COMPARISON OF OESTROGEN RECEPTOR, PROGESTERONE RECEPTOR, AND HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR-2 EXPRESSION IN PRIMARY BREAST CANCER AND LYMPH NODE METASTASIS AT THE KORLE-BU TEACHING HOSPITAL, ACCRA, GHANA.

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DECLARATION BY THE CANDIDATE

I hereby declare that this is the product of my own research undertaken under supervision and has neither been presented in whole nor in part for another degree elsewhere. I am responsible for any flaws in this work.

Signature…… Date……. /……../……..

(Bernice Anane Mawuli)

DECLARATION BY THE SUPERVISORS

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

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Co-supervisor

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(Professor Andrew Anthony Adjei)

DEDICATION

I dedicate this work to the following individuals;

My parents, Mr. Francis Anane Mawuli and Madam Mercy Koduah.

My siblings, Emelia Ankamah, William A. Mawuli, King Gideon Safo and King Kwadwo Safo.

All women with breast cancer and their various families.

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LIST OF ABBREVIATIONS.

AF1- Growth factor binding domain

AF2- Ligand binding domain

Akt 1- Protein kinase B

ASCO/CAP- American Society of Clinical Oncology/College of American Pathologists

BRCA 1- Breast cancer gene 1

BRCA 2- Breast cancer gene 2

COOH- Carboxyl

DNA- deoxyribonucleic acid

ELISA- Enzyme-linked immunosorbent assay

ESR1- Oestrogen receptor 1

ESR2- Oestrogen receptor 2

FISH-Fluorescence in situ hybridization

Ig-Immunoglobulin

LNER- Oestrogen receptor status in lymph node

LNPR- Progesterone receptor status in lymph node

LNHER2- HER2/neu status in lymph node

n- Sample size

MAPK- Mitogen activated protein kinase

PCR- polymerase chain reaction

PELP- Proline, glutamate and leucine rich protein 1

PLN- Percentage of lymph node involvement

PTER- Oestrogen receptor status in primary tumour

PTPR- Progesterone receptor status in primary tumour

PTHER2- HER2/Neu status in primary tumour

SD- Standard deviation

STAT- Signal transducers and activators of transcription

SISH- Silver in situ hybridization

TNBC- Triple negative breast cancer

ABSTRACT

Introduction: Determination of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (Her-2/neu) has become part of the diagnostic tests for breast cancer. Results of ER and PR are used to select breast cancer patients who are most likely to respond to hormonal therapy whereas Her-2/neu results are used to select patients with invasive breast cancer most likely to respond to targeted therapy. Immunohistochemical (IHC) determination of ER, PR and HER-2/neu is usually done on the primary breast cancer tissue. Re-evaluation of ER, PR and HER-2/neu on metastatic breast tumour is sometimes done when metastasis occurs. The axillary lymph nodes are the most common site of metastasis of breast cancer. Characteristics of primary breast cancer may change when metastasis occurs. This may affect the expression of proteins including ER, PR and HER-2/neu. The choice of the appropriate sample for the IHC determination of ER, PR and HER-2/neu when metastasis occurs may therefore be clinically relevant.

Aim: The aim of this research was to compare the expression of ER, PR and the over-expression of HER- 2/neu between primary breast cancer and metastatic deposits in corresponding lymph nodes in women with breast cancer at the Korle-Bu Teaching Hospital.

Methodology: The study involved 54 archived tissue blocks of primary breast cancer tissue and corresponding metastatic deposits in lymph nodes of women with invasive breast cancer. The cases were submitted to the Pathology Department of The Korle-Bu Teaching Hospital between January and December 2009. Sections were taken from the tissue blocks and stained immunohistochemically with antibodies for ER, PR and HER-

2/neu. Stained sections were reported as either positive or negative according to the guidelines of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP).

Results: The mean age ± SD of women diagnosed with breast cancer with lymph node metastases was 49.89 ± 10.70 years with a median age of 51 years. The percentage of retrieved axillary lymph nodes that were involved with breast cancer cells ranged from 8% to 100%. The mean percentage of lymph nodes retrieved from the axilla was 57.87 with a median of 62.71 and a range of 92. About 22% (12 out of 54) of the cases had 100% lymph nodes involved with breast cancer cells and 3.7% (2 out of 54) had 8% of retrieved lymph nodes involved with breast cancer cells. About 20% (11 out of 54) had grade I, 42.6% (23 out of 54) had grade II, and 37% (20 out of 54) had grade III breast tumours respectively. In the primary breast cancer tumour, 31.5% (17 out of 54) of the cases were ER positive, 68.5% (37 out of 54) were ER negative, 25.9% (14 out of 54) were PR positive and 74.1% (40 out of 54) were PR negative. HER-2/neu was positive in 25.9% (14 out of 54) and negative in 74.1% (40 out of 54) of the cases. In the corresponding lymph nodes, 33.3% (18 out of 54) were ER positive, 66.7% (36 out of 54) were ER negative, 29.6% (16 out of 54) were PR positive, and 70.4% (38 out of 54) were PR negative. HER-2/neu was positive in 29.6% (16 out of 54) and negative in 70.4% (38 out of 54) of the cases. There was 94.4% and 92.5% concordance for ER and PR respectively between primary tumour and lymph nodes. The concordance rate for HER-2/neu was 96.3%. There was no statistical difference ($p > 0.05$) in ER, PR and HER-2/neu between primary breast cancer and metastatic deposits in lymph nodes.

Conclusion: There were minor changes in the expression of ER, PR and HER-2/neu between primary breast tumour and metastatic deposits in lymph nodes.

CHAPTER 1: INTRODUCTION

1.0 Background

Breast cancer is the growth of malignant cells in the breast. It is the most common malignancy among women (23% of all cancers), and the second leading cause of cancer deaths in women (10.9% of all cancers), excluding non-melanoma skin cancers (GLOBOCAN 2008 Cancer Fact Sheet). The International Agency for Research on Cancer (IARC), based on the GLOBOCAN 2008 report (Ferlay *et al*., 2010), estimated that in 2008, 1.38 million new cases of breast cancer were diagnosed worldwide. The same report indicated that, the incidence of breast cancer in the developed countries is higher (80 per 100,000) compared to that of developing countries (40 per 100,000). However, the mortality rate of breast cancer in the developing countries is more than half (269,000) of the estimated 458,000 deaths. This difference may be attributed to late stage at diagnosis and limited access to timely standard treatments (Berry *et al*., 2005).

In Ghana, breast cancer is the leading malignancy in women (Badoe and Baako, 2000), accounting for 15.4% of all malignancies. The treatment and/or management options for breast cancer include surgery and/or radiotherapy or chemotherapy, hormonal therapy and targeted therapy. The use of hormonal therapy depends on the presence of oestrogen receptor (ER), and/or the progesterone receptor (PR) in the primary breast tumour (Barnes and Hanby 2001; Haider *et al*., 2001). Breast cancer patients whose tumours are HER-2/neu positive will benefit from anti-HER-2/neu targeted therapy (Dawood *et al*., 2010).

1.1 Problem statement

Assessment of ER, PR status and HER-2/neu in breast cancer patients is usually made using the primary breast cancer tissue (Zidan, 2005; Idirisinghe *et al*., 2010). For patients who have developed distant metastases, ER, PR expression and HER-2/neu over expression are determined on metastatic tumour deposits (Azam *et al*., 2009; Hoefnagel *et al*., 2010). Several studies have suggested a difference in ER, PR expression and HER-2/neu over-expression between primary breast cancer tissue and metastatic tumour deposits (Tanner *et al*., 2001; Gipponi *et al*., 2004; Azam *et al*., 2009). Receptors may be lost due to tumour heterogeneity (Nedergaard *et al*., 1995; Azam *et al*., 2009; Almendro and Fuster, 2011).

At the Histopathology Department of the Korle-Bu Teaching Hospital, Accra, Ghana, expression of ER, PR and the over-expression of HER 2/neu are determined using the primary breast cancer tissue. Metastatic deposits in the lymph nodes are used for receptor expression when the primary breast cancer specimen is not available, or when the tissue characteristics of the primary breast tumour is altered due to inadequate fixation. The primary breast cancer tissue may also have undergone necrosis as a result of neo-adjuvant chemotherapy. This could lead to inappropriate choice of hormone therapy and targeted therapy if there is a significant difference in receptor expression between primary breast cancer and metastatic deposits in lymph nodes.

1.2 Aim

The aim of this research was to compare the expression of ER, PR and the over-expression of HER- 2/neu between primary breast cancer and metastatic deposits in lymph nodes in women with breast cancer at the Korle-Bu Teaching Hospital.

1.3 Objectives

1. To determine the expression of ER, PR and HER- 2/ neu over-expression in primary breast cancer tissue.

2. To determine the expression of ER, PR and HER- 2/ neu over-expression in metastatic deposits corresponding axillary in lymph nodes.

3. To compare the expression of ER, PR and HER-2/neu between primary breast cancer tissue and metastatic deposits in the lymph nodes.

1.4 Justification

The study proposal aims at a better understanding of the appropriateness of the use of metastatic tumour deposits in the lymph nodes for the determination of ER, PR and HER-2/neu over-expression in women with breast cancer with axillary lymph node involvement. The in-depth study of those cases might provide a better understanding of the appropriate treatment in women with breast cancer. The outcome of this study will provide relevant information on the expression of ER, PR and the over expression of HER-2/neu in metastatic lymph nodes in the management and/ or treatment of breast cancers.

1.5 Hypothesis

There is no difference in oestrogen receptor (ER), progesterone receptor (PR) expression and human epidermal growth factor 2 (HER-2/neu) over expression between primary breast cancer and metastatic deposits in lymph nodes.

CHAPTER 2: LITERATURE REVIEW

2.0 Incidence of breast cancer.

Breast cancer is a global health issue. It constitutes 23% of all cancers and is the second leading (10.9% of all cancers) cancer worldwide (GLOBOCAN 2008 Cancer Fact Sheet). More than half of deaths (269,000 out of 458,000) from breast cancer worldwide occurs in developing countries (Ferlay *et al*., 2008), including Ghana (Wiredu and Armah, 2006). Studies conducted by Biritwum *et al*., (2000) at the Korle-Bu Teaching Hospital revealed that 12.8% of all admissions for malignant neoplasms were for breast cancer, and also most women report with advanced disease to hospitals (Archampong, 1977; Clegg-Lamptey *et al*., 2007).

2.1 Risk factors of breast cancer.

The greatest risk factor for developing breast cancer is gender (female) and the second is increasing age (Yardley, 2000; American Cancer Society, 2011-2012). Other risk factors include: inherited mutations in the BRCA1 and BRCA2 genes (Struewing *et al*., 1997; American Cancer Society, 2011-2012), a personal or family history of breast cancer (Colditz *et al*., 1993; Johnson *et al*., 1995; Yang *et al*., 1998; Lim *et al*., 2011; American Cancer Society, 2011-2012), high breast tissue density (American Cancer Society, 2011-2012), high-dose radiation to the chest wall (Ng and Travis, 2009), and biopsy-confirmed atypical hyperplasia (Marshall *et al*., 1997; American Cancer Society, 2011-2012). Some reproductive factors such as early menarche and late menopause, never having given birth or giving birth for the first time after age 30 are known to increase a woman's risk

of having breast cancer (Antoniou *et al*., 2006; American Cancer Society, 2011-2012). Other factors that are associated with increased risks for breast cancer are modifiable. These include: postmenopausal obesity, use of combined oestrogen and progestin menopausal hormones, alcohol consumption, and physical inactivity (American Cancer Society, 2011-2012).

2.2.0 Symptoms of breast cancer.

The signs and symptoms of breast cancer include any one or more of the following: lump in the breast, breast pain, nipple discharge, nipple retraction, breast skin changes like skin tethering or ulceration.

2.2.1 Diagnosis of breast cancer.

Methods of assessment of a breast abnormality include clinical examination, imaging, (mammography, ultrasound, Magnetic Resonance Imaging, Positron Emission Tomography) and fine needle aspirate for cytology or biopsy for histology. The diagnostic technique employed depends on the clinical manifestation and the age of the woman.

2.3.0 Types of breast cancer.

Breast cancer may arise from the lobules, ducts or any of the connective tissue components of the breast such as blood vessels. Ductal carcinomas are the most common types of breast cancer. Rarely, breast cancer may arise from the connective tissue component of the breast. Breast cancer may be confined to the lobules, ducts or

connective tissue components of the mammary gland (that is carcinoma in-situ) or the cancer may spread to normal tissues inside or outside of the mammary gland (that is invasive carcinoma). Invasive ductal carcinoma (IDC) is the most frequently diagnosed breast cancer in women worldwide (Harris *et al*., 2000). Pagets' disease is a rare type of breast cancer that directly involves the nipple.

2.3.1 Grading/Staging of breast cancer.

Staging and grading of cancers, including breast cancer, provide information that is used to predict the clinical behaviour of the cancer, establish appropriate therapies and facilitate exchange of precise information between clinicians (Sobin, 2003; Cowherd, 2012). The Elston-Ellis modification of the Scarff-Bloom-Richardson (SBR) grading is used in the United States (Simpson *et al*., 2000). This grading system combines nuclear grade, tubule formation, and mitotic rate. Each element is given a score of 1 to 3 (1 being the best and 3 being the worst). The lowest possible score (3) is given to well differentiated tumours that all form tubules and have low mitotic rate (< 10/10 HPF). The highest possible score is 9. A histological grade of III is assigned any tumour with a Nottingham score of 8 or 9.

The Tumour (tumour size and local growth) - Node (extent of lymph node metastases) - Metastasis (occurrence of distant metastasis) (TNM) classification system describes the anatomical extent of breast cancer and allows the grouping of breast cancer into stages (that is Stage I, Stage II, Stage III and Stage IV). Stages III and IV are the advanced stages of the cancer. Metastatic breast cancer describes breast cancer that has spread from the breast to other parts of the body. Metastasis is usually to the axillary lymph nodes.

The most common sites of metastases from breast cancer are the lungs, bones, liver and brain, according to the National Cancer Institute. Once metastases are detected, the adoption of systemic therapy is mostly palliative (Muss *et al*., 1991) which, according to Andre *et al*., (2004) has improved over time. Early detection, optimal surgery and adjuvant therapy are the key strategies to improving good prognosis of breast cancer.

2.3.2 Prognostic indicators of breast cancer.

The Stage at diagnosis of breast cancer, lymph node metastasis (Rack *et al*., 2010), the histological type of the primary breast cancer (Nagao *et al*., 2012), hormone receptor status and HER-2/neu over expression (Rampaul *et al*., 2001), biological tumour markers such as CEA (Hegg *et al*., 1990) LDH and ALP and proliferation assays (Oktay *et al*., 2012) have been recognized as prognostic factors in breast cancer. Assessment of these factors is important for clinical management of breast cancer patients because these factors provide information for oncologists for best therapeutic options. It is standard practice that hormone receptor status and HER-2/neu over expression are determined prior to the commencement of treatment for breast cancer patients because there has been an established positive correlation between ER and PR with the degree of tumour differentiation (Mori *et al*., 2001). Well differentiated breast carcinomas are more likely to be ER and PR positive and have a better prognosis (Hilf *et al*., 1980).

2.4.0 Treatment and/ or Management options for breast cancer patients.

There are two groups of treatment modalities for breast cancer; the local and the systemic treatment options. Local treatment includes surgery and radiation. Systemic treatment includes chemotherapy, hormone therapy and targeted therapy. Breast cancer patients who have developed distant metastases usually undergo systemic therapy with chemotherapy, hormonal therapy and/or human epidermal growth factor receptor- 2(HER 2/neu) targeted therapy. Most breast cancer patients at the Korle-Bu Teaching Hospital are given neo-adjuvant chemotherapy: About 60% of patients report to the Hospital with advanced Stage of the disease (Clegg-Lamptey *et al*., 2007). Neo-adjuvant endocrine therapy, according to Saigal *et al.*, (2011), may be a good alternative to neo-adjuvant chemotherapy for post menopausal women with ER-Positive breast cancer including locally advanced breast cancer.

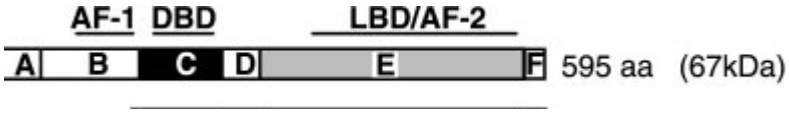
ER, PR and HER-2/neu determination has become part of the diagnostic tests for breast cancer. The ER and PR results help clinicians in selecting breast cancer patients most likely to receive benefit from hormonal therapy (Haider *et al*., 2001). HER-2/neu provides prognostic information on recurrence and survival (Mori *et al*., 2002; Azam *et al*., 2009). The presence of these markers, according to Haider *et al*., (2001), Mori *et al*., (2002) and Azam *et al*., (2009) are related to the degree of the tumour differentiation.

2.5.0 Estrogen and the estrogen receptor.

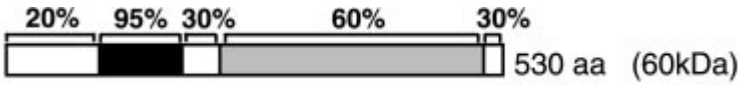
Estrogen is a lipophilic hormone with low-molecular weight (approximately 300g/mol.) (Hammond and Soule, 2004). It occurs naturally and plays a key role in the regulation of sexual and reproductive processes (Heldring *et al*., 2007). 17- oestradiol (E2) is the most

potent oestrogen produced in the body, probably because the oestrogen receptor preferentially binds it over oestriol (2x) and oestrone (3x) (Ruh *et al*., 1973). E2 also binds to the oestrogen receptor within the nucleus longer than oestriol and oestrone (Gorski *et al*., 1974; Anderson *et al*., 1975).

Figure 1: a) Schematic representation of the human oestrogen receptor-alpha.



b) Schematic representation of the human oestrogen receptor-beta



(Source: Heldring *et al*., 2007).

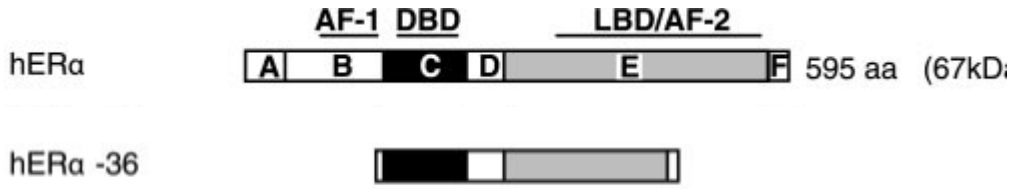
There are two different forms of the oestrogen receptor; α and β, each encoded by a separate gene ESR1 and ESR2, respectively. ERα and ERβ are co-expressed in many cell types. Hormone-activated oestrogen receptors form dimers. The dimers may be ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers. Results of studies conducted by Li *et al*., (2004) suggests that ERα is the functionally dominant partner in the ERαβ (αβ) heterodimer. The central and most conserved domain of the ER is the DNA-binding domain (DBD), which is involved in DNA recognition and binding. Ligand binding occurs at the multifunctional COOH- terminal of the ER (Nilsson *et al*., 2001). ERα and ERβ share a conserved structure with six functional domains, A to F (Enmark *et al*., 1997). (Figures 1a and 1b). The constitutively active AF-1 of the transcriptional functional domain is located at the NH2 terminus of the ER and the ligand-dependent

AF-2 resides in the COOH-terminal of the LBD (Nilsson *et al*., 2001). ERβ is homologous to ERα at the ligand-binding domain (58%) and DNA-binding domain (95%). The remaining domains are not well conserved (Enmark *et al*., 1997). ERα is located on chromosome 6 and it contains eight exons that encode a 66-kDa full-length receptor (Moriarty *et al*., 2006). ERβ is located on chromosome 14 (Enmark *et al*., 1997).

2.5.1 A 36kiloDalton variant of the oestrogen receptor-alpha.

Shi *et al*., (2009) identified a 36kDa variant of the oestrogen receptor-alpha (ER-α) (66kDa) that may contribute to tamoxifen resistance in ER-positive breast cancer patients (Fowler *et al*., 2009; Shi *et al*., 2009). A Study conducted by Zhang *et al*., (2011) concludes that ER-α36 mediates non-genomic oestrogen signaling and is highly expressed in ER-Negative breast cancer cells.

Figure 2: Schematic representation of the human ERα as compared with the 36kDa variant.



(Source: Heldring at al., 2007).

2.5.2 Oestrogen signaling.

In the circulation, oestrogen is bound to sex steroid binding globulin or to albumin. Free active oestrogen diffuses across the cell membrane and binds to a cytoplasmic receptor protein to form oestrogen-oestrogen receptor complex (McCarty, 1977). Oestrogen-oestrogen receptor complex undergoes a temperature-dependent alteration in its physical properties prior to, or simultaneously with, its translocation to the nucleus (De Sombre, 1975) where it binds with deoxyribonucleic acid (DNA) and specific chromosomal proteins, altering the pattern of gene expression and the transcription of messenger ribonucleic acids (mRNAs) (Maurer, 1974; Williams and Gorski, 1974). Studies during the past few years have demonstrated that transcriptional regulating functions of ER-α66 in the nucleus can be inhibited at the same time as its extranuclear (membrane) functions are being activated (Levin and Pietras, 2008). This may be useful in the provision of new targets for therapeutic interventions (Giretti *et al*., 2008).

2.5.3 Oestrogen signaling and breast cancer.

The oestrogen receptor, which has no trans membrane and kinase domains is known to initiate E2 rapid signaling with the formation of complexes that activates the MAPK1/3 and AKT1 in breast cancer cells (Song and Santen, 2006). Other protein complexes that are required for the initiation of the rapid actions of oestrogen include ESR1, CSK, SHC1 and PELP1. PELP1 protein expression is an independent prognostic predictor of shorter breast cancer specific survival and disease free survival in breast cancer and its elevated expression is positively associated with markers of poor outcome (Habashy *et al*., 2010).

In ER- positive breast cancer patients, PELP1 appears to have a potential application in assessing their clinical outcome.

2.5.4 Anti-oestrogens

Anti-oestrogens, designed to block ER-α, are widely and effectively used clinically in the treatment of breast cancer (Early Breast Cancer Trialists' Collaborative Group, 2005; Clarke, 2006). There are two classes of anti-oestrogens: Selective Oestrogen Receptor Modulators (SERMs) which are used for estrogen inhibition or suppression to prevent recurrence of tumours, and aromatase inhibitors prescribed after primary treatment (surgery, chemoradiation) to prevent recurrence of breast cancer in postmenopausal women. Tamoxifen is the most common SERM used. Anastrazole-oral (Arimidex) is the most common aromatase inhibitor used at the Korle-Bu Teaching Hospital (personal communication with Mr. Clegg-Lamptey, Head of Department Surgery). Other aromatase inhibitors used are, letrozole and exemestane. The overall effects of anti-estrogens on breast cancer cells include tumour shrinkage (Dhingra, 1999), a decrease in the numbers of cells in the S-phase of the cell cycle (Dalvai and Bystricky, 2010) and the induction of cellular apoptosis (Riggins *et al*., 2005).

2.5.5 Cell migration and breast cancer metastasis

Cell migration is a key first step in the metastasis of breast cancer. Mechanisms underlying the effects of oestrogen on metastasis may be different from that of tumour regression. Inhibition of oestrogen synthesis in breast cancer results in tumour regression and reduction of new metastasis. Estrogen (E2) interacts with ESR1 and several other receptors to facilitate E2 induced cell migration. The pathways that involve these

receptors have been studied to develop models that will inhibit tumour cell migration and metastasis (Li *et al*., 2010).

2.6.0 Progesterone and Progesterone receptor.

Progesterone is a C-21 steroid hormone. It is lipophilic and diffuses across the cell membrane of its target cell where it binds to a cytoplasmic receptor (McCarty, 1977) to form a progesterone-progesterone receptor complex. Progesterone-progesterone receptor complex is translocated into the nucleus (De Sombre, 1975) where it binds to specific proteins in the nucleus of its target cell and alters the expression of genes in the target cell (Maurer and Chalky, 1974; Williams and Gorski, 1974). The progesterone receptor is also known an NR3C3. In humans, PR is encoded by a single gene located on the long arm of chromosome 11 (11q22) (Law *et al*., 1987).

2.6.1 Isoforms of the Progesterone receptor.

There are two main nuclear isoforms of the human PR; A and B (Graham *et al*., 1995). Aside the lack of amino-terminal 164 amino acids that form the third transactivation domain of PRA, both isoforms are structurally identical (Kastner *et al*., 1990). Studies conducted by Mote *et al*., (2002) revealed that PRA predominance was evident in a high proportion of ductal carcinomas in situ (DCIS) and invasive breast lesions. Over abundance of PRA is related to Tamoxifen resistance (Hopp *et al*., 2004), while excess production of PRB is associated with breast cancer risk (Osborne *et al*., 2005) and poorer outcome of chemotherapy. Some protein products from PR target genes are associated with mammary gland and breast cancer development, including the transcription factors

STAT5A and C/EBPβ (Cork *et al*., 2008). There exists a cytoplasmic PR protein, PRC which lacks a full length DNA binding domain (Wei *et al*., 1990). PRC in humans has been proposed to play a potential physiological role in pregnancy (Condon *et al*., 2006).

2.6.2 The Progesterone receptor and breast cancer.

According to Cork *et al*., (2008), there exist other variants of PR with loss of specific functional domains which may alter the progestin responsiveness of a tissue and contribute to the abnormal growth associated with breast cancer. PR is an estrogen responsive gene hence PR positivity indicates not only the presence of ER but also a functioning ER pathway (Horwitz and McGuire, 1975; Osborne *et al*., 2005). Some PR negative tumours (loss of PR due to HER-2 over expression which down regulates the PR gene) correlate with response to aromatase inhibitors but not SERMs. This could be due to the existence of crosstalk between ER, PR and HER-2/neu (Cui *et al*., 2005; Osborne *et al*., 2005). Patients with ER-positive/PR-positive tumours have a better prognosis than patients with ER-positive/PR-negative tumours, who have a better prognosis than patients with ER-negative/PR-negative tumours (Gown *et al*., 2008; Lin *et al*., 2012).

2.6.3 Hormone replacement therapy (HRT) and breast cancer.

Progesterone through the PR is of significance in the development of breast cancer (Stahlberg *et al*., 2004). The National Toxicology Program in its December 2002 press release declared oestrogen as a carcinogen. There is increased risk of breast cancer in women on HRT with combined oestrogen-progesterone compared to oestrogen alone (Campagnoli *et al*., 2005).

2.7.0 The Human Epidermal Growth Factor Receptor-2

Human Epidermal Growth Factor Receptor-2 (HER-2/neu) is a member of the Erythroblastic Leukemia Viral Oncogene (ErbB) protein family. The HER-2/neu, encoded by the ERBB2 gene is a proto-oncogene located at 17q12-21.32 (Popescu *et al*., 1989). Transmembrane Her-2/neu molecules are expressed as inactive monomers on the cell surface. Ligand binding results in dimerization of the monomers (Alroy and Yarden, 1997; Schlessinger, 2000) leading to the phosphorylation of specific tyrosine residues in the receptor cytoplasmic tails (Schlessinger, 2000). Subsequent activation of signal transduction pathways leads to cell growth and differentiation (Olayioye, 2001).

In about 10% to 40% of primary breast cancers, the HER-2/neu gene is amplified, with subsequent over expression of the HER-2/neu protein and is associated with more aggressive disease (Salmon *et al*., 1987; Rubin and Yarden, 2001; Paluch-Shimon *et al*., 2005). Several studies have been done to correlate HER-2/neu gene amplification and protein over expression with disease outcome. HER-2/neu protein over expression by immunohistochemistry (IHC) has been found to have significant correlation with disease outcome on univariate analysis but has failed to demonstrate independent predictive status for immuno reactivity on multivariate analysis (Noguchi et *al*., 1992; Hartmann *et al*., 1994). HER-2/neu gene amplification and HER-2 protein over expression has been observed in many human cancers including endometrial (Hetzel *et al*., 1992), uterine cervix (Mitra *et al*., 1994), head and neck (Beckhardt *et al*., 1995), breast (Kaptain *et al*., 2001), bladder (Eltze *et al*., 2005), colon (Schuell *et al*., 2006), oesophageal (Reichelt *et al*., 2007), and gastric (Gravalos *et al*., 2008).

2.7.1 HER-2/neu over expression and breast cancer.

Her-2/neu is expressed at low levels in normal non-neoplastic epithelia, including breast duct epithelium, and is over-expressed in breast cancers (Slamon *et al*., 1987; Paluch-Shimon *et al*., 2005). Breast cancers can have up to 25-50 copies of the HER-2/neu gene, and up to 40-100 fold increase in HER-2/neu protein resulting in 2 million receptors expressed at the tumour cell surface (Kallioniemi *et al*., 1992). The HER-2 pathway involves a complex biological network comprising of ligands, coming from outside the cell that bind to membrane receptors, a number of protein kinases transmitting the signal to the nucleus and transcription factors regulating genes that affect various cellular functions (Citri and Yarden, 2006). The HER-2/neu signaling pathway, as well as the ER signaling pathway are the dominant drivers of cell proliferation and survival in about 85% of all breast cancers (Gutierrez and Schiff, 2011). Among the HER family members (HER1 to 4), HER-2 is the dominant tyrosine kinase in breast cancer. HER-2 does not have a ligand and relies on heterodimerization with another family member or homodimerization with itself when expressed at very high levels to be activated (Citri and Yarden, 2006). HER-2 exists in an open conformation exposing its dimerization domains making it the dimerization partner of choice among the family members. When HER-2/neu is over-expressed, tyrosine kinase is constitutively activated (Moasser *et al*., 2001), resulting in mitogenic transduction and poor prognosis (Zhang *et al*., 2004). HER-2/neu over expression has been associated with significantly shortened disease-free and overall survival (Seshadri *et al*., 1993). The insulin-like-growth factor receptor 1, can complex with HER-2 leading to HER-2 activation (Nahta *et al*., 2005). Oestrogen, working via the non-genomic activity of ER outside the nucleus has been shown to

activate HER-2 signaling (Shou *et al*., 2004). P95, an aberrant form of the HER-2 has been found in some breast cancers (Molina *et al*., 2002; Scaltriti *et al*., 2007). P95 is related to the extent of lymph node involvement and is enhanced in nodal tissue suggesting an important role as a marker or cause in breast cancer metastasis (Molina *et al*., 2002). P95 is constitutively active since the external domain of these receptors acts as an inhibitor until they are bound by ligand. P95 lacks the external domain of the HER-2, so it is not detected by antibodies that target the external domain of HER-2. P95 can cause resistance to transtuzumab (Carolina *et al*., 2011).

2.8.0 Significance of ER, PR and HER-2/neu in breast cancer.

Women with breast cancer, as part of their treatment and/or management, undergo surgery and/or radiotherapy, chemotherapy or hormone/endocrine therapy depending on the age of the individual and stage of the breast cancer at diagnosis. The addition of radiotherapy to surgery and adjuvant systemic treatment reduces the risk of any recurrence of breast cancer (Whelan *et al*., 2000), and those who suffer a recurrence usually do not have aggressive liver or central nervous system involvement (Maki and Grossman, 2000) mainly as a result of an increase in loco-regional control.

Presence of ER and PR in breast cancer tissues as determined by immunohistochemistry correlates well with response to endocrine therapy and chemotherapy (Early Breast Cancer Trialists' Collaborative Group, 1998; Barnes and Hanby 2001; Haider *et al*., 2001), and provides prognostic information on recurrence and survival since their expression is related to the degree of the tumour differentiation (Makki and Grossman, 2000). Studies conducted in the United States show that about 70-80% of breast cancers

are oestrogen receptor-positive (Keen and Davidson, 2003; Gown, 2008). These tumours tend to grow more slowly, are better differentiated, and are associated with a slightly better overall prognosis (Clark, 2000). Approximately 30% of breast tumours are ER-Negative (Lacroix *et al*., 2004; Simpson *et al*., 2005). These cancers are generally more aggressive than ER-positive breast cancers. Approximately 40% of breast cancers are ER positive and PR positive (Margolese *et al*., 2000).

HER2/neu status is known to be a prognostic as well as predictive marker in both node-negative (Andrulis *et al*., 1998) and node-positive breast cancer patients (Muss *et al*., 1994). The most successful example of targeted therapy in metastatic breast cancer is the targetting of human epidermal growth factor receptor - 2 (HER2) by trastuzumab (Barton and Swanton 2011). Trastuzumab is a monoclonal antibody that targets the extra cellular domain of the HER-2/neu protein. Trastuzumab does not block autophosphorylation of HER-2, however it inhibits HER-2 downstream signaling (Nahta *et al*., 2006). Other mechanisms of action of trastuzumab may include disruption of the HER-2/Src interaction, internalization and down regulation of the receptor and enhancement of antibody mediated cytotoxicity. Studies conducted by Dawood *et al*., (2010) revealed that transtuzumab improved prognosis in HER-2/neu positive breast cancer women. About twenty-five percent of metastatic breast cancer patients receiving first line treatment respond to trastuzumab alone (Vogel *et al*., 2002). Several clinical trails (Joensuu *et al*., 2006; Perez *et al*., 2007; Smith *et al*., 2007) have shown that trastuzumab in combination with different chemotherapy has significantly improved disease free survival and overall survival with reductions in the risk of recurrence of breast cancer ranging from 40-50%.

Studies conducted by Makki and Grossman (2000) concluded that ER/PR status can help the radiologist in detecting metastatic breast cancer: patients with ER-positive/PR-positive (ER+/PR+) breast cancer tend to develop bone metastases; whiles patients with ER-negative/PR-negative (ER-/PR-) tumours tend to develop metastases in the brain. This will help the clinician to estimate the likelihood of metastases to various organ systems, as well as to potentially target therapy.

Molecules involved with ER signaling pathway to a large extent influence endocrine responsiveness of breast cancer (Rastelli and Crispino, 2008). C1orf64 or ER-related factor (ERRF), an ER signaling molecule, may be targeted for therapeutic purposes (Su *et al*., 2012).

2.9.0 Axillary lymph nodes as a possible alternative for ER, PR and HER-2/neu determination.

Endocrine therapy and targeted therapy for breast cancer patients are based on the hormone receptor status and the over expression of HER-2/neu of the primary breast cancer tissue (Lower *et al*., 2005; Guarneri *et al*., 2008; Rack *et al*., 2010). Tumour deposits in the metastatic site are used to determine the response to endocrine therapy and targeted therapy when metastasis occurs (Dawood, 2010). It is sometimes however difficult to access metastatic tumour (Aktas *et al*., 2011). Breast cancer commonly spreads through lymph nodes. Seventy-five percent of all breast tumour and greater than ninety-five percent of all larger breast tumour generate lymph node metastases (Harrell *et al*., 2006). Presence of axillary lymph node metastasis, according to Rack *et al*., (2010), is the most important predictor for disease-free and overall survival of breast cancer

patients. Harrel *et al*., in 2006 developed metastasis models using ZsGreen labeled MCF-7 and T47D human breast cancer cells in nude mice. They observed that proliferation was higher in lymph nodes than in primary breast tumour. Harrell *et al*., observed also that unlike in the primary tumour where high levels of ER are down-regulated by E2, there is partial failure of ER down-regulation in lymph nodes associated with reduced PR expression. The presence of hormone receptors and HER-2/neu in lymph nodes may therefore be important in the endocrine and targeted therapy treatment of breast cancer patients with positive lymph node metastasis. According to Harrel *et al*., in the lymph node microenvironment, ER may however be dysfunctional and perhaps hormone resistant.

Several studies have been conducted to compare the expression of ER, PR and HER-2/neu over expression in primary breast cancer tissue and tissue from different metastatic sites. There however exist some differences in the results from the various studies. Factors suggested to be responsible for the change in hormone receptor status and HER-2/neu in metastatic breast cancer include: tumour heterogeneity (Nedergaard *et al*., 1995; Azam *et al*., 2009; Arslan *et al*., 2011), clonal selection of tumour cell subpopulations, genetic instability of tumour cells (Edgerton *et al*., 2003; Prat and Perou, 2009), local or systemic treatments, the time interval between primary tumour and metastasis, receptor status determination techniques (Prat and Perou, 2009), and the site of metastasis (Arslan *et al*., 2011).

Unmekita *et al*., (1998) and Bogina *et al*., (2011) in their study observed that loss of PR in recurrent and metastatic (site not stated) breast cancer was frequent and correlated with a worse prognosis; a change in ER was infrequent and a change in HER-2/neu was rare.

Bogina *et al*., 2011, therefore suggested the reassessment of HER-2/neu with both IHC and FISH in metastatic breast cancer when there is a change in HER-2/neu between the primary tumour and Metastatic tumour.

Studies conducted by Lower *et al* in 2005 showed a correlation between ER and PR between primary and Metastatic (site not stated) breast cancer. Out of the 200 cases analyzed for ER, 39(19.5%) were positive in the primary tumour and negative in the metastatic tumour. 21 (10.5%) cases had ER negative primary tumours and ER positive metastatic tumours. There was discordance, (30%), between primary and metastatic ER status. Tumours from 68 of 173 (39.3%) showed discordance for PR. Results of studies of Gancberg *et al*., (2002) did not support routine determination of HER-2 on metastatic sites, particularly when FISH results from the primary tumour are available. Guarneri *et al*., (2008) and Sari *et al*., (2011) however concluded in their study that biopsy of metastatic breast cancer is recommended, if feasible with minimal invasiveness because treatment options might change for a significant proportion of breast cancer patients with metastasis (Guarneri *et al*., 2008; Sari *et al*., 2011).

Broom *et al*., in 2009 found that significant discordance exits for hormone status between primary and metastatic breast cancer with a frequent loss of PR. Broom *et al*. showed 17.7% and 37.3% (n=100) discordance between primary and metastatic breast cancer for ER and PR respectively. 9.7% of primary tumours changing from ER-positive to ER-negative and 8.0% changing from ER-negative to ER-positive. No significant discordance for Her-2/neu was found. Simmons *et al*., (2009) also showed the presence of substantial discordance in hormone receptors between primary breast cancer and metastases, which led to altered management on 20% of cases.

Aktas *et al*., in 2011 compared ER and PR expression in primary breast cancer with circulating tumour cells. Breast cancer patients with ER-positive and PR positive tumours had circulating tumour cells (CTCs) which were ER-negative and PR- negative. Treatment based on the ER and PR status of the primary breast cancer may therefore be ineffective in metastatic disease. Holdaway *et al.* in 1983 showed concordance of 46% between primary and metastatic breast cancer. Six out of nine of the cases that were ER-positive in the primary tumour were ER-negative in the metastatic disease. Four out of five PR-positive cases in the primary tumour were PR-negative in the metastatic tumour. Nine out of nineteen ER-positive and three out of fifteen PR-positive cases in the primary tumour were negative in the metastatic tumour respectively. Holdaway *et al.* attributed some of the changes in receptor expression to treatment that the patients had received during the course of the disease. Azam *et al.* in 2009 compared ER and PR expression in primary breast cancer with corresponding metastases in lymph nodes. About 28% (28 out of 100) patients had ER-positive primary tumours. ER expression in the corresponding lymph nodes was 25%. The percentage of PR positivity in the primary breast tumour was reduced from 28% to 22%. HER-2/neu expression however, remained almost the same between the primary tumour and tumour metastases.

With regards to the above mentioned studies, there seem to be a change in receptor expression in the metastatic disease. Small as this proportion may be, it is necessary to identify women with metastatic breast cancer who belong to this group, since survival time may depend on receptor expression in the metastatic tumour (Holdaway *et al*., 1983; Azam *et al*., 2009; Aktas *et al*., 2011). There exist phenotypic changes between primary and metastatic breast cancer (Jacot *et al*., 2011). This has been hypothesized to be the

cause of difficulty faced in the treatment and/ or management of breast cancer patients with metastasis. Treatment (neoadjuvant, surgery, radiation, chemotherapy, adjuvant therapy and HER-2-targeted therapy) may modify ER, PR and HER-2/neu between primary and metastatic disease (Liu *et al*., 2012).

2.9.1 Molecular subtypes of breast cancer

Growing evidence suggests that breast cancer is a heterogeneous disease defined by ER, PR, and HER-2/neu expression with distinct aetiological pathways and prognoses (Kwan *et al*., 2009). Breast cancer can be subtyped according to hormone receptor status and HER-2/neu status (Bauer *et al*., 2007): ER+ and/or PR+, HER-2/neu – (luminal A), ER+ and/or PR+, HER-2/neu + (luminal B), ER-, PR-, HER-2/neu- (Basal or Triple negative) and ER-, PR-, HER-2/neu + (HER-2 over expressing). Considerable differences exist in survival, demographic and tumour characteristics between ER, PR and HER-2 breast cancer subtypes (Parise *et al*., 2009). Parise *et al*., 2009 recommended reporting breast cancer as an ER/PR/HER-2 subtype and documenting precisely demographic and tumour characteristics. Some of the results of studies conducted by Bauer *et al*., (2010) include the following; ER negativity appear to be a stronger predictor of poor survival than HER-2 positivity, HER-2 positivity did not always translate to worse survival as noted when compared triple positive subtype to triple negative subtype, the heterogeneity of the high risk category was most evident in the ER/PR/HER-2 subtype with four or more positive axillary lymph nodes. Bauer *et al*., therefore recommends the correlation of ER/PR/HER-2 subtype with molecular classification. According to Troester and Swift-Scalan, (2009), regional variations in breast cancer subtypes contribute to racial disparities of the disease.

2.9.2 Triple negative breast cancers.

Triple negative breast cancers are breast cancers that are immunohistochemically classified as ER negative, PR negative and HER-2/neu negative. Triple negative breast cancer (TNBC) constitute about 15% of all breast cancers worldwide (Lara-Medina *et al*., 2011). Black women have a higher incidence of triple negative breast cancers than white women regardless of age and body mass index (Stead *et al*., 2009; Rais *et al*., 2012). Studies have shown that this subtype of breast cancer occurs primarily in younger women of African American or Hispanic descent (Griffiths and Olin, 2012; Dawood, 2010). Results of studies conducted by Stark *et al*. in 2010, showed that among the 75 cases of Ghanaian women with breast cancer at The Komfo Anokye Teaching Hospital, 61 (81%) had triple negative breast cancer. In the same study, 26% (n=581) African Americans and 16% (n=1008) White Americans had triple negative breast cancer. In a similar study conducted by Adisa *et al*. (2012) in Nigeria, 65% (n=17) of Nigerian women with breast cancer were triple negative and had high grade cancers (100% grade III). Triple negative breast cancer is characteristically aggressive with high recurrence, metastatic, and mortality rates (Brown *et al*., 2008; Dawood, 2010) but initially responds to traditional systemic cytotoxic chemotherapy (Dawood, 2010). Potential future therapies for TNBC include targeted molecular strategies including poly ADP ribose polymerase (PARP) and epidermal growth factor receptor (EGFR) inhibitors and anti angiogenic agents (Duffy *et al*., 2012; Griffiths and Olin, 2012). Over expression of EGFR has been shown to be associated with TNBC and poor survival in Tunisian women (Kallel *et al*., 2012). Systemic evaluation of EGFR in breast cancer could benefit triple negative subgroup

from EGFR targeting agents. Women with metastatic triple-negative breast cancers have a much shorter median time from relapse to death (Dent *et al*., 2007).

19p13.1 is the first triple-negative-specific breast cancer risk locus and the first locus specific to a histological subtype defined by ER, PR, and HER2 to be identified (Stephens *et al*., 2012).

Results of studies from three locations in Nigeria and one location in Senegal published in the Science in Africa (Olopade, 2005) reveal that breast cancer in African women may be genetically different from breast cancer in White women. This, according to the study author may explain the absence of the molecular targets that form the basis of many standard therapies: 80% of breast cancer in Caucasian women have ERs, 23% of breast cancer in African women are ER+. Breast cancer in Caucasian women are more likely to be HER-2/neu + (23%) than breast cancer in African women (19%). Hence the worse prognosis of breast cancer in African women as compared to the Whites (Science in Africa, 2005).

CHAPTER 3: MATERIALS AND METHODS

3.0 Study design.

The study was a retrospective study which involved the use of archived formalin fixed, paraffin wax embedded primary breast cancer tissue blocks of women, and their corresponding axillary lymph node metastases submitted to the Pathology Department of The University of Ghana Medical School (UGMS), Korle-Bu, between January and December 2009.

3.1 Study site.

This study was carried out between the months of January and December 2012 at The Pathology Department, of The Korle –Bu Teaching Hospital (KBTH), Accra, Ghana. The KBTH, situated in the nation's capital, Accra is the leading tertiary hospital and the major referral centre in the country. It also serves as the leading teaching hospital of The University of Ghana Medical School (UGMS). The Pathology Department is the biggest tertiary care centre in the country. More than 75% of the total number of breast cancer cases seen in the country are processed through this department. Thus the demographics of the patients that were tested in this study were not limited to a specific social group. The patients in this study originated from various social and ethnic groups as well as geographically distinct areas from each region of Ghana.

3.2 Subjects/target population.

Subjects for the study were female patients with invasive breast cancer with axillary lymph node metastases obtained from the histopathology data entry book (log book) for the year 2009.

3.3.0 Inclusion criteria.

Breast specimen of women diagnosed with invasive breast cancer with lymph node metastasis submitted to the Department of Pathology of the Korle-Bu Teaching Hospital between January and December, 2009.

3.3.1 Exclusion criteria.

1- Cases of breast cancer without axillary lymph node involvement.

2- All cases that fitted into the above mentioned inclusion criteria but whose primary breast cancer was either;

A- Not available or

B- Not properly fixed or

C- Had features of tissue necrosis.

3.4 Sample size determination.

From the formula $n= z^2(p)(1-p) / (error^2)$, where n is sample size, z is standard-score at 95% confidence interval=1.96, p is the prevalence of breast cancer, and error = 5% (Significant difference). Using a prevalence rate of 4.03% (estimated) obtained from the 2009 annual report from the Department of Pathology, UGMS, Korle-Bu in Ghana, a minimum of 59 breast cancer patients were needed for this study.

3.5 Informed consent.

The study was given approval by the Ethical and Protocol Review Committee of the University of Ghana Medical School, College of Health Science, Korle - Bu. The protocol identification number of the ethical clearance for this work was MS-Et/M.1-p 5.1/2011-12 (Appendix III). The reference number was MS-AA/C.2/Vol.16A. Consent was sought from the Head of Department of Pathology, University of Medical School for the use of archived patients' formalin-fixed paraffin embedded breast tissue blocks.

3.6.0 Use of control samples.

3.6.1 Positive controls

For Oestrogen Receptor and Progesterone Receptor

Archived formalin fixed paraffin wax embedded tissue blocks of a histologically diagnosed fibroadenoma of a 12 year old female.

For Human Epidermal Growth Factor Receptor -2:

Archived formalin fixed paraffin wax embedded tissue blocks of a histologically diagnosed invasive breast cancer tissue.

3.6.2 Negative controls.

For ER, PR and HER-2/ neu: Sections from same samples used as positive controls with the omission of the primary antibody incubation step.

3.7.0 Sample preparation.

Selected formalin-fixed, paraffin wax embedded tissue blocks of patient and controls were re-embedded and trimmed to expose the full surface of the embedded tissues. Blocks were put on ice for 30 minutes to allow the embedded tissues as well as the paraffin wax attains an equal cool temperature. Using the microtome at a predetermined gauge of 4 microns, sections were cut and allowed to float on water bath at a temperature of 60^0 C to spread the folds in the sections. Sections were picked onto microscope slides and labeled with patient histopathology number and arranged into staining racks. Subsequently, sections were kept in the oven at 60^0C and then stained with haematoxylin and eosin (H&E) for confirmation of diagnosis by the principal supervisor. After confirmation, one section each for ER, PR and HER-2/neu as well as controls were taken and kept in staining racks in the oven at 32^0C overnight to ensure proper adherence of sections onto pre-coated slides. Commercially prepared pre-coated slides obtained from Leica Microsystems were used.

3.7.1 Haematoxylin &Eosin staining technique.

Sections were removed from the oven and dewaxed in 3 changes of xylene for 2 minutes each. Sections were taken through descending grades (80%, 70% and 60%) of ethanol for 2 minutes each. Subsequently, sections were placed under running tap water for 10 minutes. Nuclear staining was done by incubating sections in aqueous haematoxylin for 5 minutes with subsequent bluing at pH 6.8. Eosin was used for a minute as a counterstain to stain the cytoplasm of the cell in the section. Dehydration through ascending grades (60%, 70% and 80%) of ethanol to absolute ethanol for 2 minutes each was done. Sections were subsequently cleared in 2 changes for 2 minutes each in xylene and mounted in Distrene, Plasticiser, Xylene (DPX) mountant and coverslipped appropriately. Examination of the stained slides by the Principal Supervisor was done under the light microscope using the X10 as well as the X40 objectives of Olympus microscope, CX41RF. X10 objective was for scanning through the section and X40 objective for detailed evaluation to confirm diagnosis of patients' cases.

3.7.2 Immunohistochemistry.

This is based on the principle that antibodies bind specifically to antigens that induced the production of the antibodies. The method provides a valuable means of visualization of antigens on frozen sections as well as paraffin wax embedded sections. The introduction and use of polymeric labeling has led to increased sensitivity of the IHC method.

3.7.3 Immunohistochemical staining procedure.

Prior to undertaking this procedure, the dilutions of the primary antibodies were validated on a series of known positive and negative controls. All staining procedures were carried out at 25 ^{o}C.

3.7.4 Hydration of sections.

Sections of each case for ER, PR and HER-2/neu as well as the positive and negative control slides, which have been kept in the oven at 32 ^{o}C were transferred into a 60 ^{o}C oven for 30 minutes. The sections were removed from the oven, dewaxed in 2 changes of xylene for 2 minutes each. Sections were taken through 80% alcohol, 70% alcohol and 60% alcohol for 2 minutes each and finally to distilled water.

3.7.5 Epitope retrieval.

This is achieved through the breaking of protein cross-links formed by formalin fixation thereby uncovering hidden antigenic sites. The heat induced antigen retrieval method was used. A pyrex bowl was filled with 500ml of 0.01M citric acid at PH 6.0 (retrieval solution), covered with its lid and then immersed into a metallic bowl filled with water. Enough water was used to ensure that its level was at the same level as the retrieval solution in the pyrex bowl. A hot plate was used to serve as a source of heat. The retrieval solution was heated to a temperature of 95 ^{o}C without boiling. The lid of the pyrex bowl was carefully removed and with the help of a holder, the hydrated slides in its rack was carefully immersed into the heated retrieval solution. The lid of the pyrex bowl was replaced and sections were allowed to heat for 2 minutes. The pyrex bowl was

subsequently removed from the water bath and allowed to cool to room temperature, after which the slides were removed and placed in a bowl of distilled water.

3.7.6 Incubation with antibodies and other reagents.

Sections were rinsed in 50mM Tris-buffered saline (TBS) at PH 7.6, and arranged onto staining racks, after which endogenous peroxidase activity from, for example blood in the tissue, was blocked using 3% hydrogen peroxide solution for 5 minutes. Sections were rinsed in TBS after which any protein, apart from ER, PR and HER-2/neu were blocked with a protein block solution provided for 5 minutes. Sections were then rinsed in TBS. Sections were incubated with 100 ul of primary antibodies at the pre-determined concentrations (Appendix I) for 60 minutes. The staining racks were kept moistened to provide enough moisture that did not allow the sections to dry. After the primary antibody incubation period, the slides were rinsed in TBS for 5 minutes and then incubated with 100 ul of the post primary block provided for 30 minutes. Following the rinsing of the slides in TBS for 5 minutes, slides were incubated with 100 ul of NovoLink Polymer provided for 30 minutes. Slides were washed in TBS for 5 minutes with gentle rocking. Peroxidase activity was developed with Diaminobenzidine (DAB) for 5 minutes. The DAB reaction was stopped with distilled water after which slides were rinsed under running tap water. Sections were then counterstained in Haematoxylin, washed under running tap water, dehydrated in alcohols of increasing grades, cleared in xylene and mounted in Distrene, Plasticiser, Xylene (DPX). After the mountant had been allowed to set, the slides were examined under the light microscope (Olympus microscope CX41RF) at X10. The slides were subsequently reviewed by the Pathologist. The stained mounted

slides were confirmed by reviewers using the Olympus microscope CX41RF. X10 objective was used to scan through the sections and X40 objectives used for detailed evaluation of ER, PR and HER-2/neu positivity.

3.8 Staining reaction and reporting criteria for immunostains.

Breast tumours were defined as positive for ER or PR if there was ≥1% tumour nuclear staining in the presence of expected reactivity in internal and external controls. Absence of tumour nuclear staining or presence of < 1% tumour nuclear staining in the presence of expected reactivity in internal and external controls was considered negative. This reporting criterion is in accordance with recommendations made by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP). (Hammond *et al*., 2010).

HER-2/neu was considered positive if more than 30% of tumour cells showed strong complete membranous staining. Weak, incomplete membrane staining or cytoplasmic staining was considered negative.

All the immunostained slides were reviewed by the Pathologist, after the principal investigator had reported/scored them.

3.9 Statistical analyses.

Data obtained were entered into and analysed by the Statistical Programme for the Social Sciences (SPSS) Version 16.0. Frequencies, percentages, means and standard deviations were used to summarize data collected. The paired sample t-test was used to test for significant differences in means. The McNemar test was used to determine any statistically significant differences in ER, PR and HER-2/neu between primary and corresponding lymph nodes. A probability value less than 0.05 was considered statistically significant.

CHAPTER 4: RESULTS

4.0 Total number of cases.

The histopathology log book was used to retrieve patient information such as age and the type of specimen. Information with regards to diagnosis, tumour grade, number of lymph nodes involved with metastatic breast tumour were retrieved from the duplicate reports of patients' results kept at the department. The total number of surgical cases received at the Department of Pathology in the year 2009 was 7,115. About 11% (786 out of 7,115) of these cases were breast cases. Out of the total number of breast cases submitted, 36.5 % (287 out of 786) were positive for breast cancer. The rest of the breast cases were mostly fibroadenomas. About 40% (114 out of 287) of the breast cancer specimens were tru cut biopsies and 39% (112 out of 287) were breast cancer cases without lymph node metastases. About 21% (61 of 287) of the breast cancer cases were mastectomy specimens with axillary contents, and were diagnosed as invasive ductal carcinomas (IDC) with lymph node metastases. About 11% (7 out of 61) of the breast cancer cases with axillary lymph node metastases were excluded because the primary breast cancer tissue showed extensive necrosis. The total number of cases that were recruited into the study was therefore 54. These patients received neo adjuvant chemotherapy as part of their treatment and/ or management plan.

4.1 Pathological characteristics of cases.

The youngest patient enrolled in this study was 25 years and the oldest was 72 years. The mean age ± SD was 49.89 ± 10.70. The median age was 51 years. About 30% (16 out of 54) of the cases were between the ages of 49 and 56 (Table not shown). About 20.4% (11 out of 54) of the patients had grade I breast cancer, 42.6% (23 out of 54) had grade II and 37% (20 out of 54) had grade III breast cancer respectively. All of the cases were invasive ductal carcinomas. The number of axillary lymph nodes that were retrieved from the axillary contents ranged from 1 to 13. The percentage of retrieved axillary lymph nodes that were involved with breast cancer cells ranged from 8% to 100%. About 22% (12 out of 54) of the cases had 100% lymph nodes involved with breast cancer cells and 3.7% (2 out of 54) had 8% of retrieved lymph nodes involved with breast cancer cells. The mean percentage of lymph nodes that were involved with breast cancer was 57.87%.

Table 1 shows the summary of ER, PR and HER-2/neu statuses between primary breast tumour and corresponding lymph nodes.

Table 1: Summary of ER, PR and HER-2/neu statuses in primary tumour and lymph nodes.

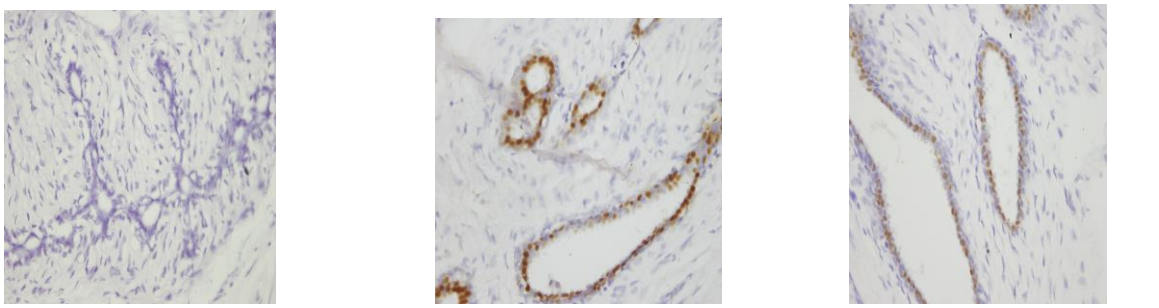
Receptor status	Primary tumour		Corresponding Lymph node	
	Frequency	Percentage (%)	Frequency	Percentage (%)
ER positive	17	31.5	18	33.3
ER negative	37	68.5	36	66.7
PR positive	14	25.9	16	29.6
PR negative	40	74.1	38	70.4
HER-2 positive	14	25.9	16	29.6
HER-2 negative	40	74.1	38	70.4

n =54

There was a slight increase in receptor positivity as well as an increase in receptor negativity in the lymph nodes (Table 1). However, there was no statistical difference, $p>0.05$, between them.

Figure 3 shows micrographs of control slides.

A B C



D

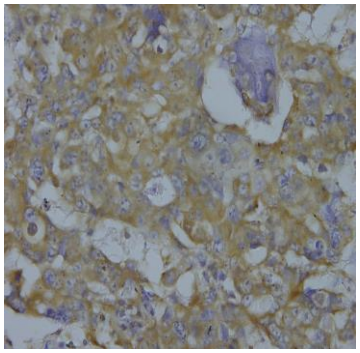
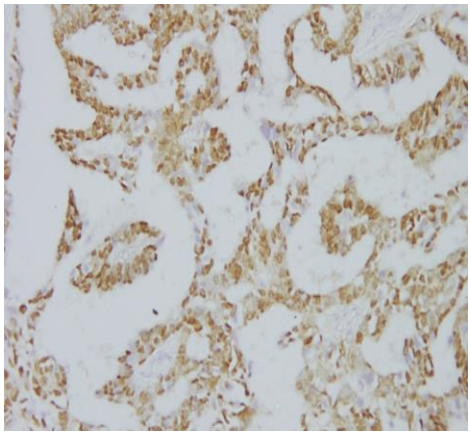


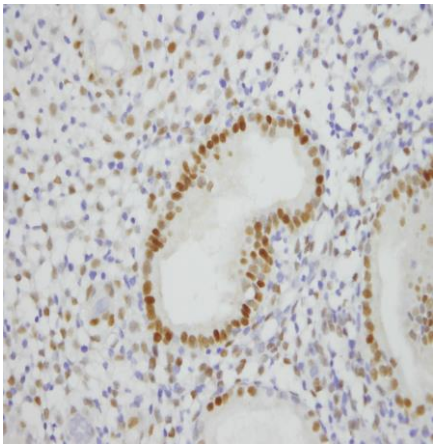
Figure 3: Slides of a fibroadenoma tissue showing; A: negative control, B: PR positive control, C: ER positive control and D: HER-2/neu positive control in an invasive ductal carcinoma tissue(X400 for all slides).

Figures 4 A and B show micrographs of ER positive slides of a primary breast tumour and a lymph node respectively.

Figure 4

A: ER positive primary tumour (X400). **B: ER positive lymph node(X400).**

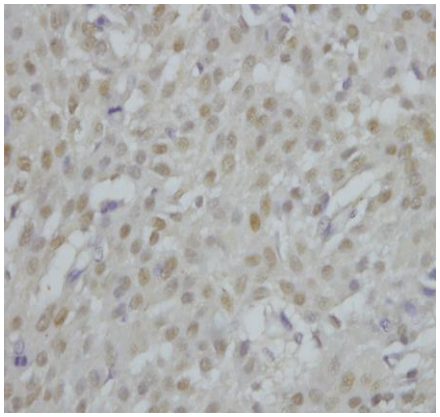


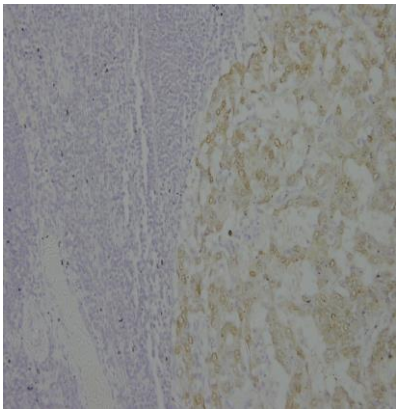


Figures 5 A and B show micrographs of PR positive slides of a primary breast tumour and lymph node respectively.

Figure 5

A: PR positive primary tumour (X400) **B: PR positive lymph node (X400)**

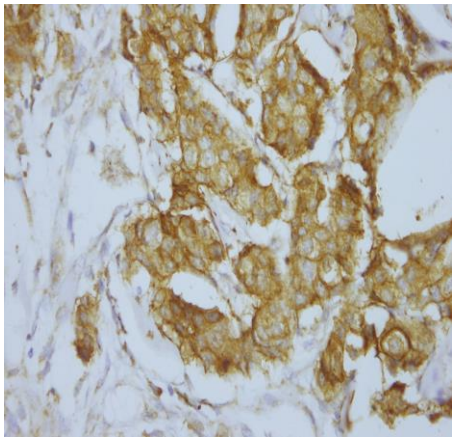


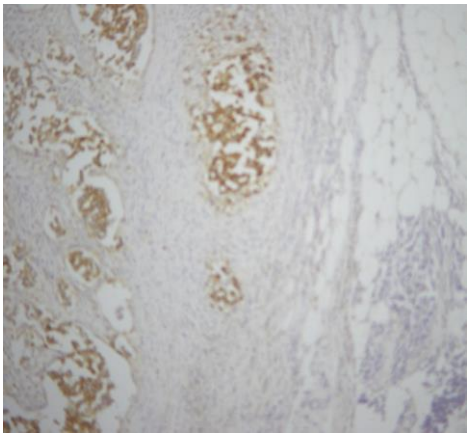


Figures 6 A and B show micrographs of HER-2/neu positive slides of a primary breast tumour and lymph node respectively.

Figure 6

A: HER-2 positive primary tumour(X400). **B: HER-2 positive lymph node(X400).**





Case to case comparison of ER, PR and HER-2/neu between primary breast cancer and corresponding lymph nodes (Table not shown) showed that, of the 54 cases analysed, 1.9% (1 out of 54) was positive for ER in the primary breast tumour and negative in the lymph nodes. About 4% (2 out of 54) of the cases were negative for ER in the primary breast tumour and positive in the lymph nodes. The discordant rate for ER was 5.6%. There was 94.4% (51 out of 54) concordance for ER between primary breast cancer and metastatic deposits in lymph nodes. The discordant rate for PR was 7.5%. About 2 % (1 out of 54) of the cases that were PR positive in the primary tumour was negative in the corresponding lymph node metastasis. About 6% (3 out of 54) of the cases which were PR negative in the primary tumour were positive in the corresponding metastatic deposits

in the lymph nodes. There was 92.6% (50 out of 54) concordance for PR between primary breast cancer and metastatic deposits in lymph nodes. None of the HER-2/neu positive primary breast tumour was negative in the metastatic deposits in the corresponding lymph nodes. About 4% (2 out of 54) of the HER-2/neu negative primary tumor were positive in the corresponding axillary lymph nodes. There was 96.3% (52 out of 54) concordance for HER-2/neu.

Table 2 shows the subtypes of breast cancer as determined by ER, PR and HER-2/neu statuses.

Table 2: Subtypes of breast cancer according to receptor status.

Subtype	**Frequency**	**Percentage (%)**
Triple negative (ER-/PR-/HER-2/neu-)	26	48.1
HER-2/neu type (ER-/PR-/HER-2/neu+)	6	11.1
Luminal A (ER+/PR+-/HER-2/neu-)	12	22.2
Luminal B (ER+/PR+-/HER-2/neu+)	5	9.3
ER-/PR+/HER-2/neu+/-	5	9.3
Total	54	100

Triple negative breast cancer was the most frequent followed by the Luminal A type and then the HER-2 type breast cancers. Hormone receptor positive and HER-2/neu positive breast cancer (ER+/PR+/HER2+), as well as ER negative breast cancers were infrequent.

4.2 Pathological characteristics among triple negative breast cancer cases.

About 48% (26 out of 54) of the cases that were recruited in this study had triple negative breast cancer (TNBC). The age range among this subtype of breast cancer was 29 to 72 years. The mean age ± SD was 49.89 ± 10.77. The median age of the patients with TNBC was 50 years. About 15% (4 out of 26) had grade I breast cancer, 50% (13 out of 26) and 34.6% (9 out of 26) had grade II and grade III breast cancer respectively. The percentage of lymph node involvement among this group of breast cancer was 8% to 100%. About 39% (10 out of 26) patients had 100% lymph nodes involved with breast cancer cells.

There was no statistical difference in age ($p=0.853$), tumour grade ($p=0.731$) and percentage of lymph nodes involved with metastatic breast cancer ($p=0.325$) between the TNBC subgroup and the other groups of breast cancer.

Table 3 shows the comparison of the differences in ER, PR and HER-2/neu between primary breast cancer and corresponding lymph nodes.

Table 3: Comparison of differences in ER, PR and HER-2/neu between primary breast cancer and corresponding lymph nodes.

Receptors	Primary tumour	Corresponding lymph node	P value
ER			
Positive	17 (31.5)	18 (33.3)	
Negative	37 (68.5)	36 (66.7)	1.000
PR			
Positive	14 (25.9	16 (29.6)	
Negative	40 (74.1)	38 (70.4)	0.625
HER-2/neu			
Positive	14 (25.9	16 (29.6)	
Negative	40 (74.1)	38 (70.4)	0.500

The McNemer test was used to test for any significant differences in the expression of ER, PR and the over expression of HER-2/neu between the primary breast cancer and the corresponding metastatic deposits in the axillary lymph nodes. There were no statistically significant differences ($p>0.05$) in ER, PR and HER-2/neu between primary breast cancer and corresponding lymph nodes (Table 3).

CHAPTER 5: DISCUSSION

The incidence of breast cancer varies globally. Estimated worldwide incidence of breast cancer ranges from 16.8 per 100,000 women in West Africa to 89.7 per 100,000 women in Western Europe (GLOBOCAN 2008). In Africa, the estimated incidence of breast cancer ranges from 16.8 per 100,000 in Guinea to 41 per 100,000 in South Africa.

The estimated breast cancer incidence in Ghana as reported in the GLOBOCAN Cancer Fact Sheet is 25.8 per 100,000. However, the study presented herein showed that out of the 786 breast cases, 287 (36.5%) were histologically diagnosed as breast cancer.

Breast cancer occurs at a relatively younger age in African women as compared to White women. The mean age of breast cancer at diagnosis among African women is 48 years (Rambau *et al*., 2011). Similarly, in this study, the mean age of breast cancer was 49.87± 10.70 years, which is consistent with findings in the African medical literature. In Europe (Whites), the median age at diagnosis of breast cancer is reported to be 67 years (Finnish Cancer Registry, Cancer stat fact sheets, 2011). The median age at diagnosis of breast cancer as reported in this study was 51 years. The factors responsible for this difference in age at diagnosis of breast cancer are not fully understood, although it could be due to mutations in the breast cancer susceptibility genes BRCA 1 and BRCA 2 and their variants which are more common among Black (African) women (American Cancer Society, 2011-2012).

About 80% of the cases accessed in this study were grade II and III breast cancers. In addition, the average lymph node involvement by metastatic breast cancer was 60%. This is consistent with reports that African women present late to hospitals with advanced

breast cancer due to late attendance or reporting to the hospital as reported by Harris *et al*., (2000) and Clegg-Lamptey *et al*., (2007).

In this study, the expression of ER was 32% and is similar to ER expression of 23% in African women as reported by Olopade (2005). The expression of ER in breast cancer among women in the United States is 70-80% (Gown, 2008). The lower prevalence of ER expression in African women as compared to ER expression in Whites, coupled with the high prevalence (48.1%) of triple negative breast cancer in African women as observed in this study, tends to make breast cancer in African women more aggressive (Rambau *et al*., 2011) than breast cancer in Whites. The prevalence of triple negative breast cancer worldwide is 15% (Lara-Medina, 2011). The reason(s) for the high prevalence of triple negative breast cancer could not be discerned from the study, although according to Stark *et al*., (2010), genetics may play a role. Further studies need to be done to define the high prevalence of triple negative breast cancer among Ghanaian and other African women with breast cancer.

5.0 Comparison of ER, PR and HER-2/neu.

Breast cancer is considered as a heterogeneous disease (Perou *et al*., 2000). As breast cancer progresses, clonal expansion of breast cancer cells occur and these cells may become genetically altered leading to tumour heterogeneity (Almendro and Fuster, 2011). These genetic alterations may contribute to the differences in hormone receptor expression and HER-2/neu over expression between primary breast cancer and metastatic deposits in corresponding lymph nodes as observed in this study. During breast cancer

progression, there is also the adaptation of clones of tumour cells in changing microenvironments. In the lymph node microenvironment, according to Dawood (2000) clonal selection of tumour cell sub populations occurs and this could account for some of the metastatic deposits in the lymph nodes being hormone receptor negative whiles the primary tumour is hormone receptor positive and vice versa.

Literature has revealed varying results from the comparison of ER, PR and HER-2/neu between primary breast cancer and metastatic tumour deposits. Factors that may contribute to differences in the expression of ER, PR and the over expression of HER-2/neu between primary breast cancer and metastatic tumour deposits may include: tumour heterogeneity (Nedergaard *et al*., 1995; Azam *et al*., 2009; Arslan *et al*., 2011), clonal selection of tumour cell subpopulations, genetic instability of tumour cells (Edgerton *et al*., 2003; Prat and Perou, 2009), local or systemic treatments, the time interval between primary tumour and metastasis, receptor status determination techniques (Prat and Perou, 2009), and the site of metastasis (Arslan *et al*., 2011).

Simmons *et al*., 2009 and Broom *et al*., 2009 reported in their studies that there was no significant concordance for ER, PR and HER-2/neu between primary breast tumour and distant metastases. Contrary to the findings of Simmons *et al*., (20009) and Broom *et al*., (2009), the findings reported herein indicate a significant concordance in ER, PR and HER-2/neu between primary breast tumour and metastatic deposits in lymph nodes. Similar findings were reported by Holdaway *et al*., (1983) and Azam *et al*., (2009).

5.1 Conclusion.

There are minor changes between ER, PR and HER-2/neu status between primary tumour and metastatic deposits in lymph nodes. These changes are however, statistically not significant. In the absence of the primary breast cancer tissue, the tumour deposits in the resected axillary lymph nodes will give a fair reflection of the ER, PR and HER-2/neu status of the primary tumour in the majority of cases to inform further management decision.

5.2 Recommendation.

In the absence of the primary breast cancer tissue, the tumour deposits in the resected axillary lymph nodes will give a fair reflection of the ER, PR and HER-2/neu status of the primary tumour in the majority of cases to inform further management decision.

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APPENDIX I

DESCRIPTION OF ANTIBODIES.

ESTROGEN RECEPTOR:

Product Code: NCL-L-ER-6F11

LOT: L1151211

Clone: 6F11

Immunogen : Prokaryotic recombinant protein corresponding to the full length alpha form of the human estrogen receptor molecule.

Immunoglobuin: Mouse monoclonal

Immunoglobulin class: IgG1

Antibody concentration: Greater than or equal to 67.5mg/L as determined by ELISA

Total protein concentration: 4.1g/L

Total antibody concentration: 75mg/L

Dilution factor of antibody used: 1/60

Concentration of antibody used: 75mg/L X 1/60 = 1.25mg/L

PROGESTERONE RECEPTOR:

Product Code: NCL-L-PGR-312

LOT: 6008675

Clone: 16

Immunogen : Prokaryotic recombinant protein corresponding to the N-terminal region of the human progesterone receptor molecule.

Immunoglobuin: Mouse monoclonal

Immunoglobulin class: IgG1

Antibody concentration: Greater than or equal to 342.0mg/L as determined by ELISA

Total protein concentration: 4.6g/L

Total antibody concentration: 360mg/L

Dilution factor of antibody used: 1/100

Concentration of antibody used: 360mg/L X 1/100 = 3.6mg/L

HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR-2:

Product Code: NCL-L-CB11

LOT: 6009335

Clone: CB11

Immunogen : Synthetic peptide corresponding to a site on the internal domain of the human c-erbB-2 oncoprotein.

Immunoglobulin class: IgG1

Antibody concentration: Greater than or equal to 23.4mg/L as determined by ELISA

Total protein concentration: 3.9g/L

Total antibody concentration: 26mg/L

Dilution factor of antibody used: 1/40

Concentration of antibody used: 26mg/L X 1/40 = 0.65mg/L

APPENDIX II

REAGENT PREPARATION

50mM Tris Buffered Saline (TBS):

The following were dissolved in 800ml of distilled water;

8g Sodium chloride

0.2g potassium chloride

6g Tris Base

PH was then adjusted to 7.6 with concentrated hydrochloric acid

Total volume was made up to 1L with distilled water.

0.01M Citric acid:

2.1g of citric acid was dissolved in 1L of distilled water

PH was adjusted to 6.0

APPENDIX III

UNIVERSITY OF GHANA MEDICAL SCHOOL
COLLEGE OF HEALTH SCIENCES
ACADEMIC AFFAIRS OFFICE

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Your Ref. No.

P O Box 4236
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27th October 2011

Ms. Bernice Anane Mawuli
Department of Pathology
UGMS
Korle-Bu, Accra.

ETHICAL CLEARANCE

Protocol Identification Number: MS-Et/M.1 – P 5.1/2011-12

The Ethical and Protocol Review Committee of the University of Ghana Medical School on 19th October, 2011 unanimously approved your research proposal.

TITLE OF PROTOCOL: "**Comparison of Estrogen Receptor Alpha (ERa) Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor-2 (HER 2/NEU) Expression in Primary Breast Cancer and Lymph Node Metastasis at Korle-Bu Teaching Hospital"**

PRINCIPAL INVESTIGATOR: Ms. Bernice Anane Mawuli

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed:.....
PROFESSOR Y. TETTEY
(AG. CHAIRMAN, ETHICAL AND PROTOCOL REVIEW COMMITTEE)

cc: Dean
Head of Department
Research Office