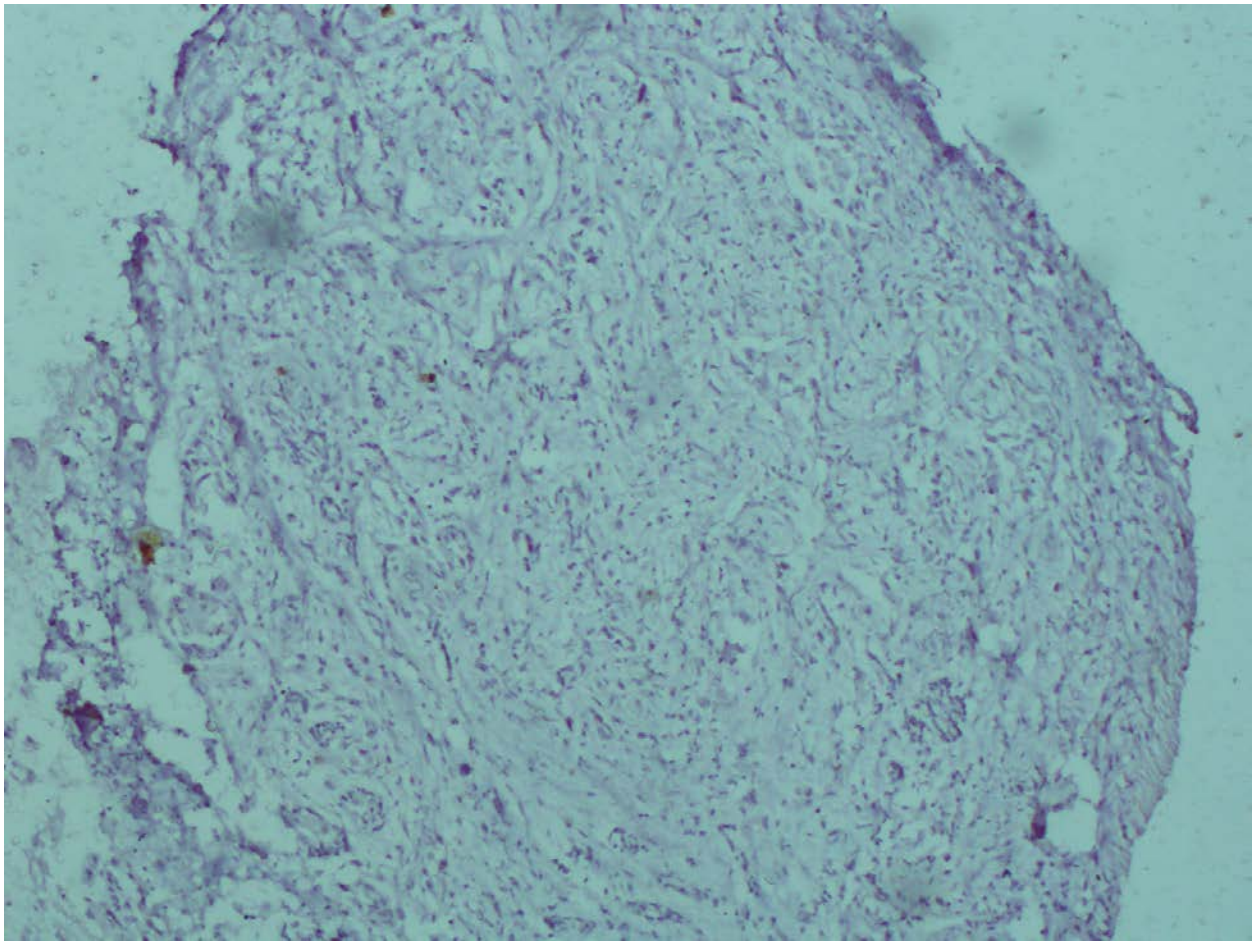

Figure 4.4: Photomicrograph of PR Negative (X400)

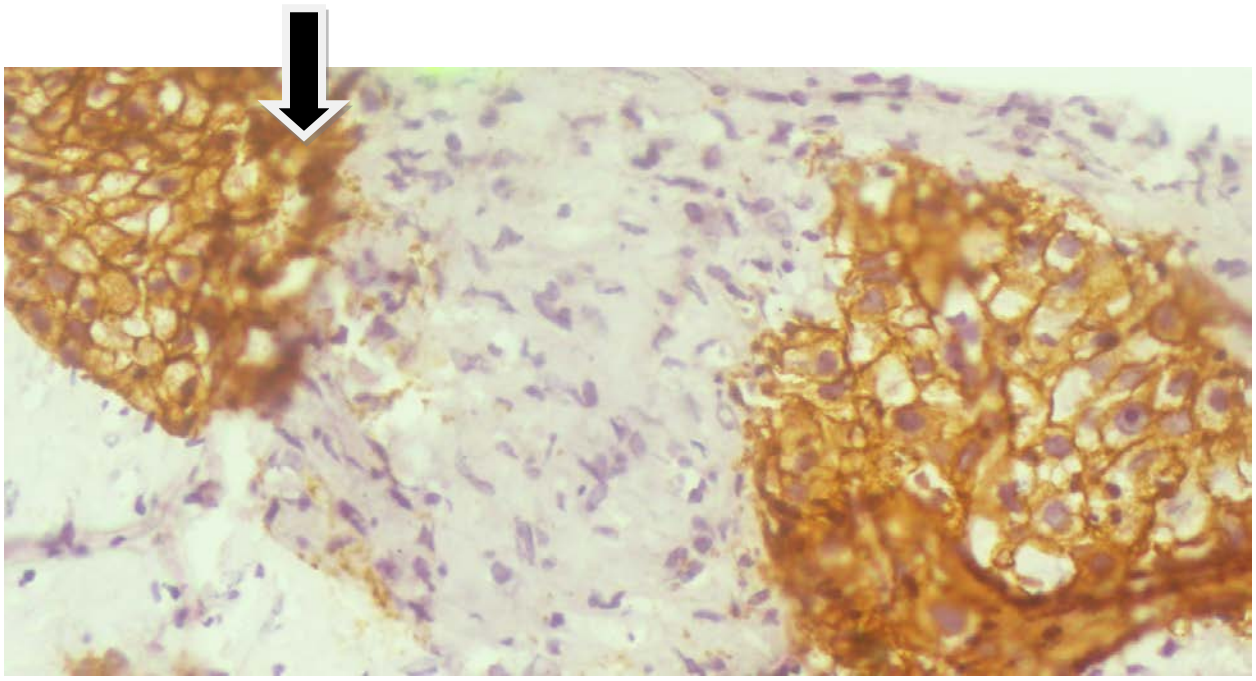

Figure 4.5: Photomicrograph of HER 2 Positive (X400). Marked area represents stained cells

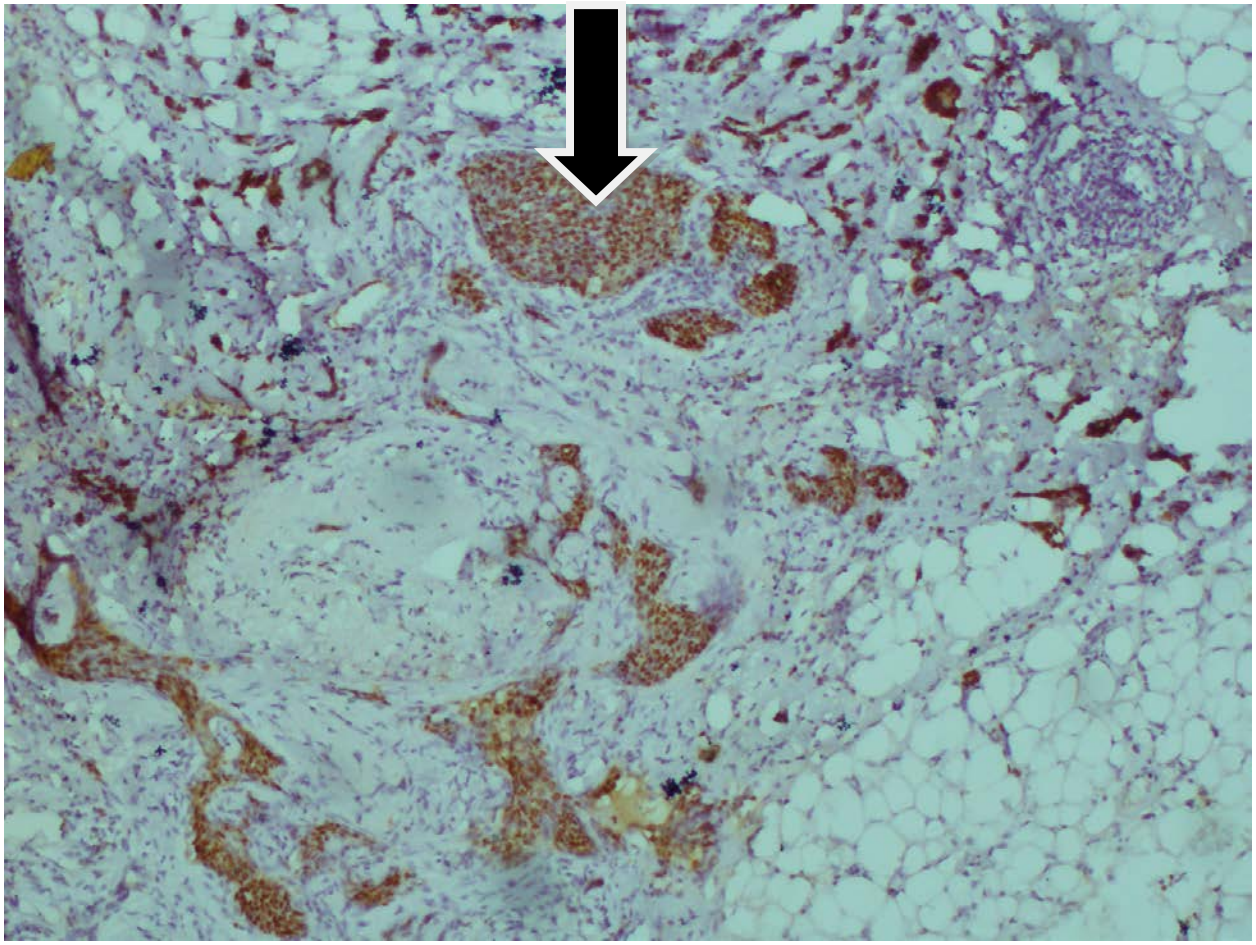

Figure 4.6: Photomicrograph of HER 2 Negative (X400). Marked area represents stained cells

Table 4.14 shows the expression pattern of oestrogen receptor (ER), progesterone receptor (PR), HER 2 and co-expression pattern of oestrogen receptor and progesterone receptors (ER/PR) of breast tumours. Participants with oestrogen receptor positive breast cancer were ten (12.7%) while sixty-nine participants (87.3%) had oestrogen receptor negative breast cancer. Eight (10.1%) had progesterone positive breast cancer, while seventy-one (89.9%) had progesterone receptor negative breast cancer. Fifteen participants (19.0%) had HER 2 positive breast cancer, while sixty-four (81.0%) had HER 2 negative breast cancer.

Table 4.15 shows the frequency and percentages of oestrogen, progesterone and HER 2 receptors in participants with breast cancer. Fifty-five (69.62%) had triple negative breast cancer i.e. negative expressions of the markers; ER, PR and HER 2. Two individuals (2.53%) were positive for the three receptors (triple positive breast cancer). Two (2.53%) were positive for oestrogen and progesterone receptors but negative for HER 2. Five (6.33%) were positive for oestrogen receptor but negative for progesterone receptor and HER 2. An individual (1.27%) was positive for oestrogen receptor and HER 2 but negative for progesterone receptor. Ten (12.66%) had negative oestrogen and progesterone receptors but positive HER 2. Two (2.53%) had negative oestrogen receptor and HER 2 but positive progesterone receptor. Two (2.53%) had negative oestrogen receptor but positive progesterone receptor and HER 2.

Table 4.14 Distribution of Hormone Receptor Positivity and Negativity in Women with Breast Cancer

Marker	Frequency(n)	Percentage (%)
Oestrogen Receptor		
ER+	10	12.7
ER-	69	87.3
Progesterone Receptor		
PR+	8	10.1
PR-	71	89.9
HER 2		
HER 2+	15	19.0
HER 2-	64	81.0

ER=Oestrogen receptor, PR= Progesterone receptor, HER 2=Human epithelial receptor 2

Table 4.15 Different Expression Patterns according to the Positivity and Negativity of ER, PR and HER2 in women with Breast Cancer

Number of Cases, n=79 (%)	ER	PR	HER 2
2 (2.53%)	+	+	+
2 (2.53%)	+	+	-
5 (6.33%)	+	-	-
1 (1.27%)	+	-	+
55 (69.62%)	-	-	-
10 (12.66%)	-	-	+
2 (2.53%)	-	+	-
2 (2.53%)	-	+	+
Premenopausal, n=52 (%)			
46 (88.5%)	-	-	-
6 (11.5%)	-	-	+
0(0%)	+	+	+
Postmenopausal, n=27 (%)			
2 (7.4%)	+	+	+
2 (7.4%)	+	+	-
4(14.8%)	+	-	-
1 (3.7%)	+	-	+
9 (33.3%)	-	-	-
4 (14.8%)	-	-	+
2 (7.4%)	-	+	-
3 (11.1%)	-	+	+

ER=Oestrogen receptor, PR= Progesterone receptor, HER 2=Human epithelial receptor 2

Table 4.16 Multiple regression of endocrine disruptors with hormones in women with breast cancer. Oestradiol significantly predicted Pb (β=0.374, p=0.027), progesterone significantly predicted Cd (β=0.348, p=0.039) and FT_3 significantly predicted BPA (β=0.404, p=0.036). However, FT_4 inversely predicted As (β=-0.337, p=0.002).

Table 4.17 shows the multiple of endocrine disruptors and hormones with anthropometric indices and blood pressure and hormone receptors in women with breast cancer. WC significantly predicted Pb (β=5.830, p=0.031), Cd (β=5.855, p=0.029). Diastolic blood pressure significantly predicted Cd (β=0.299, p=0.021). Waist circumference significantly predicted As. (β=-7.074, p=0.010). Hip circumference and WHR positively predicted As (β=3.832, p=0.011; β=2.732, p=0.007 respectively). Waist height ratio and height positively and significantly predicted BPA (β=8.786, p=0.047; β=3.046, p=0.045, respectively).

Table 4.18 shows the multiple regression of endocrine disruptors with Hormones in premenopausal women with breast cancer. Oestradiol positively and significantly predicted Pb (β=0.464, p=0.022) and Cd (β=0.423, p=0.038). FT_4 and TSH inversely and significantly predicted As (β=-0.277, p=0.046; β=-0.323, p=0.036, respectively). Progesterone significantly predicted PCBs (β=1.106, p=0.019), FT_3 inversely predicted PCBs (β=-0.605, p=0.033).

Table 4.19 shows the multiple regression of endocrine disruptors with anthropometric indices and blood pressure in premenopausal women with breast cancer. Hip circumference, height and WHR significantly predicted As (β=6.848, p=0.000; β=0.620, p=0.038; β=4.195, p=0.000, respectively). Waist circumference and SBP inversely and significantly predicted As (β=-9.861, p=0.001; β=-0.361, p=0.012, respectively).

Table 4.16 Multiple Regression of Endocrine Disruptors with Hormones in Women with Breast Cancer (HCBCa)

Dependent	Predictors	Beta	t	p
Lead				
R^2=0.103, F=1.259,p=0.282	Progesterone	-0.240	-1.448	0.152
	Oestradiol	0.374	2.253	0.027*
	LH	0.150	1.008	0.317
	FSH	0.116	0.733	0.466
	FT_3	0.147	1.265	0.210
	FT_4	-0.083	-0.760	0.449
	TSH	-0.074	-0.642	0.523
Cadmium				
R^2=0.106, F=1.300, p=0.262	Progesterone	-0.240	-1.447	0.152
	Oestradiol	0.348	2.096	0.039*
	LH	0.226	1.514	0.134
	FSH	0.025	0.156	0.876
	FT_3	0.148	1.280	0.204
	FT_4	-0.072	-0.656	0.514
	TSH	-0.087	-0.754	0.453
Arsenic				
R^2=0.154, F=1.997, p=0.066	Progesterone	-0.230	-1.428	0.157
	Oestradiol	0.154	0.957	0.342
	LH	-0.091	-0.629	0.531
	FSH	0.079	0.515	0.608
	FT_3	0.004	0.032	0.974
	FT_4	-0.337	-3.162	0.002*
	TSH	-0.124	-1.108	0.271
BPA				
R^2=0.371, F=2.698, p=0.026	Progesterone	0.107	0.389	0.700
	Oestradiol	-0.080	-0.311	0.758
	LH	-0.301	-0.964	0.342
	FSH	0.019	0.058	0.954
	FT_3	0.404	2.192	0.036*
	FT_4	-0.054	-0.366	0.716
	TSH	-0.198	-1.338	0.190

*=significant at $p<0.05$, beta= Standardized coefficient, p=Probability value.

Table 4.17 Multiple Regression of Endocrine Disruptors with Anthropometric Indices, Blood Pressure and Hormone Receptors in Women with Breast Cancer (HCBCa)

Dependent	Predictors	Beta	t	p
Lead				
R^2=0.223, F=1.745, p=0.082	Waist Circumference	5.830	2.199	0.031*
	Hip Circumference	-1.574	-1.090	0.280
	Waist Hip Ratio	-0.858	-0.877	0.384
	Waist Height Ratio	-4.532	-1.900	0.062
	Systolic Blood Pressure	0.059	0.474	0.637
	Diastolic Blood Pressure	0.225	1.770	0.081
	Height	-1.502	-1.803	0.076
	Body weight	-0.177	-1.233	0.222
	BMI	0.163	0.105	0.917
	ER	-0.226	-1.794	0.077
	PR	-0.095	-0.733	0.466
	HER 2	0.144	1.260	0.210
Cadmium				
R^2=0.235, F=1.870, p=0.059	Waist Circumference	5.855	2.226	0.029*
	Hip Circumference	-1.833	-1.280	0.205
	Waist hip Ratio	-1.042	-1.073	0.287
	Waist Height Ratio	-4.225	-1.785	0.079
	Systolic Blood Pressure	-0.009	-0.075	0.941
	Diastolic Blood Pressure	0.299	2.371	0.021*
	Height	-1.440	-1.742	0.086
	Body weight	-0.190	-1.336	0.186
	BMI	0.953	0.592	0.557
	ER	-0.213	-1.705	0.093
	PR	-0.088	-0.685	0.495
	HER 2	0.160	1.419	0.160
Arsenic				
R^2=0.208, F=1.596, p=0.120	Waist Circumference	-7.074	-2.643	0.010*
	Hip Circumference	3.832	2.630	0.011*
	Waist Hip Ratio	2.732	2.765	0.007*
	Waist Height Ratio	2.751	1.142	0.257
	Systolic Blood Pressure	-0.092	-0.738	0.463
	Diastolic Blood Pressure	-0.118	-0.922	0.360
	Height	0.969	1.153	0.253
	Body weight	0.180	1.246	0.217
	BMI	-1.594	-1.072	0.290
	ER	0.095	0.747	0.458
	PR	-0.233	-1.776	0.080
	HER 2	0.149	1.299	0.199
BPA				
R^2=0.322, F=1.164, p=0.356	Waist Circumference	-5.251	-1.055	0.301
	Hip Circumference	-2.697	-1.032	0.311
	Waist Hip Ratio	-1.721	-0.889	0.382
	Waist Height Ratio	8.786	2.083	0.047*
	Systolic Blood Pressure	0.135	0.678	0.503
	Diastolic Blood Pressure	-0.172	-0.852	0.401
	Height	3.046	2.106	0.045*
	Body weight	0.245	1.209	0.237
	BMI	-3.668	-0.811	0.433
	ER	-0.057	-0.177	0.861
	PR	-0.018	-0.063	0.950
	HER 2	0.038	0.199	0.844

*=significant at $p<0.05$, beta= Standardized coefficient, p=Probability value.

Table 4.18 Multiple Regression of Endocrine Disruptors with Hormones and HER 2 in Premenopausal women with Breast Cancer (Premenopausal-HCBCa)

Dependent	Predictors	Beta	t	p
Lead				
R^2=0.190, F=1.150,p=0.350	Progesterone	-0.346	-1.692	0.097
	Oestradiol	0.464	2.379	0.022*
	LH	0.185	1.229	0.226
	FSH	0.049	0.347	0.730
	FT_3	0.221	1.514	0.137
	FT_4	-0.092	-0.675	0.503
	TSH	-0.045	-0.300	0.765
Cadmium				
R^2=0.158, F=1.234, p=0.304	Progesterone	-0.329	-1.587	0.119
	Oestradiol	0.423	2.136	0.038*
	LH	0.184	1.199	0.237
	FSH	0.052	0.358	0.722
	FT_3	0.215	1.455	0.152
	FT_4	-0.085	-0.620	0.538
	TSH	-0.058	-0.383	0.703
Arsenic				
R^2=0.192, F=1.565, p=0.170	Progesterone	-0.390	-1.921	0.061
	Oestradiol	0.268	1.385	0.173
	LH	0.022	0.145	0.885
	FSH	0.149	1.053	0.298
	FT_3	0.026	0.178	0.859
	FT_4	-0.277	-2.051	0.046*
	TSH	-0.323	-2.165	0.036*
PCBs				
R^2=0.462, F=1.718, p=0.184	Progesterone	1.106	2.658	0.019*
	Oestradiol	-0.628	-1.781	0.097
	LH	-0.280	-1.208	0.247
	FSH	-0.057	-0.267	0.793
	FT_3	-0.605	-2.359	0.033*
	FT_4	0.225	1.044	0.314
	TSH	0.112	0.405	0.690

*=significant at $p<0.05$, beta= Standardized coefficient, p=Probability value

Table 4.19 Multiple Regression of Endocrine Disruptors with Anthropometric indices and Blood Pressure in Premenopausal women with Breast Cancer (Premenopausal-HCBCa)

Dependent	Predictors	Beta	t	p
Arsenic				
R^2=0.442, F=3.873, p=0.001	WC	-9.861	-3.719	0.001*
	HC	6.848	4.118	0.000*
	Height	0.620	2.135	0.038*
	Body weight	0.278	0.167	0.868
	SBP	-0.361	-2.630	0.012*
	DBP	-0.113	-0.834	0.409
	BMI	0.112	0.071	0.944
	WHR	4.195	4.273	0.000*
	WHtR	2.103	1.004	0.321

*=significant at p<0.05, beta= Standardized coefficient, p=Probability value. BMI=Body mass index, WC=Waist circumference, HC=Hip circumference. WHR=Waist hip ratio, WHtR=Waist height ratio, SBP=Systolic blood pressure, DBP=Diastolic blood pressure, TSH=Thyroid stimulating hormone.

Table 4.20 shows multiple regression of endocrine disruptors with Hormones and PR in postmenopausal women with breast cancer. FT_4 inversely predicted As (β=-0.484, p=0.009).

Table 4.21 shows multiple regression of endocrine disruptors with anthropometric indices and blood pressure in postmenopausal women with breast cancer. Weight and WC significantly predicted Pb (β=4.993, p=0.037; β=9.560, p=0.027, respectively). BMI, height and WHtR inversely predicted Pb (β=-4.183, p=0.035; β=-6.460, p=0.005; β=-8.326, p=0.037, respectively). Waist circumference significantly predicted Cd (β=8.910, p=0.042). Height inversely predicted Cd (β=-5.651; p=0.015).

Table 4.22 shows the multiple regression of endocrine disruptors with hormones in postmenopausal women without breast cancer. Progesterone significantly predicted Cd and PCBs (β=0.506, p=0.031, β=0.818, p=0.019, respectively). FSH and FT_3 significantly predicted PCBs (β=0.785, p=0.030, β=0.724, p=0.043, respectively).

Table 4.23 shows multiple regression of endocrine disruptors with anthropometric indices and blood pressure in postmenopausal women without breast cancer. BMI inversely predicted Pb (β=-1.831; p=0.013), body weight significantly predicted Pb (β=2.356, p=0.007). Waist circumference and WHtR significantly predicted BPA (β=28.357, p=0.024; β=21.638, p=0.020, respectively). Waist circumference inversely predicted As (β=-20.648, p=0.034). Height inversely predicted BPA (β=-8.205, p=0.013).

Table 4.20 Multiple Regression of Endocrine Disruptors with Hormones and PR in Postmenopausal Women with Breast Cancer (Postmenopausal-HCBCa)

Dependent	Predictors	Beta	t	p
Arsenic				
R^2=0.416, F=2.336, p=0.056	Progesterone	-0.199	-1.071	0.295
	Oestradiol	-0.108	-0.581	0.567
	LH	0.034	0.177	0.861
	FSH	-0.084	-0.394	0.697
	FT_3	-0.086	-0.524	0.605
	FT_4	-0.484	-2.857	0.009*
	TSH	0.238	1.392	0.177

*=significant at p<0.05, beta= Standardized coefficient, p=Probability value.

Table 4.21 Multiple Regression of Endocrine Disruptors with Anthropometric indices and Blood Pressure in Postmenopausal Women with Breast Cancer (Postmenopausal-HCBCa)

Dependent	Predictors	Beta	t	p
Lead				
R^2=0.453, F=1.934, p=0.102	BMI	-4.183	-2.259	0.035*
	Height	-6.460	-3.099	0.005*
	Body weight	4.993	2.221	0.037*
	WC	9.560	2.384	0.027*
	WHtR	-8.326	-2.221	0.037*
	HC	-1.726	-0.889	0.384
	WHR	-1.445	-0.875	0.391
	SBP	0.044	0.219	0.829
	DBP	0.298	1.529	0.141
Cadmium				
R^2=0.427, F=1.740, p=0.142	BMI	-3.501	-1.847	0.079
	Height	-5.651	-2.648	0.015*
	Body weight	4.183	1.818	0.083
	WC	8.910	2.170	0.042*
	WHtR	-7.336	-1.912	0.070
	HC	-1.935	-0.974	0.341
	WHR	-1.598	-0.946	0.355
	SBP	0.031	0.154	0.876
	DBP	0.322	1.616	0.121

*=significant at p<0.05, beta= Standardized coefficient, p=Probability value. BMI=Body mass index, WC=waist circumference, HC=Hip circumference. WHR=Waist hip ratio, WHtR=Waist height ratio, SBP=Systolic blood pressure, DBP=Diastolic blood pressure, LH=Luteinizing hormone, TSH=Thyroid stimulating hormone

Table 4.22 Multiple Regression of Endocrine Disruptors with Hormones in Postmenopausal Women without Breast Cancer (Postmenopausal-AHWB)

Dependent	Predictors	Beta	t	p
Cadmium				
R^2=0.355, F=1.727, p=0.154	Progesterone	0.506	2.305	0.031*
	Oestradiol	0.221	1.112	0.278
	LH	0.154	0.605	0.551
	FSH	0.171	0.695	0.494
	FT_3	0.331	1.556	0.134
	FT_4	0.049	0.278	0.783
	TSH	0.314	1.675	0.108
PCBs				
R^2=0.700, F=2.667, p=0.096	Progesterone	0.818	2.941	0.019*
	Oestradiol	-0.135	-0.449	0.665
	LH	-0.635	-1.910	0.092
	FSH	0.785	2.638	0.030*
	FT_3	0.724	2.397	0.043*
	FT_4	-0.102	-0.452	0.663
	TSH	0.137	0.585	0.574

*=significant at p<0.05, beta= Standardized coefficient, p= Probability value

Table 4.23 Multiple Regression of Endocrine Disruptors with Anthropometric Indices and Blood Pressure in Postmenopausal Women without Breast Cancer (Postmenopausal-AHWB)

Dependent	Predictors	Beta	t	p
Lead				
R^2=0.372, F=1.382, p=0.257	BMI	-1.831	-2.718	0.013*
	Height	-0.089	-0.038	0.970
	Body weight	2.356	2.959	0.007*
	WC	-8.611	-1.000	0.329
	WHtR	2.605	0.377	0.710
	HC	6.028	1.856	0.078
	WHR	3.676	1.859	0.077
	SBP	-0.021	-1.000	0.921
	DBP	-0.252	-1.104	0.282
Arsenic				
R^2=0.301, F=1.004, p=0.467	BMI	-0.682	-0.959	0.348
	Height	4.145	1.689	0.106
	Body weight	1.076	1.281	0.214
	WC	-20.648	-2.273	0.034*
	WHtR	13.995	1.921	0.068
	HC	6.390	1.865	0.076
	WHR	4.058	1.945	0.065
	SBP	-0.014	-0.064	0.949
	DBP	-0.072	-0.297	0.749
BPA				
R^2=0.820, F=3.546, p=0.055	BMI	0.702	1.007	0.347
	Height	-8.205	-3.284	0.013*
	Body weight	0.218	0.275	0.792
	WC	28.357	2.862	0.024*
	WHtR	21.638	3.011	0.020*
	HC	-6.274	-1.700	0.133
	WHR	-3.558	-1.792	0.116
	SBP	0.210	0.903	0.396
	DBP	0.294	1.451	0.190

*=significant at $p<0.05$, beta= Standardized coefficient p=Probability value. BPA=Bisphenol-A, BMI=Body mass index, WC=Waist circumference, HC=Hip circumference. WHR=Waist hip ratio, WHtR=Waist height ratio, SBP=Systolic blood pressure, DBP=Diastolic blood pressure.

CHAPTER FIVE

5.0 DISCUSSION

Breast cancer is the most common type of cancer among women worldwide with a noticeable fatality rate (Wang *et al*., 2009). An increase in premenopausal breast cancer accounting for between 57 and 67% of breast cancer has been reported. This represents a higher proportion of premenopausal than postmenopausal breast cancer. Postmenopausal breast cancer accounts for about 20% of breast cancer in indigenous African women (Okonofua, 1999; Adesunkanmi, 2006; Oluwatosin and Oladepo, 2006; Okobia *et al*., 2006; Abdulkareem, 2009; Kene, 2010; Sule, 2011). These observations are similar to the findings in this present study. Fifty four (63.5%) of the HCBCa were premenopausal while 31 (36.5%) were postmenopausal, illustrating the prominence of premenopausal breast cancer in Nigeria.

In this study, the mean age at presentation by HCBCa was 48.32 ±1.3 years. This is consistent with other studies (Elumelu *et al*., 2011; Popoola *et al*., 2012). The reduced life expectancy in Nigeria and other developing countries has been attributed to young people constituting a large percentage of the population (Adebamowo and Ajayi, 2000). Late presentation in the clinic of advanced breast cancer in stages 3 and 4 is a peculiar feature that has been widely reported, particularly in indigenous Nigerian women (Ntekim *et al*., 2009; Elumelu *et al*., 2011). In this study, 83.5% participants presented at advanced stages of the disease (stages 3 and 4). This has been adduced to lack of adequate knowledge of the disease, fear of mastectomy and poverty (Ajekigbe, 1991; Oluwole *et al*., 2003; Elumelu *et al*., 2011).

Unilateral breast cancer is more frequent in the left breast than in the right (Tulinius *et al*., 1990). Contrarily, in this study, there was no association between left and right breast cancer site in both pre and postmenopausal-HCBCa. This confirms the study of Ohanaka (2007), who did not observe an association between the left and the right breasts of young women with breast cancer. Emerging information suggests that breast density rather than anatomical site is a strong quantitative risk factor for breast cancer (Hennessey *et al*., 2014). Breast density reflects fibro-glandular tissue which comprises of epithelial and stromal tissues in the breast (McCormack and DosSantos-Silva, 2006).

Observations in this study showed that reproductive factors such as number of previous pregnancies, number of live births and number of induced abortions were significantly higher in premenopausal-HCBCa compared with premenopausal-AHWB ($p<0.05$). Induced abortion was significantly associated with increased risk of breast cancer among Chinese females (Huang *et al*., 2014). Age at menarche was also significantly higher in premenopausal-HCBCa compared with premenopausal-AHWB ($p<0.05$). This is at variance with an earlier report of an association between early age at menarche and increased risk of breast cancer attributed to increased exposure to oestrogens (Orgeas *et al*., 2008).

Endogenous sex steroid hormones have been reported to play a major role in the aetiology of breast cancer (Bernstein and Ross, 1993; Clemons and Goss, 2001). Both premenopausal and postmenopausal women secrete steroid hormones throughout their lives with a difference in the pattern of secretion. The hormones are mainly regulated by the ovary in premenopause while they are regulated in postmenopause by the adrenal gland. In the development of breast cancer, the tumour grows within a hormonal milieu which has a decisive influence on its growth. (Harnandez *et al*., 2005). In spite of the multiple epidemiological studies that have investigated the association of serum sex hormones and premenopausal breast cancer risk, the results have been inconsistent (Wysowski *et al*., 1987; Key and Pike, 1988; Helzlsouer *et al*., 1994; Rosenberg *et al*., 1994; Thomas *et al*., 1997; Kabuto *et al*., 2000; Haslam *et al*., 2002; Yu *et al*., 2003; Micheli *et al*., 2004; Missimer *et al*., 2004; Kaaks *et al*., 2005; Eliassen *et al*., 2006; Ho *et al*., 2009).

Comparison of E_2 level between premenopausal-HCBCa and premenopausal-AHWB in this study showed no significant difference ($p>0.05$). These findings have been reported by others (Sturgeon *et al*., 2004; Ho *et al*., 2009). However, E_2 and progesterone levels were higher in postmenopausal-HCBCa compared with postmenopausal-AHWB ($p<0.05$) in this present study. Positive association of E_2 with breast cancer risk in postmenopausal women has previously been observed (Hankinson *et al*., 1998).The underlying mechanisms of action of E_2 in the aetiology of breast cancer include the alkylation of cellular molecules, generation of active radicals and genotoxicity of oestrogen metabolites which are involved in initiation, promotion and progression of breast cancer (Nandi *et al*., 1995; Clemons and Goss, 2001; Yager and Davidson, 2006; Drabsch*et al*., 2007). Wang *et al*. (2009) also observed high levels of progesterone in

postmenopausal breast cancer. Increased postmenopausal progesterone levels have also been implicated in dementia, with unknown reasons but may relate to small subclinical cerebral thrombosis (Yaffe, 2003; Zhu and Brinton, 2012). It is uncertain if the elevated progesterone in the postmenopausal-HCBCa in this study is related to menopause or breast cancer.

Follicle stimulating hormone controls E_2 level by negative feedback mechanism in premenopausal women (Fabian*et al*., 2015). Serum LH and FSH were significantly higher in premenopausal-HCBCa compared with premenopausal-AHWB ($p<0.05$). High serum LH and FSH were reported to be associated with a significantly worse breast cancer prognosis in premenopausal breast cancer patients (Pujol *et al*., 2001). The ability of FSH to activate adenylyl cyclase thereby resulting in increased cAMP levels could be associated with its ability to induce breast cancer cell proliferation, differentiation and metastasis (Tunizicker-Dunn and Maizels, 2006; Zreik *et al*., 2006, Zhou *et al*., 2013). These findings implicate gonadotropin exposure in premenopausal breast carcinogenesis. Serum FSH level was significantly lower in postmenopausal-HCBCa compared with postmenopausal-AHWB ($p<0.05$) in this study. Although, the reasons are not clear, low FSH level has also been observed in postmenopausal women with ovarian cancer (Arslan *et al*., 2003; McSorley *et al*., 2009). However, breast and ovarian cancers are hormone-dependent cancers with genomic similarities (CGAN, 2012). Mechanisms involving FSH reduction and increased E_2 may underlie postmenopausal breast cancer in this study.

Oestrogen receptor, PR and HER 2 play important roles in the growth and differentiation of breast cancers making them important prognostic markers (Patel *et al*., 2013; Mohamed *et al*., 2015; Deepti*et al*., 2015). Women with ER+ breast cancer can benefit from endocrine therapy explaining their better survival outcomes (Makanjuola *et al*., 2014). In this study ER-, PR- and HER 2- were observed in 69 (87.3%), 71 (89.9%), 64 (81.0%) HCBCa respectively. Huo *et al.* (2009) reported the predominance of hormone receptor negative breast cancer in indigenous African women. Oestrogen receptor negative and PR- were observed in all premenopausal-HCBCa.These findings suggest the involvement of genetics in the aetiology of breast cancer. Young women are diagnosed with breast cancer with more aggressive tumour and are associated with higher mortality, shorter disease-free survival and more likely to recur after treatment both

loco regionally and at distant sites than in older women (Nixon *et al*., 1994; Gajdosc *et al*., 2000; Foxcroft *et al*., 2004; Ntekim *et al*., 2009).

Hormone receptor positive expressions were however observed in less than 20% of postmenopausal-HCBCa in this study. These findings were similar to other studies in Africans (Huo*et al*., 2009; Stark *et al*., 2010). Contrarily, observations in blacks residing in the United States of America and United Kingdom showed a higher proportion of positive receptors expressions (Chu and Anderson, 2002; Bowen *et al*., 2008; Ahmed *et al*., 2011; Ali *et al*., 2012). These reports implicate geographic or environmental factors beyond genetics. Most evidence regarding the prognostic role of PR is based on the assumption that PR expression indicates a functioning ER pathway (Ravdin *et al*., 1992). Hence, PR+ and ER- tumours have a better response to endocrine therapy than ER+ and PR- (Payne *et al*., 2008).

Triple negative breast cancers are poorly differentiated and are characterized by an aggressive clinical history. No specific treatment guidelines are currently available for this breast cancer sub-type. However, they are managed with standard treatment, which leaves them with a high rate of local and systemic relapse (Cleator *et al*., 2007). In this study, 55 (69.62%) HCBCa were triple negative. Forty six (88.5 %) HCBCa were premenopausal while 9 (11.5 %) were postmenopausal. Stark *et al*. (2010) and Makanjuola *et al.* (2014) reported a high prevalence of triple negative hormone receptors in their different studies in indigenous African women.

Thyroid hormones may be critical in the pathogenesis and progression of diseases due to their regulatory role on cell maturation (Mourouzis *et al*., 2013; Mourouzis *et al*., 2015). Thyroid signalling may be altered in cancer as a result of the activation of growth kinase signaling which may be of physiological relevance (Pallud *et al*., 1999; Casula and Bianco, 2012). Several studies which compared levels of peripheral thyroid hormones in women with breast cancer and women without breast cancer are inconclusive regarding associations between thyroid hormones and breast cancer risk (Goldman, 1990; Smyth, 1997; Sarlis *et al*., 2002, Tosovic *et al*., 2012).

In this present study, there was an association between FT_4 and HCBCa (pre and postmenopausal). Guigon *et al*. (2011) reported an association between FT_4 and breast cancer. Thyroid hormones appear to stimulate lobular development, contributing to the differentiation of breast tissue (Neville *et al*., 2002). It is postulated that the thyroid gland interacts with the breast

tissues based on the common property of the mammary and thyroid epithelial cells to concentrate iodine by a membrane active transport mechanism. Additionally, TSH receptors in fatty tissues which are abundant in the mammary gland have been reported to be a possible reason for this interaction (Turken *et al*., 2003; Ali *et al*., 2011). However, serum levels of the thyroid hormones in the study participants were within the normal reference interval (FT_3, 3.2-6.0pmol/L; FT_4, 10.6-21.0 pmol/L; TSH, 0.38-4.31mIU/L). Emerging reports show that changes in thyroid hormone levels within normal range may be associated with proliferative activity of breast tumours in euthyroid patients with breast cancer (Milionis and Milionis, 2013).

The increase in breast cancer incidence in women has been related to industrialization consequent upon the widespread contamination of the soil, air and water by the toxic metals (Jarup and Akesson, 2009; Julin *et al*., 2012; Ragab *et al*., 2014). Breast cancer is a multistep process involving both genetic and epigenetic changes (Lustberg and Ramaswamy, 2009) such as differential DNA methylation and altered histone modifications. Hypermethylation blocks the promoter region of a gene and results in gene silencing. Identification of epigenetic changes and their correlation with other factors could lead to improvements in cancer diagnosis and treatment (Sunami *et al*., 2008). Metals act as catalyst in the oxidative deterioration of biological macromolecules, induce reactive oxygen species, which accumulate and induce epigenetic factors (Hou *et al*., 2012).

In this present study, there was an association between Cd and breast cancer (pre and postmenopausal). The ability of Cd to induce cell proliferation, differentiation, apoptosis and signal transduction by enhancement of protein phosphorylation, activation of transcription and translation factors suggests its ability to induce breast cancer (Joseph *et al*., 2001; Jin *et al*., 2003; Shih *et al*., 2004; Martinez-campa *et al*., 2006; Sun *et al*., 2007; Templeton and Liu, 2010; Yu *et al*., 2010; Siewt *et al*., 2010). Hypermethylation and repression of DNA repair genes appear to be an early signature of cadmium-induced cancer and may constitute part of the mechanisms by which the toxicant induces tumorigenesis (Zhou *et al*., 2008). Additionally, Cd has the potential to disrupt endocrine function by behaving like sex hormones (Enmark and Gustafsson, 1999; Stoica *et al*., 2000b; Thomas and Dong, 2006). At low concentrations, the metal mimics the effects of E_2 and binds with high affinity to the hormone-binding domain of ERα. This binding involves several amino acids, suggesting that Cd activates the receptor

through the formation of a complex with specific residues in the hormone-binding domain (Johnson *et al*., 2003; Benbrahim-Tallaa, 2009).

Lead is of concern due to its wide use (Florea and Busselberg, 2011). However, results of epidemiologic studies investigating the association of Pb with cancers are inconsistent and vary according to the type of cancers reported (Steenland *et al*., 1992; Wong and Harris, 2000). Direct DNA damage as a result of oxidative stress, clastogenicity, inhibition of DNA synthesis or repair has been reported as the mechanisms of Pb carcinogenicity (Martin *et al*., 2003; Ragab *et al*., 2014). In this present study, Pb was associated with pre and postmenopausal breast cancer. This is consistent with the findings of Siddiqui *et al*. (2006) in which blood Pb level was significantly higher in breast cancer patients than their controls. Lead adversely affects steroidogenesis by substituting for zinc in the DNA binding zinc (Zn^{2+})-finger motif of steroidogenic enzymes, resulting in their decreased expression. These enzymes are steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage enzyme (CYP450cc) and 3 beta hydroxysteroid dehydrogenase (3β HSD). (Lutzen *et al*., 2004).

Arsenic exposure constitutes one of the most wide-spread environmental carcinogens and is associated with increased risk of different types of cancers (Florea *et al*., 2007; Florea and Busselberg, 2008; Ying *et al*., 2009). However, few studies have focused on the association of environmental exposure to As and breast cancer risk. Information on the association of As with breast cancer in sub-Saharan Africa is sparse. In this present study, As was associated with pre and postmenopausal breast cancer. Low dose As represses tumour suppressor genes (Li *et al*., 2010). Transcription factors in human MDA-MB-435 breast cancer and rat H4IIE hepatoma cells were reportedly sensitive to low dose As (Kaltreider *et al*., 1999; Stoica *et al*., 2000a). Arsenic is thought to induce carcinogenicity by inducing DNA hypomethylation leading to aberrant gene expression (Zhao *et al*., 1997; Verma and Srivastave, 2002) or by DNA methylation silencing genes associated with controlling tumourigenesis (Vaissiere *et al*., 2008).Arsenic competes with DNA methyl transferase genes (DNMT) for S adenosylmethionine (SAM), potentially limiting the availability of SAM to be used by DNMT to catalyze methylation of CpG. This could result in hypomethylation and reactivation of silenced tumour suppressor genes (Vo An and Millis, 2012; Pogrinby and Rusyn, 2013). Altered histone modification associated with arsenic-induced gene expression in carcinogenesis has been suggested (Zhou *et al*., 2008).

Humans are exposed daily to a variety of compounds.It is thus likely thatthe combination or mixture of chemicals may become dangerous even whennone of the chemicals reaches an effective level. These chemicals enter the food chain and accumulate in animals and eventually humans (Lubrano *et al*., 2013). Bisphenol-A, a breakdown product of coatings in food and beverage containers, may act as oestrogen receptor agonist (Meerts *et al*., 2001; Fernandez and Russo, 2010). Bisphenol-A promotes the proliferation of both ER+ and ER- breast cancer cells (Song *et al*., 2015). Recent report suggests that BPA enhances the growth of triple negative breast cancer cells via oestrogen related receptor gamma (ERRγ) and matrix metalloproteinases (MMPs). This involves the activation of extracellular signal regulated kinases and protein kinases B (Akt) which inhibit apoptosis (Zhang *et al*., 2016). In this present study, BPA was associated with pre and postmenopausal breast cancer.

Environmental exposure to PCBs has been suggested as potential causes of breast cancer (Davis *et al*., 1993; Wolff *et al*., 1993). Polychlorinated biphenyls are weak oestrogens in vitro, have tumour promoting ability and are able to induce metabolic enzymes (Norback and Weltman, 1985; McKinney and Waller, 1994). In this study, an association was observed between PCBs and breast cancer (pre and postmenopausal). Polychlorinated biphenyls exposure may lead to formation of DNA adduct through a pathway involving cytrochrome P450 1A1 (CYP1A1) (Oakley *et al*., 1996). Cytochrome P450 1A1 is important in the metabolism of potentially genotoxic chemicals (Pelkonen and Nebert, 1982). The interaction of PCBs with CYP1A1 polymorphisms in the aetiology of breast cancer is therefore suggested. Polychlorinated biphenyls activate aryl hydrocarbon receptor (AhR). The mechanisms through which AhR regulates energy metabolism are not clearly established, although ER has been implicated (Lubrano *et al*., 2013). Emerging evidence indicates that BPA and PCBs can affect mitochondrial function and cause pro-oxidative conditions leading to pathological conditions like cancer (Valavinides *et al*., 2006; Albers *et al*., 2010; Farahat *et al*., 2011). Oxidative stress may thus be one of the the mechanisms, whereby environmental toxicants cause breast cancer.

Cadmium has the potential of disrupting endocrine function by behaving like sex hormones (Yu *et al*., 2003; Siewt *et al*., 2010). Cadmium and Pb were positively related with E_2 in premenopausal-HCBCa in this study. There was also a positive relationship between PCBs and progesterone in premenopausal-HCBCa. Polychlorinated biphenyls activate aryl hydrocarbon

receptor (AhR) which has been associated with ER (Lubrano *et al*., 2013). Polychhlorinated biphenyls are also associated with ER-negative tumours which have a faster rate of progression (Carpenter *et al*., 2005).

Arsenic was inversely related with FT_4 in pre and postmenopausal-HCBCa. An inverse relationship was also observed between As and TSH as well as PCBs and FT_3 in premenopausal-HCBCa. These observations suggest the possible interference of thyroid hormones by As and PCBs in women with breast cancer. This could be due to the binding of As to the thyroid hormone receptors which blocks the binding of the thyroid hormones (Davey *et al*., 2008). Bisphenol-A was positively related with FT_3 in HCBCa. In vitro studies demonstrate that BPA binds to thyroid receptors with relatively low affinity and mediate growth stimulatory effect via T_3- receptors (Meerts *et al*., 2001).

Adiposity is a prognostic factor of breast cancer as well as an independent risk factor of postmenopausal breast cancer (Chan and Norat, 2015). Waist circumference, waist hip ratio and waist height ratio are indicators of visceral adiposity. Body weight and BMI are indicators of general adiposity while hip circumference is an indicator of subcutaneous adiposity (Charles-Davies *et al*., 2012; Amadou *et al*., 2013). Although the women in this present study were matched for age and menstrual status, increased adiposity (waist circumference, hip circumference, body weight, height, waist hip ratio and waist height ratio) was observed in premenopausal-HCBCa compared with premenopausal-AHWB. Fagherazzi *et al.* (2012) showed an association between hip circumference and premenopausal breast cancer. Increased adiposity (body weight and height) was also significantly higher in postmenopausal-HCBCa compared with postmenopausal-AHWB in this study.

Increased visceral adiposity and insulin resistance characterise metabolic syndrome (MS), which predisposes individuals to chronic diseases-cancer, cardiovascular diseases and type 2 diabetes mellitus. It is associated with the female gender and is prevalent in 44.5% of apparently healthy women in Ibadan. Increased visceral adiposity, a strong metabolic risk factor was the most frequent component in these women while reduced high density lipoprotein cholesterol was the most frequent component in males (Charles-Davies *et al*., 2014; Chan and Norat, 2015).

Sex hormones-testosterone and oestrogen are synthesised from cholesterol. Increased conversion of testosterone to oestradiol by aromatase in increased adipose tissue has been reported in pre-menopausal women with MS (Fabian *et al*., 2015). Thus, increased adiposity alone may not underlie the aetiology of breast cancer. Ogundiran *et al*. (2010) showed no association between body weight and the risk of breast cancer in indigenous African women with breast cancer irrespective of their menstrual status.

However, endocrine disruptors are known to accumulate in adipose tissue (Grun and Blumberg, 2009). It thus appears that increased adiposity may enhance the accumulation of endocrine disruptors in the pathology of breast cancer (Ajayi *et al*., 2014). This hypothesis is corroborated in this study as height had a positive relationship with As in premenopausal-HCBCa. However, menopause may define the role of endocrine disruptors in increased adipose tissue, in breast cancer as body weight had a positive relationship with Pb in both postmenopausal-HCBCa and postmenopausal-AHWB. In postmenopausal women, Pb from prolonged environmental exposure may accumulate in adipose tissue without causing breast cancer. Height had a negative relationship with Cd and Pb in postmenopausal-HCBCa.

Mechanisms in the pathogenesis of breast cancer may differ between pre and postmenopause and may involve different endocrine disruptors and fat depots. Previous studies showed increased height in apparently healthy premenopausal women with metabolic syndrome than without metabolic syndrome (Charles-Davies *et al*., 2012). Short term exposure to As rather than increased height may be a breast cancer risk factor in pre-menopause. Long term exposure to Pb and Cd may be involved in breast cancer pathogenesis without the contribution of height in postmenopause.

Body mass index was inversely related with Pb in both postmenopausal-HCBCa and postmenopausal-AHWB. This suggests that BMI and Pb may not be important as breast cancer risk factors in postmenopause. Moreover, waist height ratio (a strong index of visceral obesity) was inversely related with Pb in postmenopausal-HCBCa. Although, visceral adiposity appeared not important in postmenopausal breast cancer, waist circumference was positively related with Cd and Pb in HCBCa and postmenopausal-HCBCa. These findings suggest that Cd and Pb may accumulate in increased abdominal adiposity in postmenopausal women with breast cancer. In HCBCa in this study, waist hip ratio was positively related with As while an inverse relationship

existed between waist circumference and As in HCBCa. Hip circumference was positively related with As in HCBCa and premenopausal-HCBCa. Arsenic is lipophilic and probably has preference for subcutaneous fat (Ying *et al*., 2009).

In this present study, mean values of SBP and DBP in the HCBCa and AHWB reflect normal blood pressure. However, SBP was significantly higher in HCBCa and premenopausal-HCBCa than their respective controls. This might reflect the mild increase in visceral obesity in premenopausal-HCBCa compared with premenopausal-AHWB. Hypertension was associated with metabolic syndrome and the female gender (Fabian *et al*., 2015). Experimental studies indicate that As exposure may be involved in the development of hypertension through the activation of stress response transcription factors including activator protein and nuclear factor – kappa B (Aposhian *et al*., 2003; Balakumar *et al*., 2008). In vitro arsenite altered vascular tone in blood vessels by suppressing vasorelaxation and increased the expression of cyclooxygenase-2 in endothelial cells (Lee *et al*., 2003). In this study, SBP was inversely related with As in premenopausal-HCBCa. This reason for this observation is unclear, it is hypothesized that the influence of As on blood pressure in women with breast cancer could be menstrual phase specific. Diastolic blood pressure was positively related with Cd in HCBCa in this study. Tellez-Plaza *et al*. (2008) reported an association between blood pressure and DBP via these mechanisms; partial agonism for calcium channels, direct vasoconstrictor action, activation of the sympathetic nervous system and inhibition of vasodilator substances such as nitric oxide (Bilgen *et al*., 2003; Varoni *et al*., 2003).

In HCBCa, BPA was positively related with waist height ratio and height. In postmenopausal-AHWB, BPA was positively related with waist circumference and waist height ratio, while it was inversely related with height. These observations suggest that BPA could be involved in adiposity. Studies have shown that exposure to BPA could suppress the release of adiponectin, an adipocyte-specific hormone that increases insulin sensitivity, this could lead to insulin resistance and increased susceptibility to obesity and metabolic syndromes which have been implicated in breast cancer (Hugo *et al*., 2008; Li *et al*., 2013).

An association between diets and the risk of breast cancer has been observed. This is because environmental toxicants are present in the food chain (Reuben, 2010). Regular consumption of fruits and vegetables are associated with decrease risk of many cancers, however, results for

breast cancer risk are not conclusive (Riboli and Norat, 2003; Guadet *et al*., 2004; Hirose *et al*., 2005; WCRF, 2007; de Lima *et al*., 2008). In this present study, HCBCa consumed more vegetables but less fruits weekly. Nutrient loss occurs in the preparation and cooking processes of vegetables, particularly in Nigeria, resulting in the reduction of bio-available phytochemicals (including antioxidant vitamins) and other anticarcinogenic compounds capable of protecting against cancer (Taiwo and Akanbi, 1997; Cavagnaro and Galmarini, 2012; Czarnowska and Gujska, 2012).

Meat and dairy products contain fat with a high proportion of saturated fatty acids which have been associated with increased breast cancer risk. They may also contain insulin-like growth factor-1 (IGF-1) which has been reported to promote breast cancer cell growth and pesticides that are potentially carcinogenic (Moormar and Terry, 2004). Consumption of red meat has been associated with increased risk of breast cancer in some studies, while the association of dairy product intake with breast cancer risk is inconclusive (Zheng *et al*., 1998; Moormar and Terry, 2004; Taylor *et al*., 2007). In this present study, daily consumption of red meat was associated with HCBCa while weekly consumption of diary product was associated with AHWB. This suggests that red meat consumption may be involved in breast carcinogenesis

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATION

6.1 Summary and Conclusions

Breast cancer is the most common type of cancer among women worldwide with a noticeable fatality rate. Fifty four (63.5%) of the HCBCa were premenopausal while 31 (36.5%) were postmenopausal, illustrating the prominence of premenopausal breast cancer in Nigeria. The reduced mean age at presentation by HCBCa was 48.32 ±1.3 years reflects the reduced life expectancy in Nigeria and other developing countries. Late presentation of 83.5% of HCBCa in the clinic of advanced breast cancer in stages 3 and 4 was a peculiar feature in this study. This has been adduced to lack of adequate knowledge of the disease, fear of mastectomy and poverty.

Reproductive factors-increased number of previous pregnancies, increased number of live births and increased number of induced abortions were associated with premenopausal breast cancer. These findings corroborate earlier reports. The association of increased age at menarche with premenopausal breast cancer risk in this present study is contrary to reports by others on early age at menarche and increased risk of breast cancer which was attributed to increased exposure to oestrogens.

Endogenous sex steroid hormones have been reported to play a major role in the aetiology of breast cancer. Thyroid hormones appear to stimulate lobular development, contributing to the differentiation of breast tissue. Elevated FT_4 level was associated premenopausal breast cancer while elevated oestradiol and progesterone levels and FT_4 levels were associated with postmenopausal breast cancer in this study, also corroborating previous findings. Although the serum levels of the thyroid hormones in the study participants were within the normal reference interval, emerging reports show that changes in thyroid hormone levels within normal range may be associated with proliferative activity of breast tumours in euthyroid patients with breast cancer.It is however uncertain if the elevated progesterone in the postmenopausal with breast cancer in this study is related to menopause or breast cancer.

The role of gonadotropins in the aetiology of breast cancer is increasingly gaining attention. Increased levels of serum gonadotropins-LH and FSH were associated with premenopausal

breast cancer in this study probably reflecting worse breast cancer prognosis in premenopausal breast cancer patients. However, reduced serum FSH level was associated with postmenopausal breast cancer in this study similar to previous studies with unclear reasons.

Oestrogen receptor, PR and HER 2 play important roles in the growth and differentiation of breast cancers making them important prognostic markers. In this study, 52 (100%) and 46 (88.5%) of premenopausal HCBCa had ER/PR negative and triple negative expressions respectively. Hormone receptor positive expressions were observed in less than 20% of postmenopausal breast cancer in this study. This corroborates the predominance of hormone receptor negative and aggressive cancer breast cancer with high mortality particularly in younger indigenous African women contrary to observations in the Caucasians. These findings implicate geographic or environmental factors beyond genetics.

Environmental toxicants studied-Cd, Pb, As, BPA and PCBs may be breast cancer risk factors Hypermethylation and repression of DNA repair genes, disruption of endocrine function through interaction and mimicry of specific steroid hormones and their receptors, induction of metabolic enzymes and pro-oxidative conditions may underlie mechanisms that lead to breast cancer. Thus identification of epigenetic changes and their correlation with other factors could lead to improvements in cancer diagnosis and treatment. Lead adversely affects steroidogenesis by substituting for zinc in the DNA binding zinc (Zn^{2+})-finger motif of steroidogenic enzymes, resulting in their decreased expression. Arsenic is thought to induce carcinogenicity by inducing DNA hypomethylation leading to aberrant gene expression.

Adiposity has been implicated is a prognostic and independent risk factor of postmenopausal breast cancer. Increased adiposity was also associated with pre and postmenopausal breast cancer in this study. However, findings suggest that endocrine disruptors are the actual culprits as some accumulate in adipose tissue to exert their deleterious effects.

Identification of hormone receptor expression, appropriate diet rich in antioxidants, low fat and reduced red meat; physical activity and reduction of environmental pollution may be beneficial in the prevention and management of breast cancer.

6.2 Recommendation

Determination of hormone receptors status may assist in the proper management of breast cancer. Reduction of environmental pollution by appropriate government legislation, safety regulations on the use of toxic substances will reduce the incidence of breast cancer. Intake of diet rich in antioxidants will protect against oxidative stress which is involved in breast carcinogenesis. Moreover, diet low in fat, reduced consumption of red meat as well as regular physical exercise may be of benefit to the women diagnosed with breast cancer. Routine screening for thyroid status is also recommended. Overall, maintenance of a healthy lifestyle is key in the prevention and management of breast cancer.

Contributions to Knowledge in the Discipline

i. The endocrine disrupting ability of known environmental toxicants-cadmium, lead, arsenic, bisphenol-A and polychlorinated biphenyls may result in development of breast cancer.

ii. The accumulation of these endocrine disruptors in adipose tissue and their interaction with oestradiol, progesterone and thyroid hormones may be underlie mechanisms in breast cancer aetiology in Nigeria.

iii. All premenopausal breast cancer are ER/PR receptor negative. Receptor triple negative expressions (ER, PR and HER 2) are the predominant in breast cancer particularly in premenopause in Nigeria suggesting that mechanisms involved in breast cancer development may be different between receptor negative premenopausal breast cancer and receptor positive post menopausal breast cancer.

iv. Elevated FT_4 level was associated premenopausal breast cancer while elevated E_2, progesterone and FT_4 levels were associated with postmenopausal breast cancer.

v. Increased levels of serum gonadotropins-LH and FSH were associated with premenopausal breast cancer while reduced serum FSH level was associated with postmenopausal breast cancer in this study.

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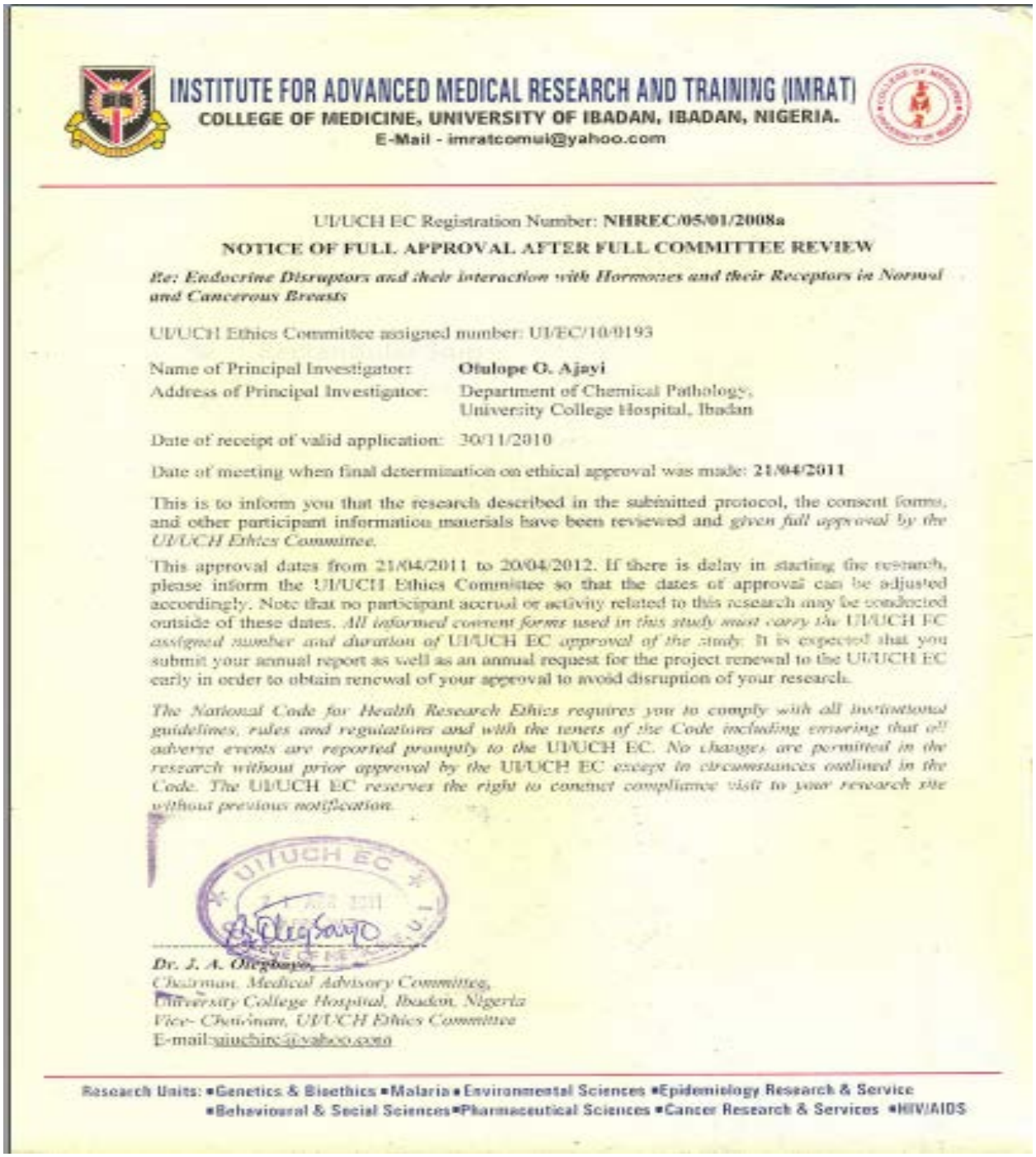
APPENDCES

APPENDIX 1(a)



INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IMRAT)
COLLEGE OF MEDICINE, UNIVERSITY OF IBADAN, IBADAN, NIGERIA.
E-Mail - imratcomui@yahoo.com

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

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Endocrine Disruptors and their interaction with Hormones and their Receptors in Normal and Cancerous Breasts

UI/UCH Ethics Committee assigned number: UI/EC/10/0193

Name of Principal Investigator: **Olulope O. Ajayi**
Address of Principal Investigator: Department of Chemical Pathology, University College Hospital, Ibadan

Date of receipt of valid application: 30/11/2010

Date of meeting when final determination on ethical approval was made: **21/04/2011**

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

This approval dates from 21/04/2011 to 20/04/2012. If there is delay in starting the research, please inform the UI/UCH Ethics Committee so that the dates of approval can be adjusted accordingly. Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed content forms used in this study must carry the* UI/UCH EC *assigned number and duration of* UI/UCH EC *approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC early in order to obtain renewal of your approval to avoid disruption of your research.

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

UI/UCH EC

Dr. J. A. Olegbayo,
Chairman, Medical Advisory Committee,
University College Hospital, Ibadan, Nigeria
Vice- Chairman, UI/UCH Ethics Committee
E-mail:

Research Units: •Genetics & Bioethics •Malaria • Environmental Sciences •Epidemiology Research & Service •Behavioural & Social Sciences •Pharmaceutical Sciences •Cancer Research & Services •HIV/AIDS

APPENDIX 1(b)

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IAMRAT)
COLLEGE OF MEDICINE, UNIVERSITY OF IBADAN. IBADAN, NIGERIA.
Director: Prof. A. Ogunniyi, B.Sc(Hons), MBChB, FMCP, FWACP, FRCP (Edin), FRCP (Lond)
Tel: 08023038583, 08038094173
E-mail: aogunniyi@comui.edu.ng

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

Notice of Approval for Amendment

Re: Endocrine Disruptors and their interaction with Hormones and their Receptors in Normal and Cancerous Breasts

UI/UCH Ethics Committee assigned number: UI/EC/10/0193

Name of Principal Investigator: **Olulope O. Ajayi**

Address of Principal Investigator: Department of Chemical Pathology, University College Hospital, Ibadan

Date of receipt of application for approval of amendment: 13/01/2015

Status: **Approval for Amendment**

This is to inform you that the UI/UCH Ethics Committee has reviewed your application for approval of amendment to your research protocol. The amendment indicates that expression of oestrogen and progesterone receptors, CerB-2 will be determined by immunohistochemistry and its procedure will be carried out at the Genetics and Bioethics Laboratory, Institute for Advanced Medical Research and Training, Biode Building, College of Medicine, University of Ibadan.

The Committee notes the amendments and having found it satisfactory, hereby approves the amended protocol.

All informed consent forms used in this study must carry the UI/UCH EC *assigned number and duration of* UI/UCH EC *approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC early in order to obtain renewal of your approval and avoid disruption of your research.

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

UI/UCH EC
12 JAN 2015
APPROVED

Dr. W. O. Balogun
Vice-Chairman, UI/UCH Ethics Committee
E-mail: uiuchirc@yahoo.com

APPENDIX 2

INFORMED CONSENT FORM

IRB Research Approval……..

This approval will elapse on……

TITLE OF RESEARCH

ENDOCRINE DISRUPTORS AND THEIR INTERACTION WITH HORMONES AND THEIR RECEPTORS IN NORMAL AND CANCEROUS BREASTS

Name and Affiliation of Researcher

This study is being conducted by AJAYI Olulope of the Department of Chemical Pathology, University of Ibadan, Ibadan..

This questionnaire is being administered to you to help in assessing the possible interaction of certain endocrine disruptors (lead, cadmium, arsenic, bisphenol-A, Polychlorinated bisphenyls) with the female reproductive hormones and their receptors.

All information provided by you will be kept very confidential. You will not be exposed to any risk or harm except the discomfort of needle prick during the collection of blood sample. Ten milliliters of blood will be collected from you using a new disposable pyrogen-free needle for some laboratory investigations. The result will be kept confidential.

This exercise might cause you minor discomfort, however, this will only last for a short space of time. You have the right to refuse participation in the research and also withdraw at any time you so desire.

If there are abnormal results, I shall contact you for necessary action. Your efforts in filling this questionnaire will be highly appreciated.

STATEMENT OF PERSON OBTAINING INFORMED CONSENT

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

DATE……..SIGNATURE…….

NAME……..

Statement of person giving consent

I have read the description of the research or have had it translated into the language I understand. I have also talked it over with the researcher to my satisfaction. I understand that my participation is voluntary. I know enough of the purpose, methods, risks and benefits of the research to judge that I want to take part in it. I have received a copy of this consent form and additional information

DATE……SIGNATURE……

NAME……

This research has been approved by the Health Research Joint Ethics Committee of the University of Ibadan/University College Hospital. The chairman of this committee can be contacted at:

Biode Building

2nd floor, room T10,

IAMRAT,

College of Medicine, University of Ibadan

E-mail:uiuchire@yahoo.com

If you have any question about participation in this research, you can contact my supervisor Dr Mabel A. Charles-Davies, Department of Chemical Pathology, College of Medicine, University of Ibadan (08023045256). Thanks.

APPENDIX 3

DEPARTMENT OF CHEMICAL PATHOLOGY

COLLEGE OF MEDICINE

UNIVERSITY OF IBADAN

QUESTIONNAIRE

Good day madam. This questionnaire is being administered to you to determine the interactions of certain chemicals in the environment (e.g. lead, cadmium e. t. c) with the female reproductive hormones in breast cancer. This will help in proper management of breast cancer. Your kind cooperation in providing correct information to the questions below will be highly appreciated. All information provided shall be kept very confidential.

Date……. Hospital Number……..

SECTION A. DEMOGRAPHIC CHARACTERISTICS

1. Gender: Female( )
2. Age:……..
3. Place of residence……
4. Ethnic group……..
5. State of Origin:……
6. Marital Status: Married ( ), single ( ), widow ( ), divorced/separated ( )
7. Highest educational attainment: None ( ), Pry School ( ), Secondary School ( ), ND/NCE ( ), HND/B.Sc. ( ), PG ( ).
8. Occupation:……..

SECTION B. DIET HISTORY

9. Dairy product (butter, cheese, milk) intake? Daily ( ), weekly ( ), occasionally ( ), never ( )

10 Vegetable intake? Daily ( ), weekly ( ), occasionally ( ), never ( )

11. Fruit intake? Daily ( ), weekly ( ), occasionally ( ), never ( )

12. Consumption of red meat? Daily ( ), weekly ( ), occasionally ( ), never ( )

13. Cereal products intake? Daily ( ), weekly ( ), occasionally ( ), never ( )

14(a). Alcohol intake? Yes ( ), No ( )

14(b) If response is yes, how? 1 bottle/day ( ), 1 bottle /week ( ), 1 bottle occasionally ( ).

14 (c) If consumption is more than 1 bottle, pls, specify……..

15(a) Cigarette smoking? Yes ( ), No ( )

15(b) If response is yes pls, indicate the number of sticks and how?.....

SECTION C. ANTHROPOMETRIC MEASUREMENTS

16. Blood pressure……..

17. Weight…….

18. Height……

19. BMI……..

20. Waist circumference……

21. Hip circumference……

SECTION D. OBSTETRICS/ GYNAECOLOGICAL AND BREAST CANCER HISTORY

22. Is your menstrual cycle regular or irregular?.....

23. Your menstruation lasts for how many days?.....

24. Duration of menstrual cycle…….

25. Present day of menstrual cycle….. (**Note**. Day 1 refers to 1st day of the last menstruation.

26. Age at menarche……

27. Age at first full pregnancy……

28. Age at menopause (if applicable)……

29. Use of contraceptive Yes ( ), No ( ). If response is yes, kindly state the type(s)

30. Any family history of breast cancer? Yes ( ), No ( ), if response is yes pls, state the relationship e.g. mother, sister…….

31. Any history of breast cancer? Yes ( ), No ( ). If response is yes, how was it managed? .....

32. Cancer stage (breast cancer subjects)……

33. Are you currently on hormone replacement therapy? Yes ( ), No ( )

APPENDIX 4

CALCULATION OF SAMPLE SIZE (COMPARISON OF TWO MEANS)

$$N = \frac{(Z_{\alpha} + Z_{2\beta})^2 (\sigma_1^2 + \sigma_0^2)}{(\mu_1 - \mu_0)^2}$$

NOTE: N=Sample size

$\mu_1-\mu_0$=Difference between the means to be detected assumed to be 10

$\sigma_1^2+\sigma_0^2$=Standard deviations

Z_{α}=Standard normal deviate corresponding to the null hypothesis i.e. 1.96

$Z_{2\beta}$=Standard normal deviate corresponding to the alternate hypothesis i.e. 1.28

α=level of significance

β=type II error

Oestradiol (pg/ml)

	Mean	Standard Deviation
Test	113.72	16.40
Control	156.00	19.80

$$N = \frac{(1.28+1.96)^2 (16.40^2+19.80^2)}{(10)^2}$$

N=69.89 (approximately 70).

N.B: The mean and standard deviation values used in the calculation of the sample size of this study were obtained from *(Egbe, 2007). He determined mean±SD of oestradiol in breast cancer patients and control.

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APPENDIX 5

Reference Intervals of Hormonal Assay

Oestradiol (E_2)

Follicular Phase: 90-1100 (pmol/L)

Luteal Phase: 90-1200 (pmol/L)

Postmenopausal: ≤170 (pmol/L)

Progesterone

Follicular: ≤2.8 (nmol/L)

Luteal: 15-80 (nmol/L)

Postmenopausal: ≤ 1.59 (nmol/L)

Luteinizing Hormone (LH)

Follicular: 1.0-13.0 (IU/L)

Luteal: 0.5-15.0 (IU/L)

Postmenopausal: 14.0-62.0 (IU/L)

Follicle-Stimulating Hormone (FSH)

Follicular: 3.0-11.0 (IU/L)

Luteal: 1.5-10.8 (IU/L)

Postmenopausal: 36.0-168.0 (IU/L)

Free Triiodothyronine (FT_3)

3.2-6.0 (pmol/L)

Free Thyroxine (FT_4)

10.6-21.0 (pmol/L)

Thyroid- Stimulating Hormone (TSH)

0.38-4.31 (mIU/L)

Reference: TOSOH enzyme immunoassay protocol leaflet

APPENDIX 6

Coexpression of ER/PR in Women with Breast Cancer

ER/PR Co-expression	Frequency (n)	Percentage (%)
ER+/PR+	4	5.1
ER+/PR-	6	7.6
ER-/PR-	65	82.3
ER-/PR+	4	5.1

APPENDIX 7

REAGENT PREPARATION FOR THE DETERMINATION OF THE ANALYTES

Reagent Preparation for Progesterone Determination

(a) Substrate Solution

All reagents were brought to 18-25 ^{O}C before preparing the working reagent. The contents of the AIA-PACK Substrate reconstituent II (100mL) were added to the lyophilized AIA-PACK Substrate reagent II and mixed thoroughly to dissolve the solid material.

(b) Wash Solution

The entire contents of the AIA-PACK concentrate (100mL) were added to approximately 2.0L of Chemical and Allied Product (CAP) Class reagent grade water and mixed well. The final volume was adjusted to 2.5L.

(c) Diluent

The entire content of the AIA-PACK Diluent Concentrate (100mL) was added to approximately 4.0L of CAP Class I reagent grade water. This was thoroughly mixed and adjusted to 5.0L.

CAP=Chemical and allied product

Calibration Procedure

The calibrators met the criteria of Institute for Reference Materials and Measurement and European Reference Materials (IRMM ERM). Calibration stability was monitored by quality control performance. The calibrator lot and concentration numbers were correctly entered into the software. Progesterone calibrators were run and were within a 10% range.

Quality Control: Manufacturer's control i.e. levels 1, 2 and 3 were run with the assay

Sensitivity: The minimal detectable concentration of progesterone by the method used is 0.318nmol/L (0.1ng/ml).

Specificity: The specificity of progesterone is 100%.

Reagent Preparation for E_2 Determination

(a) Substrate Solution

All reagents were brought to 18-25 ^{O}C before preparing the working reagent. The contents of the AIA-PACK Substrate reconstituent II (100mL) were added to the lyophilized AIA-PACK Substrate reagent II and mixed thoroughly to dissolve the solid material.

(b) Wash Solution

The entire contents of the AIA-PACK concentrate (100mL) were added to approximately 2.0L of CAP Class reagent grade water and mixed well. The final volume was adjusted to 2.5L.

(c) Diluent

The entire content of the AIA-PACK Diluent Concentrate (100mL) was added to approximately 4.0L of CAP Class I reagent grade water. This was thoroughly mixed and adjusted to 5.0L

Calibration Procedure

The calibrators met the criteria of Institute for Reference Materials and Measurement and European Reference Materials (IRMM ERM). Calibration stability was monitored by quality control performance. The calibrator lot and concentration numbers were correctly entered into the software. Oestradiol calibrators were run and were within a 10% range.

Quality Control: Manufacturer's control i.e. levels 1, 2 and 3 were run with the assay.

Sensitivity: The minimal detectable concentration of E_2 by the method used was 52.85pmol/L (14.4pg/ml).

Specificity: The specificity of E_2 is 100%**.**

Reagent Preparation for FSH Determination

(a) Substrate Solution

All reagents were brought to 18-25 ^{O}C before preparing the working reagent. The contents of the AIA-PACK Substrate reconstituent II (100mL) were added to the lyophilized AIA-PACK Substrate reagent II and mixed thoroughly to dissolve the solid material.

(b) Wash Solution

The entire contents of the AIA-PACK concentrate (100mL) were added to approximately 2.0L of CAP Class reagent grade water and mixed well. The final volume was adjusted to 2.5L.

(c) Diluent

The entire content of the AIA-PACK Diluent Concentrate (100mL) was added to approximately 4.0L of CAP Class I reagent grade water. This was thoroughly mixed and adjusted to 5.0L

Calibration Procedure

The calibrators met the criteria of Institute for Reference Materials and Measurement and European Reference Materials (IRMM ERM). Calibration stability was monitored by quality control performance. The calibrator lot and concentration numbers were correctly entered into the software. FSH calibrators were run and were within a 10% range.

Quality Control: Manufacturer's control i.e. levels 1, 2 and 3 were run with the assay.

Sensitivity: The minimal detectable concentration of follicle-stimulating hormone by the method used was 1.0 IU/L (1.0mIU/mL).

Specificity: The specificity of FSH is 100%

Reagent Preparation for LH Determination

(a) Substrate Solution

All reagents were brought to 18-25 ^{O}C before preparing the working reagent. The contents of the AIA-PACK Substrate reconstituent II (100mL) were added to the lyophilized AIA-PACK Substrate reagent II and mixed thoroughly to dissolve the solid material.

(b) Wash Solution

The entire contents of the AIA-PACK concentrate (100mL) were added to approximately 2.0L of CAP Class reagent grade water and mixed well. The final volume was adjusted to 2.5L.

(c) Diluent

The entire content of the AIA-PACK Diluent Concentrate (100mL) was added to approximately 4.0L of CAP Class I reagent grade water. This was thoroughly mixed and adjusted to 5.0L

Calibration Procedure

The calibrators met the criteria of Institute for Reference Materials and Measurement and European Reference Materials (IRMM ERM). Calibration stability was monitored by quality control performance. The calibrator lot and concentration numbers were correctly entered into the software. LH calibrators were run and were within a 10% range.

Quality Control: Manufacturer's control i.e. levels 1, 2 and 3 were run with the assay.

Sensitivity: The minimal detectable concentration of LH by the method used was 0.2 IU/L (0.2mIU/mL).

Specificity: The specificity of LH is 100%.

Reagent Preparation for FT_4 Determination

(a) Substrate Solution

All reagents were brought to 18-25 ^{O}C before preparing the working reagent. The contents of the AIA-PACK Substrate reconstituent II (100mL) were added to the lyophilized AIA-PACK Substrate reagent II and mixed thoroughly to dissolve the solid material.

(b) Wash Solution

The entire contents of the AIA-PACK concentrate (100mL) were added to approximately 2.0L of CAP Class reagent grade water and mixed well. The final volume was adjusted to 2.5L.

(c) Diluent

The entire content of the AIA-PACK Diluent Concentrate (100mL) was added to approximately 4.0L of CAP Class I reagent grade water. This was thoroughly mixed and adjusted to 5.0L

Calibration Procedure

The calibrators met the criteria of Institute for Reference Materials and Measurement and European Reference Materials (IRMM ERM). Calibration stability was monitored by quality control performance. The calibrator lot and concentration numbers were correctly entered into the software. FT_4 calibrators were run and were within a 10% range.

Quality Control: Manufacturer's control i.e. levels 1, 2 and 3 were run with the assay.

Sensitivity: The minimal detectable concentration of FT_4 by the method used was 1.29 pmol/L (0.1ng/dL).

Specificity: The specificity of FT_4 is 100%.

Reagent Preparation for FT_3 Determination

(a) Substrate Solution

All reagents were brought to 18-25 ^{O}C before preparing the working reagent. The contents of the AIA-PACK Substrate reconstituent II (100mL) were added to the lyophilized AIA-PACK Substrate reagent II and mixed thoroughly to dissolve the solid material.

(b) Wash Solution

The entire contents of the AIA-PACK concentrate (100mL) were added to approximately 2.0L of CAP Class reagent grade water and mixed well. The final volume was adjusted to 2.5L.

(c) Diluent

The entire content of the AIA-PACK Diluent Concentrate (100mL) was added to approximately 4.0L of CAP Class I reagent grade water. This was thoroughly mixed and adjusted to 5.0L

Calibration Procedure

The calibrators met the criteria of Institute for Reference Materials and Measurement and European Reference Materials (IRMM ERM). Calibration stability was monitored by quality control performance. The calibrator lot and concentration numbers were correctly entered into the software. FT_3 calibrators were run and were within a 10% range.

Quality Control: Manufacturer's control i.e. levels 1, 2 and 3 were run with the assay.

Sensitivity: The minimal detectable concentration of FT_3 by the method used was 0.77pmol/L (0.5pg/mL).

Specificity: The specificity of FT_3 is 100%

Reagent Preparation for TSH Determination

(a) Substrate Solution

All reagents were brought to 18-25 ^{O}C before preparing the working reagent. The contents of the AIA-PACK Substrate reconstituent II (100mL) were added to the lyophilized AIA-PACK Substrate reagent II and mixed thoroughly to dissolve the solid material.

(b) Wash Solution

The entire contents of the AIA-PACK concentrate (100mL) were added to approximately 2.0L of CAP Class reagent grade water and mixed well. The final volume was adjusted to 2.5L.

(c) Diluent

The entire content of the AIA-PACK Diluent Concentrate (100mL) was added to approximately 4.0L of CAP Class I reagent grade water. This was thoroughly mixed and adjusted to 5.0L.

Calibration Procedure

The calibrators met the criteria of Institute for Reference Materials and Measurement and European Reference Materials (IRMM ERM). Calibration stability was monitored by quality control performance. The calibrator lot and concentration numbers were correctly entered into the software. TSH calibrators were run and were within a 10% range.

Quality Control: Manufacturer's control i.e. levels 1, 2 and 3 were run with the assay.

Sensitivity: The minimal detectable concentration of TSH by the method used was 0.01mIU/L (0.01µIU /mL).

Specificity: The specificity of TSH is 100%

Reagent Preparation for Lead Determination

1. Five ml of triton X-100 was made up to 100 ml with deionised water to give a 5% solution (v/v). The solution of the mixture was enhanced in warm water by placing on hot plate.

2. Stock lead standard

(a) Lead standard solution was prepared by diluting 1.60g of lead nitrate ($PbNO_3$) in 100mL of de-ionized water

3. Working Standards

(a) 1.0, 2.5 and 5.0µg/L were prepared by dilution from the stock standard with acidified deionised water

(b) The working standards were used to prepare a calibration curve which was used to compare the digested samples.

Reagent Preparation for Cadmium Determination

1. Five ml of triton X-100 was made up to 100 ml with deionised water to give a 5% solution (v/v). The solution of the mixture was enhanced in warm water by placing on hot plate

2. Stock cadmium standard

Cadmium standard solution was prepared by dissolving 2.10 g of cadmium nitrate in 250 mL of de-ionized water and was made up to 1 litre.

3. Working Standards

(a) 1.0, 2.5 and 5.0µg/L were prepared by dilution from the stock standard with acidified deionised water.

(b) The working standards were used to prepare a calibration curve which was used to compare the digested samples.

Reagent Preparation for Arsenic Determination

1. Five ml of triton X-100 was made up to 100 ml with deionised water to give a 5% solution (v/v). The solution of the mixture was enhanced in warm water by placing on hot plate

2. Stock arsenic standard

Arsenic standard solution was prepared by dissolving 1.0g of arsenic powder in 50 mL concentrated nitric acid and was diluted to 1 litre with de-ionized water..

3. Working Standards

(a) 1.0, 2.5 and 5.0µg/L were prepared by dilution from the stock standard with acidified deionised water.

(b) The working standards were used to prepare a calibration curve which was used to compare the digested samples.

Quality Control of the Toxic Metals

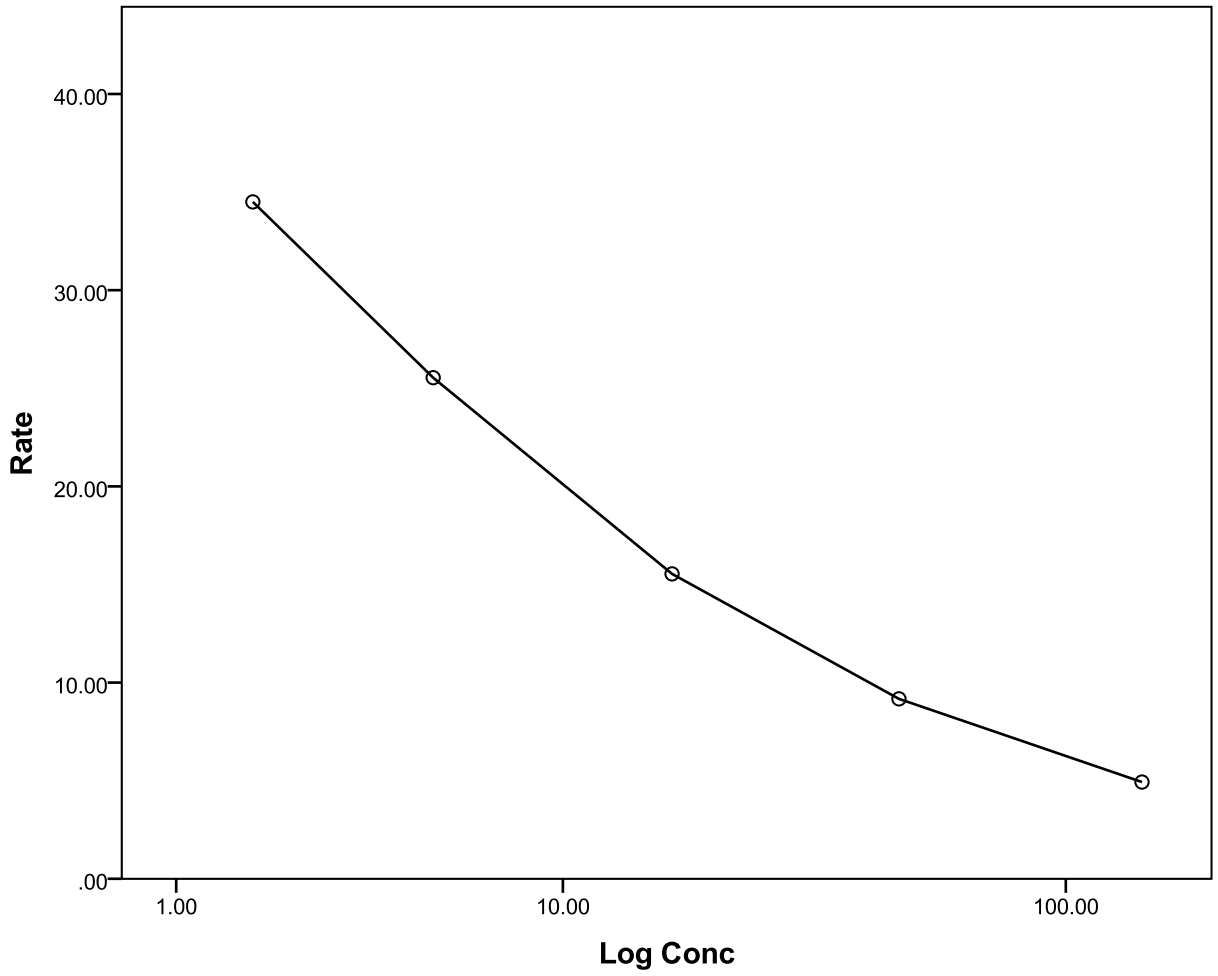
To ensure quality control of these analytes, de-ionized water samples in three different bottles were included with the serum samples and blinded to the laboratory technician. This was done to ascertain the reliability of the results.

Quality Control of the Determination of Hormone Receptors by Immunohistochemistry

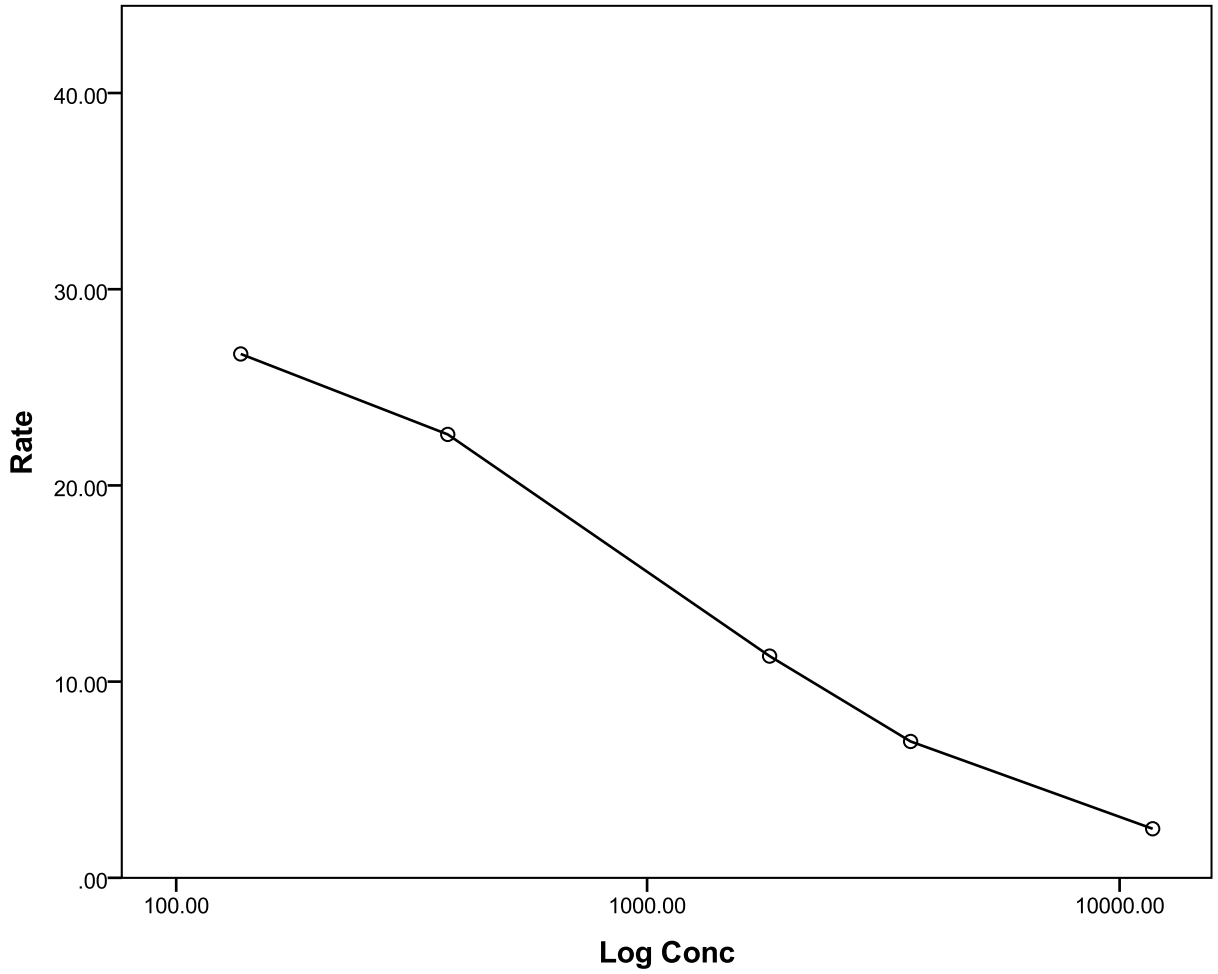
N.B-All tissue sections were performed at the same time and submitted to standard methods. Known positive and negative cases were used as external controls. ER, and PR were considered positive when >10% of the nuclei were stained in 10 high power field (HPF) (Ferrero-Pous *et al*., 2001; Pinto *et al*., 2005). The HER 2 was considered negative when with score 0 and +1, and positive with score +2 and +3. To be considered as +2, +3 the cellular membrane was completely stained in more than 10% of the tumour cells. Cells without staining, or with weak staining in part of the cell membrane and in less than 10% of the tumour cells were considered negative (Jacobs *et al*., 1999).

APPENDIX 8

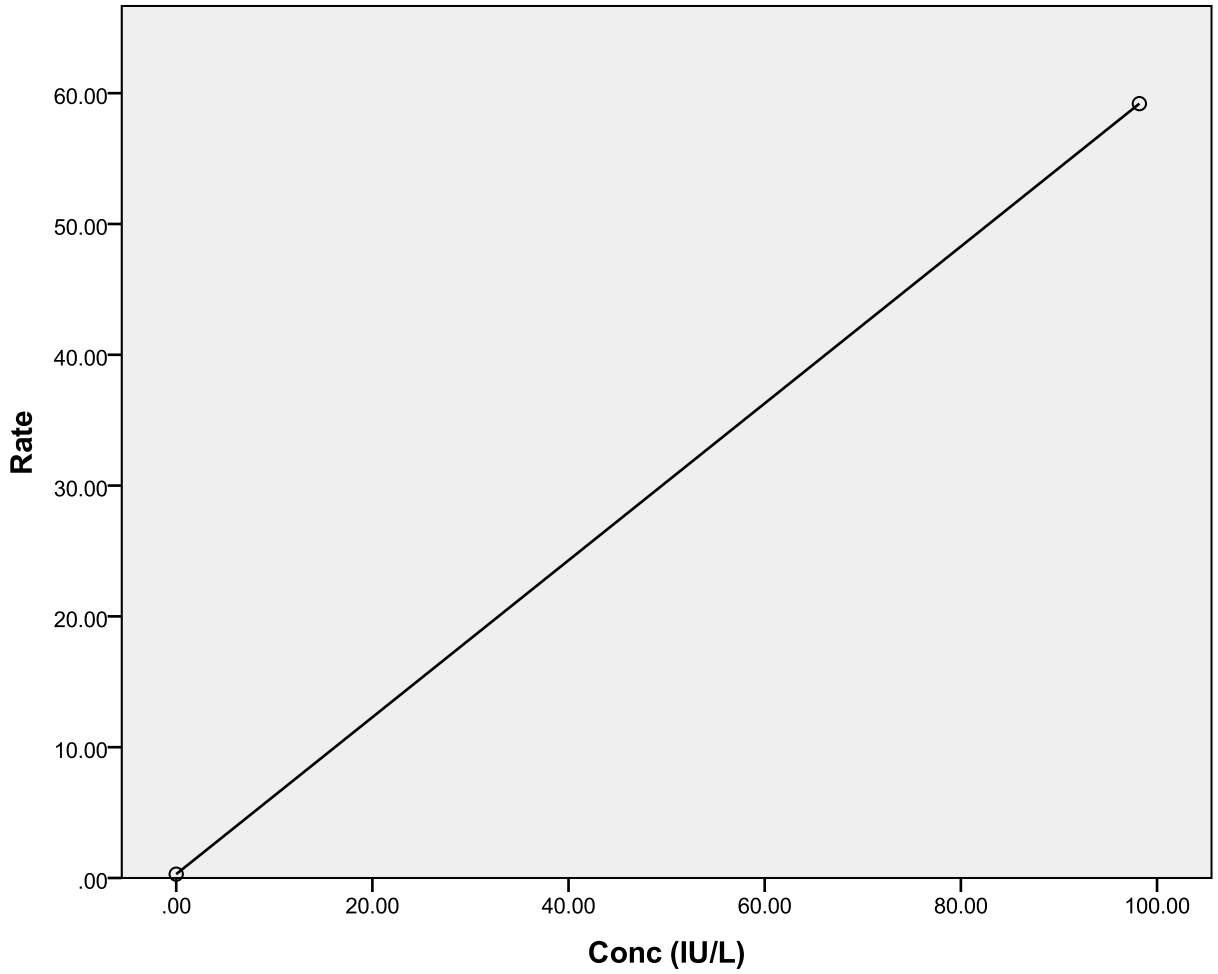
Calibration Curves of the Hormones



Calibration Curve for Progesterone Assay

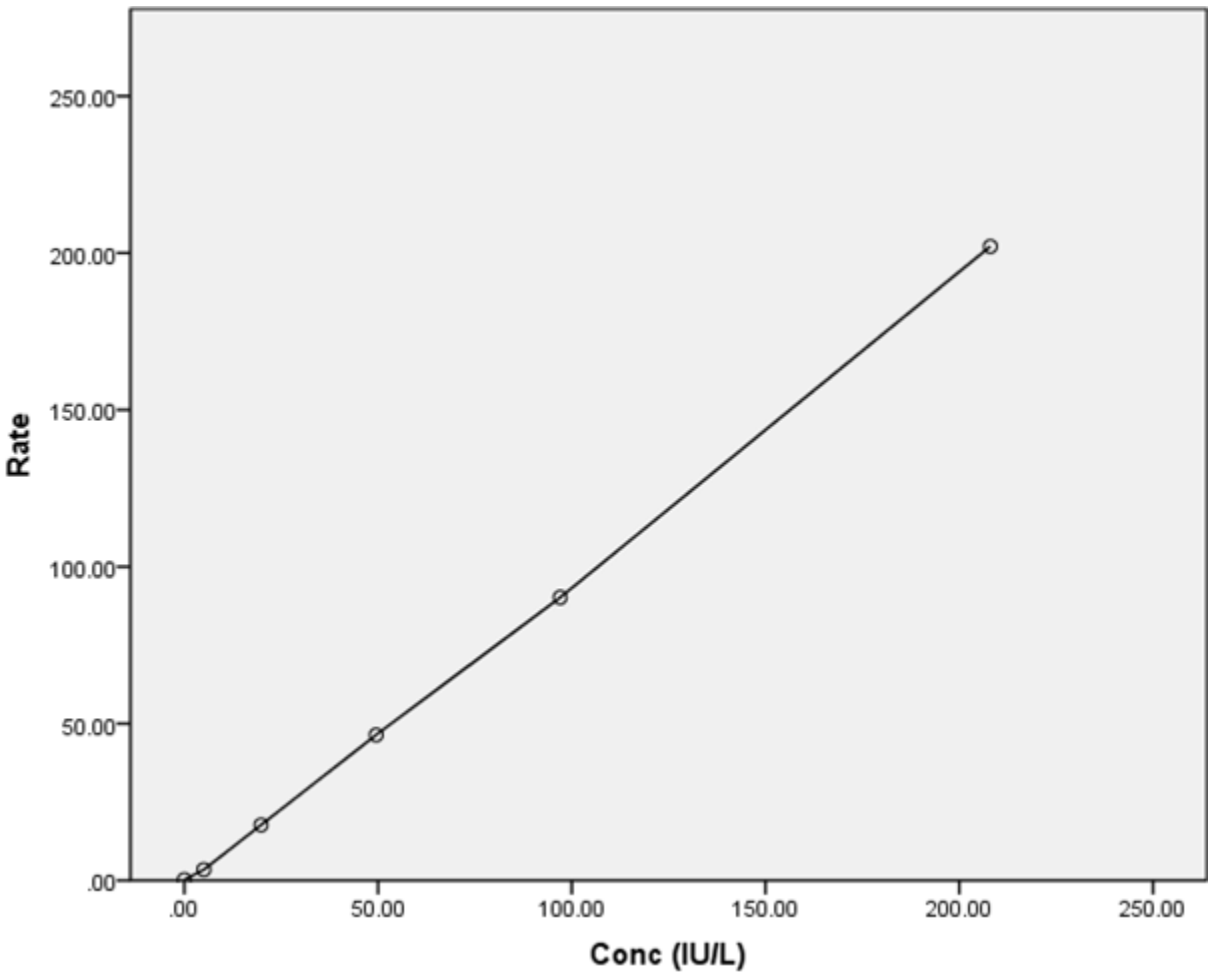


Calibration Curve for Oestradiol Assay

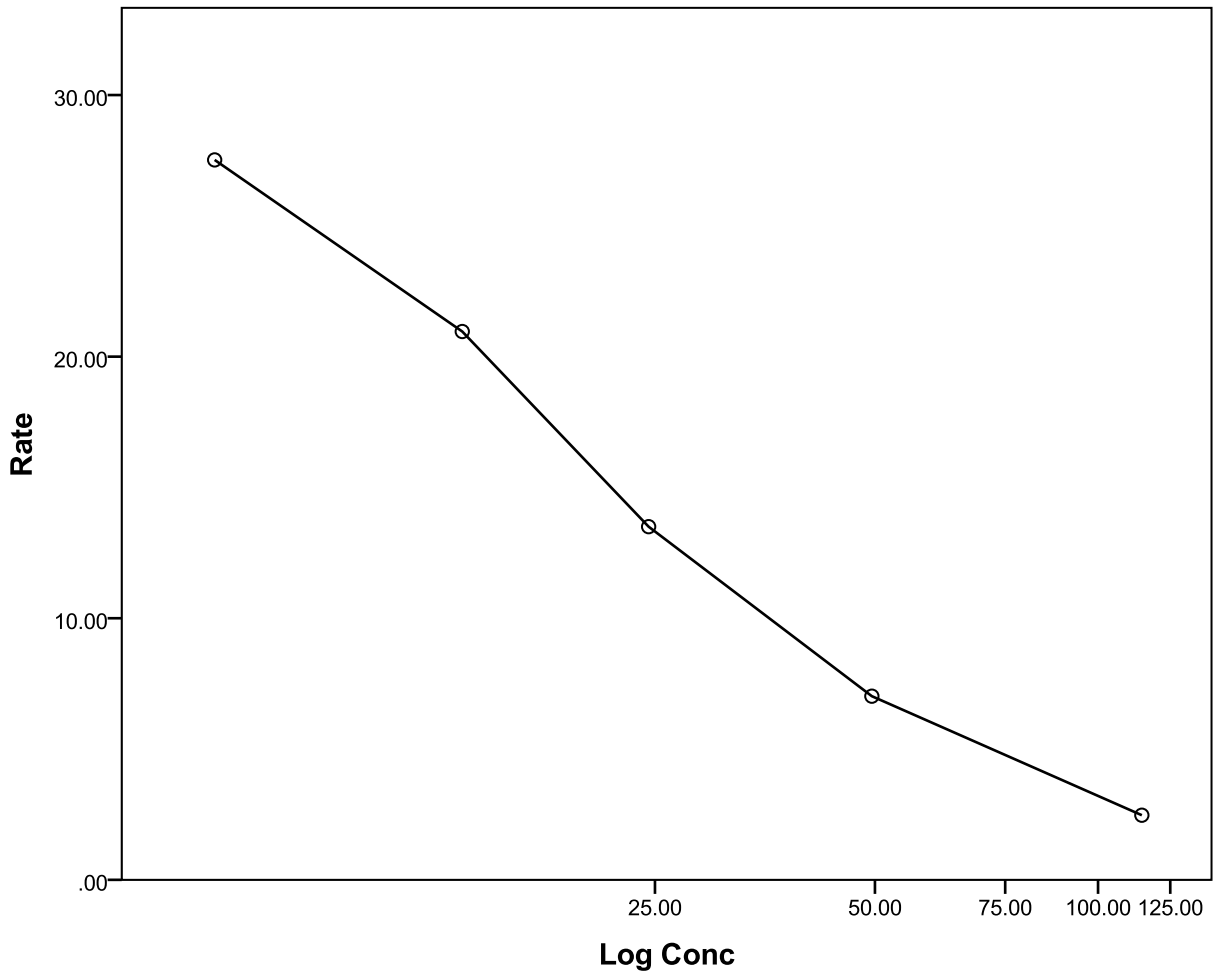


Calibration Curve for FSH Assay

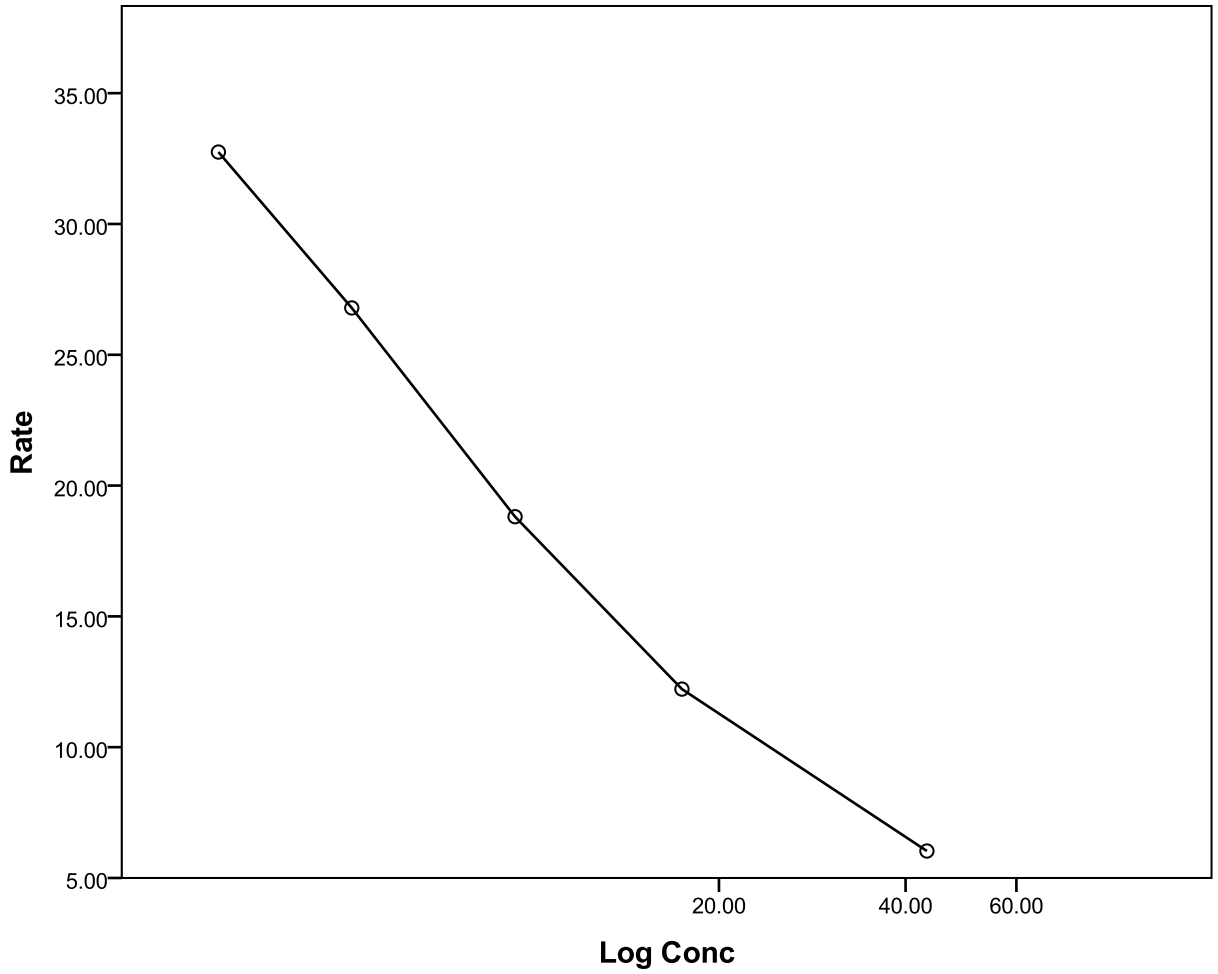
N.B: Two points were plotted on the calibration curve for FSH assay as determined by the manufacturer (Check Appendix 10)



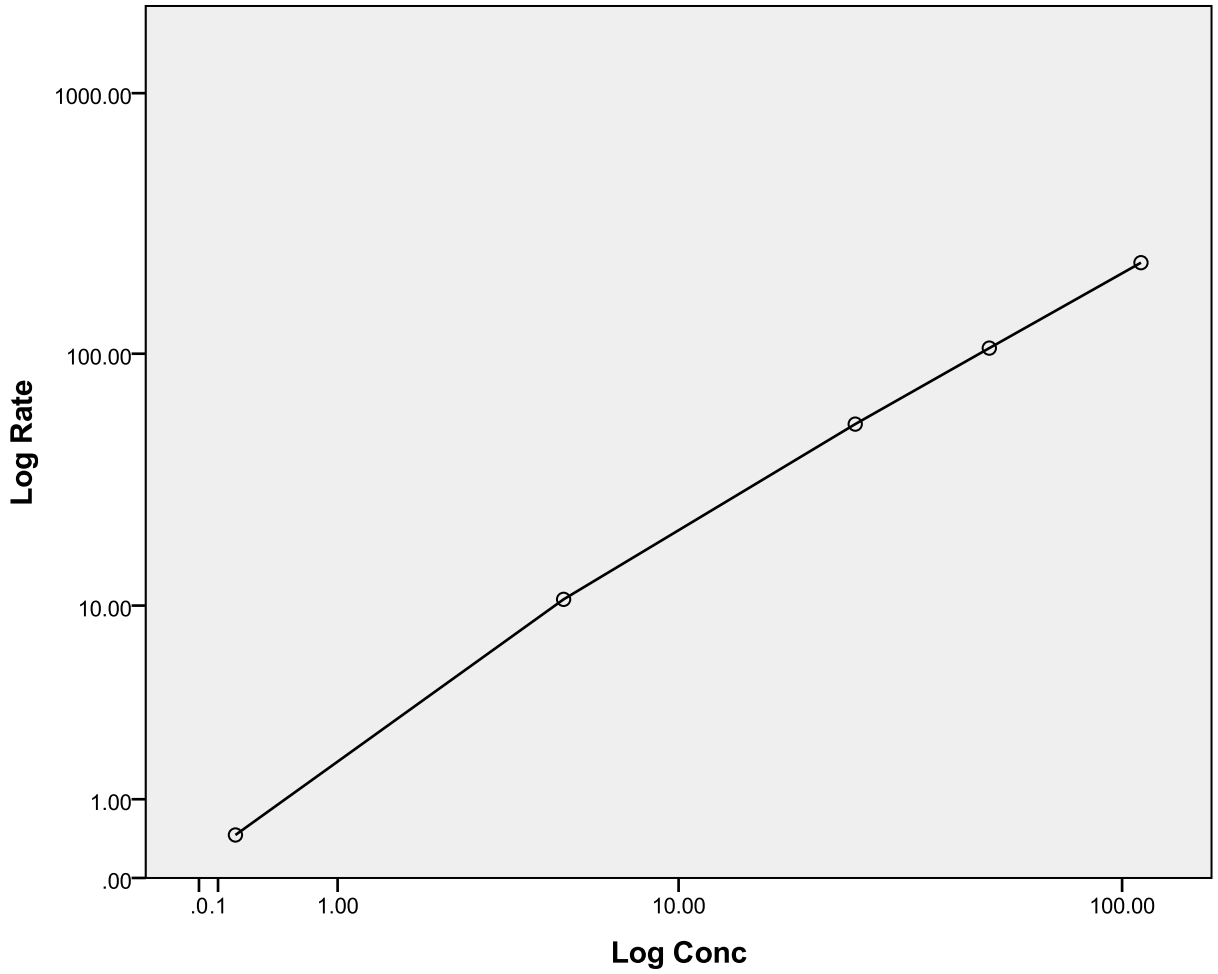
Calibration Curve for LH Assay



Calibration Curve for FT_4 Assay



Calibration Curve for FT_3 Assay



Calibration Curve for TSH Assay

APPENDIX 9

VALIDATION OF HORMONAL ASSAY

Hormone	Mean concentration	SD	CV (%)
Progesterone	A=2.07	0.23	11.3
	B=9.69	0.67	6.9
	C=25.02	1.56	6.2
Oestradiol	A=152.1	6.30	4.1
	B=623.3	15.73	2.5
	C=1993.2	57.66	2.9
FSH	A=4.94	0.28	5.6
	B=16.37	0.77	4.7
	C=60.15	2.57	4.3
LH	A=4.94	0.13	2.7
	B=25.27	0.54	2.1
	C=98.14	2.03	2.1
FT_4	A=7.727	0.41	5.3
	B=25.8	0.85	3.3
	C=59.5	1.59	2.7
FT_3	A=2.75	0.11	3.9
	B=5.10	0.16	3.2
	C=14.2	0.29	2.1
TSH	A=2.19	0.11	5.0
	B=8.26	0.39	4.8
	C=80.63	3.58	4.4

SD=Standard deviation. CV=Coefficient of variation. A=QC 1, B=QC 2, C=QC 3