THE UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE, LUSAKA.

ASSOCIATION OF BRCA 1 AND BRCA 2 PROTEIN EXPRESSION IN BREAST CANCERS WITH THEIR HISTOLOGICAL PHENOTYPE BY IMMUNOHISTOCHEMISTRY IN INDIGENOUS BLACK MALAWIANS PRESENTING AT QUEEN ELIZABETH CENTRAL HOSPITAL.

By

Nelson Chimbiya.

A Research Dissertation Submitted to the University of Zambia in partial fulfilment of the Requirements for the Master of Science in Pathology (Clinical Pathology).

2016

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DECLARATION

I Nelson Chimbiya hereby declare that this dissertation herein presented for the Degree of Master of Science in Pathology(Clinical Pathology) has not been previously submitted either in whole or in part for any other Degree at this or any other University. Various sources whose work I have utilized in my study are clearly indicated in the text and references.

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CERTIFICATE OF APPROVAL

The dissertation entitled: **Association Of BRCA 1 And BRCA 2 Protein Expression In Breast Cancers With Their Histological Phenotype By Immunohistochemistry In Indigenous Black Malawians Presenting At Queen Elizabeth Central Hospital,** has been approved as a partial fulfilment of the requirements for the award of the degree of Master of Science in Pathology (Clinical Pathology) at the University of Zambia.

EXAMINER I

SIGNED _____ DATE _____

EXAMINER II

SIGNED _____ DATE _____

EXAMINER III

SIGNED _____ DATE _____

ABSTRACT

Introduction: Breast Cancer gene 1 (BRCA1) and Breast Cancer gene 2 (BRCA2) have gained major scientific interest as potential prognostic and predictive markers for various tumours. Management of breast cancer depends on availability of robust clinical and pathological prognostic and predictive factors to guide patient decision making and the choice of treatment options. One of the prognostic factors of breast cancer is the BRCA1 and BRCA2 protein expression. Immunohistochemical studies on formalin-fixed, paraffin-embedded (FFPE) tumours have demonstrated a loss or reduction of protein expression, not only in *BRCA1* associated breast carcinomas, but also in non-*BRCA1* associated familial and sporadic breast carcinomas. Immunohistochemical information might guide therapy selection for patients.

Aim: To study association of the expression of BRCA1 and BRCA2 protein with their histological phenotype by immunohistochemistry in Malawian females presenting with breast cancer at Queen Elizabeth Central Hospital (QECH).

Materials and Methods:This was a laboratory based cross sectional study in which 63 Formalin-Fixed,Paraffin-Embeded(FFPE) breast cancer tissue samples from QECH and College of Medicine in Malawi were analysed. Tissues were cut for histological analysis and immunohistochemical staining using a microtome. Haematoxylin and eosin staining was done on slides to confirm invasive breast cancer, then underwent immunohistochemical staining with BRCA1 MS110 monoclonal antibodies and BRCA2 polyclonal antibodies**.** Data analysis was done using SPSS version 20.0 for windows. The statistical test used was Pearson chi square and fishers exact test where necessary.

Results: Sixty three FFPE breast cancer tissues were analysed from the year 2012 to 2015. The histological phenotypes that were found in the study were; invasive ductal carcinoma (IDC) 41.3% (26/63), invasive lobular carcinoma (ILC) 27% (17/63), medullary carcinoma 6.4% (4/63) and others 25.4% (16/63). The age of the patients in our study ranged from 21 to 82 years. The most affected age group was between 31 to 60 years. However, the peak age group was between 41 to 50 years. The different histological phenotypes showed a varied expression of both BRCA1 and BRCA 2 protein. Medullary carcinoma had the highest expression with 100% retention of both BRCA1 and BRCA 2.

Conclusion: The study showed the peak age for breast cancer presentation to be between 41-50years. Invasive ductal carcinoma was the predominant histological phenotype representing 41.3% of all the breast cancer samples. The overall retention for BRCA1 was 49.2% and 46% BRCA2. This shows that more than half of the breast cancer presenting at QECH had lost both the BRCA1 and BRCA 2 expression and suggests an aggressive course of these malignancies.

Key words: BRCA1, BRCA2, Breast Cancer, Invasive ductal carcinoma, Invasive lobular carcinoma.

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ABBREVIATIONS

AIDS	-	Acquired Immunodeficiency Syndrome
BC	-	Breast cancer
BRCA	-	Breast cancer
COMREC	-	College of medicine research and ethical committee
DNA	-	Deoxyribonucleic acid
ER	-	Estrogen receptor
FFPE	-	Formalin Fixed Paraffin Embedded
HER 2	-	Human Epidermal Growth Factor Receptor
HIV	-	Human Immunodeficiency Virus
IARC	-	International Agency for research on cancer
IDC	-	Invasive Ductal Carcinoma
IHC	-	Immunohistochemistry
IHME	-	Institute for Health Metrics and evaluation
ILC	-	Invasive Lobular Carcinoma
KCH	-	Kamuzu Central Hospital
LMIC	-	Low and Medium income countries
MAb	-	Monoclonal antibody
PARP1	-	poly (ADP-ribose) polymerase 1
PBS	-	phosphate buffered saline
PR	-	Progesterone receptor
QECH	-	Queen Elizabeth central hospital
TB	-	Tuberculosis
TNBC	-	Triple Negative Breast Cancer
TNM	-	Tumour Nodes Metastasis
UTH	-	University Teaching hospital
UNZABREC	-	University of Zambia Biomedical Research Ethics Committee
WHO	-	World Health Organization

DEDICATION

This study was dedicated to my beloved wife Treasa and children Chisomo, Watipatsa, Given and Tayamika who without their understanding and support this would not have been possible. They have been my source of inspiration and encouragement.

Chapter 1: Introduction

1.1 Background

The Breast Cancer gene 1(*BRCA1)* and breast cancer gene 2(*BRCA2)* and their protein products have recently been the subjects of intensive investigation because of their proven role in hereditary and putative role in sporadic human breast and ovarian cancer (Mangia, et al., 2011). Immunohistochemical studies on formalin-fixed, Paraffin-embedded (FFPE)tumours have demonstrated a loss or reduction of protein expression, not only in *BRCA1* associated breast carcinomas, but also in non-*BRCA1* associated familial and sporadic breast carcinomas (Mangia, et al., 2011).The available evidence indicate that mutated *BRCA1* and *BRCA2* genes are the underlying cause for about 5–10% of breast cancers. If mutated, *BRCA 1* gene increases an individual's chance of developing breast and ovarian cancer. (NHS Foundation Trust, 2013).

A mutation in the BRCA1 or BRCA2 gene increases an individual's risk of developing breast cancer. The *BRCA1* or *BRCA2* genes mutation does not cause cancer to occur on its own, but individuals are at great risk of developing cancer because their cells' ability to repair DNA damage are impaired by the *BRCA1* or *BRCA2* mutations (Shawky, et al., 2014). It is the accumulation of DNA damage which causes a cell to change into a cancerous cell. The decreased amount of BRCA1 protein detected in some tumours suggests a reduced expression of the gene. This does not rule out the possibility of a mutation leading to the presence of an abnormal protein or to its physical absence due to rapid degradation (Rio, et al., 1999). The reduced expression of BRCA1 detected in a high proportion of sporadic breast cancers has led to the suggestion that BRCA1 might be involved in the pathogenesis of a significant percentage of sporadic breast cancers by gene down-expression rather than mutation (Hsu, et al., 2013)

Most invasive breast cancers occurring in women with germline BRCA1 and BRCA 2 mutations are said to be Estrogen Receptor negative (ER) and Progesterone receptor negative (PR) and are negative for HER2 protein overexpression and gene amplification (Kaplan, et al., 2012). *BRCA1* gene positioned on human 17q21 chromosome encodes a protein of 220 kilodaltons consisting of 1863 amino acids. It contains 24 exons of which 22 have the coding function. This gene functions as a tumour suppressor, with loss of function of both alleles required for tumorigenic progression, (Shawky, et al., 2014). It performs many vital cellular functions, including recognition and repair of double stranded DNA breaks in cell cycles, cell-cycle checkpoint control, chromatin remodelling, transcriptional regulation of gene expression and mitosis. It especially participates in nucleotide excision repair and homologous recombination repair. Those cells with alterations in homologous recombination pathway genes are unable to repair DNA double-strand breaks, resulting in genomic instability and a predisposition to malignant transformation (Shawky, et al., 2014) Breast cancer represents an etiologically heterogeneous disease, with an intrinsic complexity in cellular–biomolecular profile and diversity in its responsiveness to treatment. BRCA1 and BRCA2-deficient cells display genomic instability due to impaired DNA repair and results in malignant transformation.

These *BRCA1* and *BRCA 2* mutated tumours are most often high-grade invasive ductal carcinomas with a high mitotic rate that frequently exhibit other characteristic pathologic features (Davila, et al., 2010). Therapies including DNA-damaging cytotoxic chemotherapies and poly adenosine diphosphate-ribose polymerase inhibitors have recently been evaluated in patients with triple-negative breast cancer (BC). These studies have demonstrated that triple-negative BC patients, particularly those with underlying *BRCA* mutations, have robust and durable responses to therapies that target underlying defects in DNA repair (Hartman, et al., 2012). In another study by Yoshikawa *et al*, it was found that reduction of BRCA1 protein expression in sporadic carcinoma was associated with solid tubular phenotype with poor tubular differentiation and with an over

expression of c-erB-2 protein which is one of the prognostic factors of breast cancer (Yoshikawa, et al., 1999). In another study by Yang et al it was found that BRCA2 non expression was associated with significantly improved primary chemotherapy response and longer platinum-free duration than were BRCA1 non expression (Yang, et al., 2011) Immunohistochemical information might guide therapy selection for patients.

Management of breast cancer depends on the availability of robust clinical and pathological prognostic and predictive factors to guide patient decision making and the choice of treatment options. Lymph node (LN) status, tumour size and histologic grade are prognostic factors that help decide administration of systemic therapy in the early stages of breast cancer. The aim of this study was to find the association between the expression of BRCA 1 and BRCA 2 proteins with their histological phenotype in indigenous black Malawian women presenting with breast cancer at Queen Elizabeth Central Hospital (QECH).

1.2 Statement of the problem

Breast cancer in limited resource countries including Malawi is an emerging health problem amongst women (Marx, 2014). According to the world health organization (WHO) by 2020, African states will account for more than one million new cases per year out of the total 16 million cases worldwide (Marx, 2014). Incidence and mortality rates are increasing throughout the world and especially in LMICs (Marx, 2014). In Southern Africa, breast cancer is the third commonest cancer following prostate and cervical cancer, and there is high mortality rate due to delayed presentation and limited therapeutic interventions (Njelekela, 2009). The increase in the observed incidence of breast cancer from 1996-2005 in Malawi is similar to that observed in Kampala, Uganda between 1991 and 2006 where it was ~5% per year (Gyorki, et al., 2012).

The available literature (table 1 below) shows that breast cancer prevalence in Malawi ranges between 6% and 7 % of the total cancer (Misiri, et al., 2012; Mlombe, et al., 2008; Msiyamboza, et al., 2012; Msyamboza, et al., 2014) . And most of the

patients who present with breast cancer are young women (Kendig, et al., 2013) .This prevalence is very high and to date the most common type of breast cancer affecting these women is not known. It is therefore important to know the role of BRCA1 and BRCA2 protein expression in these patients. This knowledge can benefit these patients because currently there are new drug derivatives i.e. PARP that are proving to be effective to carriers of BRCA1 and BRCA2 gene mutations (Khan, et al., 2010).

Table 1: **Breast cancer prevalence in Malawi**

Year of article	**Period Covered**	**Incidence location**	**Incidence of breast cancer %**	**Author**
2001	1994-1998	National	7.4% (74)	Banda et al
2008	Pre HIV era	National	6.7% (no total #given)	Mlombe et al
2012	2007-2010	National	4.6% (484)	Msiyamboza et al
2012	1996-2005	National	6% (47)	Misiri et al
2013	2011-2013	National	7% (61)	Gopal et al

The table above shows the prevalence of breast cancer in Malawi from according to the literature from 2001 to 2013.

1.3 Justification and significance of the study

Most patients presenting with breast tumours are young and often present at an advanced stage of the disease (Kendig, et al., 2013; Gyorki, et al., 2012). Higher grade carcinomas are associated with a loss of expression of BRCA1. It is important to know the role of BRCA1and BRCA2 proteins in these tumours. *BRCA1* and *BRCA2* gene proteins are known to play a major role in the molecular basis of breast cancer.

Currently no analysis of BRCA1 and BRCA2 proteins are being done in Malawi as part of the diagnosis plan to guide treatment in spite of studies conducted elsewhere showing that there is an increase of these types of breast cancers amongst women of African descent (Khan, et al., 2010). To plan treatment histologic type, grade and molecular subtype should be detected. This study has established the underlying cause of sporadic breast cancer in Malawian women and adds knowledge to the understanding of the common types of breast cancer in Malawi as well as providing ground for further research.

Chapter 2: Literature Review

Mutations within the *BRCA1* tumour suppressor gene occur frequently in familial epithelial ovarian carcinomas but they are a rare event in the much more prevalent sporadic form of the disease. However, decreased BRCA1 expression occurs frequently in sporadic tumours, and the magnitude of this decrease has been correlated with increased disease progression. (McCoy, et al., 2003). *BRCA1*, a tumour suppressor gene, encodes a 220 KDa protein to function in the response for double-strand breaks (DSBs) by homologous recombination (HR). Although *BRCA1* mutation is rare in sporadic breast cancer, reduction of BRCA1 nuclear expression in breast cancer tissue is common (Wei, et al., 2014).

Several mechanisms may be proposed to account for this decrease in expression, such as spontaneous mutations or loss of heterozygosity of the BRCA1 gene, the hyper-methylation of the BRCA1 promoter region or mutation of a gene required for BRCA1 expression (Mangia, et al., 2011) . The functions of BRCA1 protein are yet to be fully elucidated; however, BRCA1 protein participates in many signalling pathways involved in transcription, checkpoint control, and is recruited for the formation of DNA repair complexes in association with proteins such as Rad50, and BRCA2. BRCA1 is localized in the nucleolus; this explains a possible functional participation in processes of ribosomal biosynthesis, cell cycle progression, and as a reservoir for complexes formed in response to cellular stress and DNA repair.

The function of BRCA1 protein in tumorigenesis has been found to be complex, and as a tumour suppressor, it is postulated that reduced expression leads to multiple abnormalities, including a defect in the homologous recombination (HR) pathway of DNA repair. These defects are associated with a hypersensitivity to many agents that cause DNA double strand breaks, such as ionizing radiation (IR) (Tulchin, et al., 2013). Mutations in *BRCA1* and *BRCA2* genes in germline cells cause susceptibility to breast and ovarian cancer. Those cells with alterations in homologous recombination pathway genes are unable to repair DNA double-strand breaks, resulting in genomic instability and a predisposition to malignant transformation (Mahdi, et al., 2013)

In most of the studies the overlap of *BRCA1* associated breast cancers with the Triple Negative Breast Cancer (TNBC) phenotype is significant, and a substantial percentage of TNBC are said to be of African descent. It has been shown that basal like breast carcinomas frequently harbour defects in deoxyribonucleic acid (DNA) double strand break repair through homologous recombination such as *BRCA1* dysfunction. The DNA repair defects characteristic of *BRCA1* and *BRCA2* deficient cells confer sensitivity to poly (ADP-ribose) polymerase 1 (PARP1) inhibition. Both *BRCA1* and *BRCA2* are required for the homologous recombination pathway to function properly (Khan, et al., 2010).

Information on BRCA status is now important not only to address the risk of breast and ovarian cancer, but also to select therapies. Cells deficient in either BRCA1 or BRCA2 are sensitive to PARP1 inhibition, resulting in cell death and apoptosis (Khan, et al., 2010; Weberpals, et al., 2011) . A number of clinical trials have demonstrated that PARP inhibitors may be particularly useful for the treatment of patients with an inherited mutation in BRCA1/BRCA2 (Weberpals, et al., 2011). Determination of BRCA1 protein expression by immunohistochemistry (IHC) may be a clinically useful tool to provide important information on prognosis and to direct patients toward specific targeted therapies, such as poly ADP (ribose) polymerase (PARP) inhibitors, which are dependent on BRCA1 deficiency (Weberpals, et al., 2011). *BRCA1* and *BRCA2* mutations confer an extremely high risk of breast cancer, and management guidelines

for *BRCA1* and *BRCA2* mutation carriers advise close follow-up, intensive screening, and consideration of prophylactic surgery to lower this risk. (Hall, et al., 2013; Barros, 2013).

Breast carcinomas occurring in women aged below 40 years are more often in advanced stage and higher proliferation (Purnomosari, 2006). Early age of onset has been known as one of the clinical characteristics of hereditary breast cancers associated with germline *BRCA1* or *BRCA1* mutations (Purnomosari, 2006). The presence of mutations in *BRCA1* and *BRCA2* genes in young women is associated with more aggressive course of breast cancer and absence of progesterone receptor and oestrogen receptor which are unfavourable prognostic factors. In another study Zakhartseva1 *et al*. found that in cases of similar disease stages and similar histological cancer types, the clinical course of breast cancer in young age patients having BRCA1 and BRCA2 gene mutation was more aggressive (Zakhartseva, et al., 2009). The identification of these breast cancer susceptibility genes has contributed to major modifications in the treatment of women with inherited predisposition to breast cancer (Maria, et al., 2009). Studies have shown that breast cancers in women with germ-line *BRCA1* mutations are more likely to be triple-negative and high-grade (Khan, et al., 2010). It has been speculated that the BRCA1 protein may be useful in the treatment of breast carcinoma as a decision-making biomarker for aggressive treatment after operation. Immunohistochemical assessment of *BRCA1* mutation is less expensive and less time consuming than genetic testing (Maria, et al., 2009).

Tissue examination is the gold standard in tumour diagnostics. Depending on the submitted tissue material, pathology can reveal the presence and spread of the tumour as well as its biological potential as benign or malignant. Up to date techniques and integrated approaches to tissue evaluation along with other scientific methods can bring higher volumes of information with clinical relevance. The morphologic data can predict the potential effect of different treatment modalities. The pathogenesis of

tumour is also partially reflected in the neoplastic tissues (Strumfa, et al., 2012). A recent study by Hedau *et al* showed down regulation of BRCA1 protein during breast carcinogenesis, and also demonstrated that in a subset of cases; the decline in BRCA1 expression may be associated with potentially compensatory increase in BRCA2 protein, which may depend on grade of tumour as well as menopausal status of the patients (Hedau, et al., 2015).

Patients with germline mutations in the *BRCA* genes are at risk of developing breast, ovarian, pancreatic, and other malignancies. The products of the *BRCA* genes have a variety of roles, including those relating to DNA-repair mechanisms. Cells that lack a functional *BRCA1* or *BRCA2* have a deficiency in the repair of DNA double strand breaks, which is probably one of the mechanisms behind their association with increased cancer predisposition (Davila, et al., 2010)

2.2. Research Question.

What is the protein expression of *BRCA 1* and *BRCA 2* in breast cancers diagnosed in indigenous black women presenting at Queen Elizabeth Central Hospital (QECH) in Malawi.

2.3. Objectives

2.3.1 General objective

To determine association of the expression of *BRCA1* and *BRCA2* gene proteins and the histological phenotype of breast cancer in indigenous black Malawian women patients presenting with breast cancer at QECH.

2.3.2 Specific objectives.

2.3.2.1 To determine the histologic phenotypes of breast cancer in women presenting with breast cancer.

2.3.2.2 To determine the expression of *BRCA 1* and *BRCA 2* proteins in patients presenting with breast cancer.

2.3.2.3 To establish association of the expression of *BRCA1* and *BRCA2* proteins with the histologic phenotypes of breast cancer.

Chapter three: Methodology.

3.1. Research design and setting.

This was a laboratory based cross sectional study of indigenous black Malawian patients who presented to QECH with breast cancer and underwent surgery. Their biopsy samples had histological evidence of invasive breast carcinoma. Each case had the following information about the patient name, sex, age, medical record number and the tumour size, histological type, histological grade, number of axillary lymph nodes examined and number of metastatic nodes.

The study was done in the Department of Pathology and Microbiology at the University Teaching Hospital (UTH), a tertiary level hospital and national referral hospital in Lusaka, Zambia. This was because there was no immunohistochemistry laboratory at QECH at the time this study was being done. So samples were taken to Lusaka at UTH where there was a functional immunohistochemistry unit and supervisors of the principal investigator.

3.2. Sample size, sampling frame and sample selection.

The study used a convenient sample size of sixty three FFPE breast cancer tissues from January 2012 to December 2015. The 2014 samples did not meet the inclusion criteria because of incomplete data hence were excluded from the study.

3.3. Sample calculation

$N=Z^2P(1-P)/d^2$

Where N=Sample size

Z=statistical for level of confidence (1.96)

P=expected prevalence or proportion

D=Precision (5%)

$N=1.96^2*0.07(1-0.07)/0.05^2$

N=100

3.4. Study population and sample collection.

Already processed paraffin blocks diagnosed breast cancer samples were retrieved from the archives of QECH histopathology laboratory and College of Medicine, de-waxed and prepared for immunohistochemical staining to determine the presence of *BRCA1* and *BRCA2* protein as well as determine histological phenotypes.

At QECH hospital there was no guiding protocol for the storage, retrieval and disposing of breast tissue samples and other tissues. When breast tissues have been grossed are left haphazardly and the tissue blocks that are produced are kept in cartons after slides have been produced. Now these blocks are kept indefinitely but tissues are thrown away depending on how they have accumulated in the laboratory. When anyone needs specimens there was no formal channel that was being followed to inform the authority, and one would collects the blocks without any documentation. There was a need to have a standard operating procedure (SOP) on storage, retrieval, transfer and disposal of samples.

3.5. Inclusion Criteria.

All Formalin-Fixed Paraffin-Embedded (FFPE) specimens from females diagnosed with invasive breast cancer from January 2012 through December 2013 and for 2015 which had information on age, sex, invasive cancer diagnosis were included. This was so, as the FFPE available were less than 100.

3.6. Exclusion criteria.

Formalin-Fixed, Paraffin-Embedded breast cancer specimens from males and from females without invasive breast cancer and those that had missing data on sex and age though with invasive cancer diagnosis were excluded from the study**.**

3.7. Ethical issues.

Approval was sought from the University of Zambia Biomedical Research Ethics Committee (UNZABREC), and College of Medicine Research Ethical Committee (COMREC). No consent was sought from patients since these were archival samples.

But permission to use the archival histopathological tissues was sought from College of Medicine Research Ethics committee (COMREC), **S**enior medical superintendent and the Head of the Department of Pathology of the College of Medicine.

3.8. Histology

For histological procedure and staining solution preparation refer to Appendix 8.2.B

The most widely used histologic grading system of breast cancer is the Nottingham grading system, also referred as Scarff-Bloom-Richardson grading system. The Nottingham grading system combines nuclear grade, tubule formation and mitotic rate to classify invasive carcinomas into three groups that are highly correlated with survival (Kumar, et al., 2010).

The seven possible scores are condensed into three Nottingham grades shown in the table 2 below. Multiple studies have shown an independent prognostic significance of grade in breast cancer (Rakha, et al., 2008) and improved interobserver agreement with the Nottingham histologic grade method compared with other grading systems. This has been possible due to the application and endorsement of rigorous optimized and standardized methods for tissue handling, fixation, and preparation. Differences between centres can in many cases be attributed to differences in quality of tissue preparation. Suboptimal tissue fixation has been clearly demonstrated to impact adversely on ability to assess mitotic frequency resulting in a systematic downgrading of a proportion of cases. This typically results in a reduction in the proportion of cases assigned to grade.

Table 2: Nottingham Grading System

Nottingham grading scores (combined scores)	Differentiation
3,4,5	Well differentiated (Grade 1)
6,7	Moderately differentiated (Grade 2)
8,9	Poorly differentiated (Grade 3)

The table above shows histological grading criteria using Nottingham grading system

3.9. Immunohistochemical analysis of *BRCA1* and *BRCA 2* protein and histologic type.

For the immunohistochemical study, 4-µm-thick sections were cut from paraffin blocks, which contain representative histology of invasive breast carcinoma. The primary antibodies used in this study were mouse monoclonal antibody (MS110) to *BRCA1* and Rabbit polyclonal to *BRCA2* with a dilution of 1:50 and 1:200 respectively (Abcam, UK). For reagent preparation and staining refer to Appendix 8.3.C

3.10. IHC interpretation of results

For immunohistochemical evaluation of BRCA1 and BRCA 2, nuclear and cytoplasmic immunostaining of invasive breast cancer cells were compared with that of normal mammary duct cells. Nuclear staining of neoplastic cells was scored as loss, retained and equivocal for BRCA1 and BRCA2 protein. Breast cancers were scored depending on the percentage of malignant cells labelled. Breast cancers with complete absence of staining, with presence of positive internal control were scored as loss of BRCA protein. Breast cancers staining in more than 10% of tumour cell nuclei were scored as retained BRCA protein. Weak staining in 5-10% of tumour cell nuclei, in the presence of moderate to strong internal control, complete absence of staining without positive internal control were scored as equivocal staining for BRCA protein . All histologic slides were viewed by a pathologist. The tumours were typed using the World Health Organization (WHO) breast carcinoma classification.

3.11. Specimen labelling.

Each archival breast cancer specimen was given a new identification code and number for easier identification. The letters “BC” (which stands for Breast Cancer) plus a three digit numbering system (i.e. 000) was used. Hence specimen numbers 1, 2, 5, 10, 20, 25 and 30 were labelled as BC 001, BC 002, BC 005, BC 020, and BC030 respectively.

3.12. Microtomy procedure.

For sectioning procedures and microtome maintenance refer to Appendix 8.1.A

3.13. Staining.

For staining procedures (H& E) refer to Appendix 8.2.B

3.14. Cover slipping.

The slides were placed on a clean horizontal bench surface. A drop of DPX Mountant was applied to each tissue section and at the far end of the slide away from the frosted end. The cover-slip was carefully applied by placing one end of the slip on top of the drop of DPX Mountant at the far end of the slide. Slowly and gently the cover slip was rolled down the side, only using enough pressure to allow the medium to spread evenly. The slides were placed flat upon the bench for two hours to eliminate bubbles under the cover slip. When the slides were dry, they were removed from the bench and were ready for microscopic examination. Refer to Appendix 8.2.B

3.14. Data processing

The data was entered into Microsoft excel 2010. Descriptive statistics were used to provide simple summaries. The summaries formed the basis of the description of the data. Continuous variables were expressed as percentages and actual numbers. Categorical variables were expressed as percentages. Descriptive statistics were shown by graphical representation.

The Microsoft excel sheet was uploaded in the SPSS window, where data was analysed using SPSS version 20.0 software for windows. The Chi-square and Fishers exact tests were used to compare categorical data such as BRAC1 and BRCA2 protein expression, and were used to evaluate statistical significance between the *BRCA1* and *BRCA2*

protein expression patterns and the histological types. Statistical significance was shown by p-value equal to or less than 0.05.

Chapter 4: Results.

Sixty three FFPE breast cancer tissue samples were analysed for histological phenotype characteristics and BRCA1 and BRCA2 protein expressions. In terms of laterality, Breast cancer affects Malawian women more on the right breast than left breast. The prevalence of BC was found as shown in table 3 below.

4.1. Prevalence of BC.

Table 3: Prevalence of BC among breast tissue suspected tumours

T

Year	Tumour Samples	Men	Female	No age	Non BC	BC Frequency	BC Percentage
2012	99	0	99	0	77	22	22.2
2013	66	2	64	6	41	25	37.9
2015	48	0	48	1	32	16	33.3

v

Key: BC: breast cancer

The results on the prevalence of BC in the table show an increasing trend from 2012 to 2015.

4.2. Age distribution

The age of the patients in our study ranged from 21 to 82 years. The most affected age group was between 31 to 60 years. However, the peak age group was between 41 and 50 years. The results are clearly shown below.

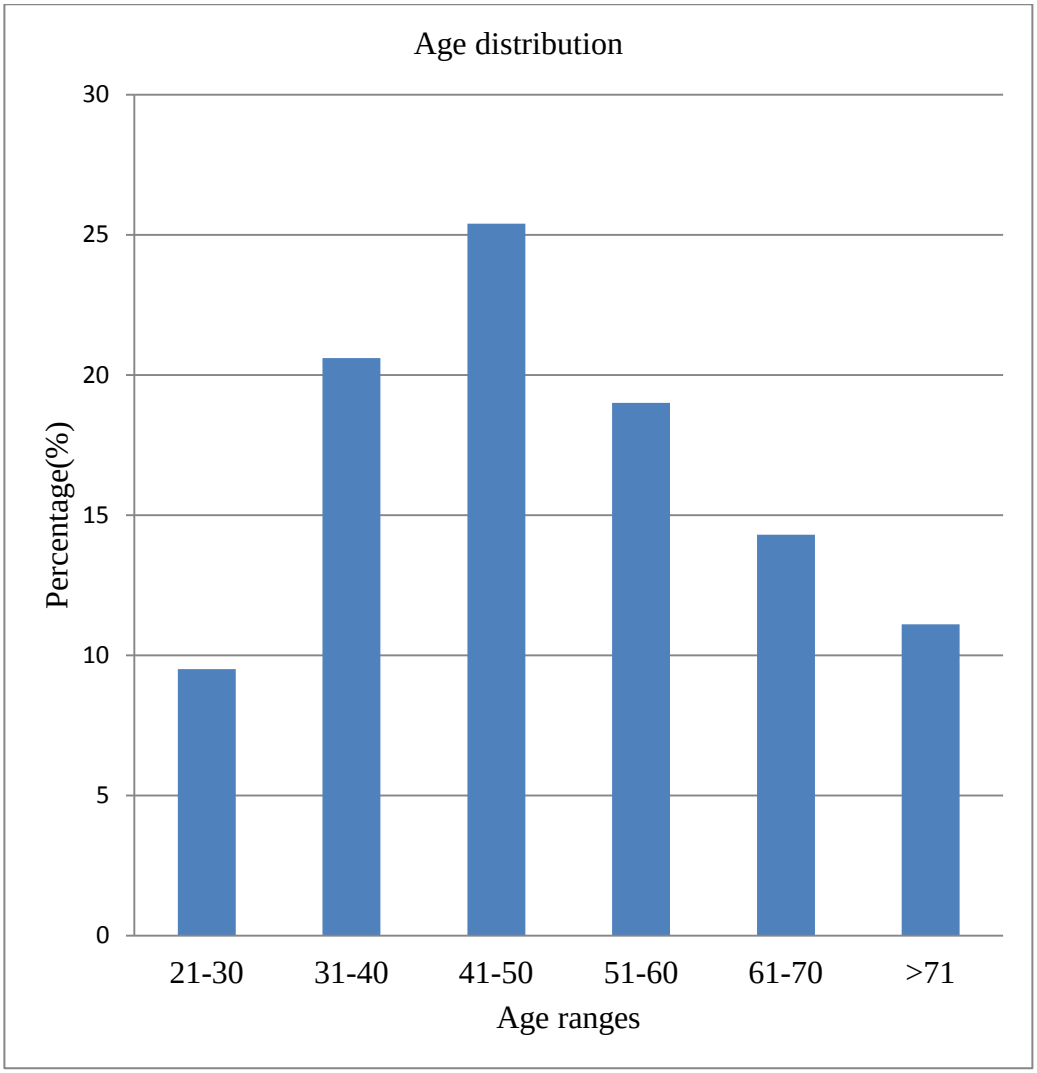

Figure 1: **Age distribution**

4.3. Histological phenotypes

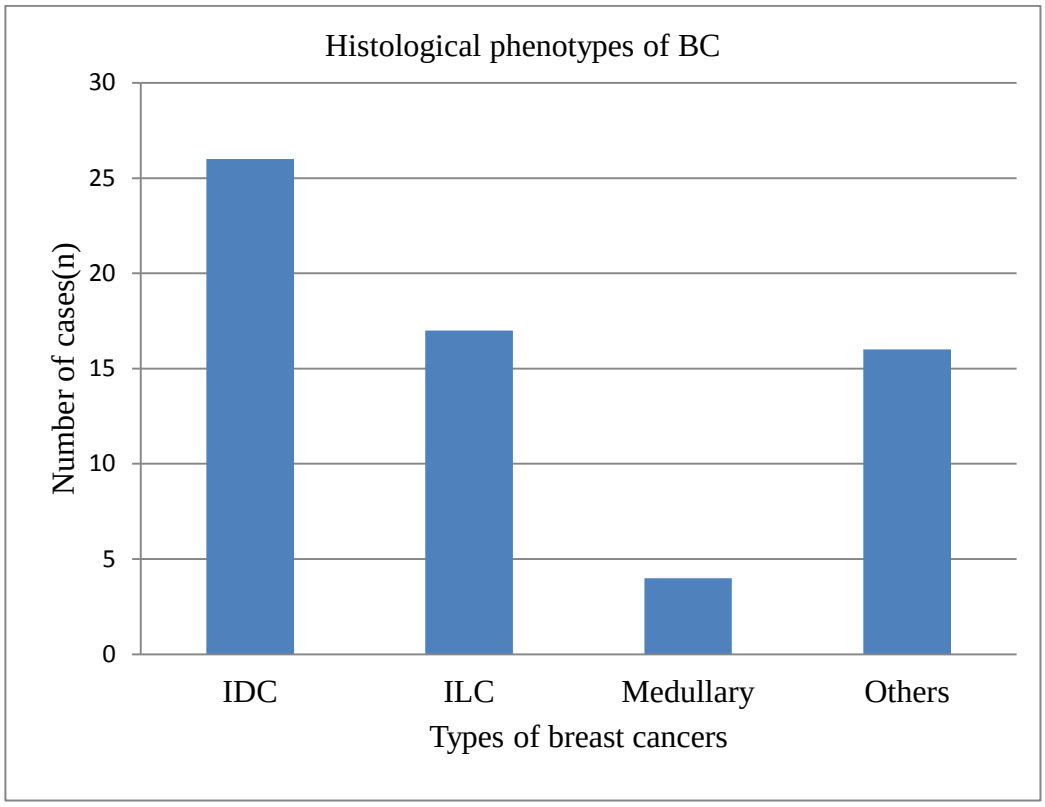

Figure 2: **Histological phenotypes of breast cancers.**

Key: IDC: Invasive ductal carcinoma, ILC: Invasive lobular carcinoma.

The figure shows that IDC was the most common breast cancer seconded by ILC.

Other rare types of BCs that were found in the study include: Ductal carcinoma combined with DCIS, Papillary intraductal carcinoma, Non-keratinizing squamous cell carcinoma, Clear cell carcinoma, Signet ring variety of mucinous carcinoma, Paget's disease and squamous cell carcinoma of surface origin ,Oncocytic carcinoma, Solitary myeloma, Malignant mesenchymal tumour and Invasive micropapillary carcinoma.

4.4 Expression of BRCA1

The expression of BRCA1 varied from one BC histological phenotype to another. Medullary carcinoma showed the highest expression of BRCA 1 with 100% retention. The results are illustrated in the figure 3 below.

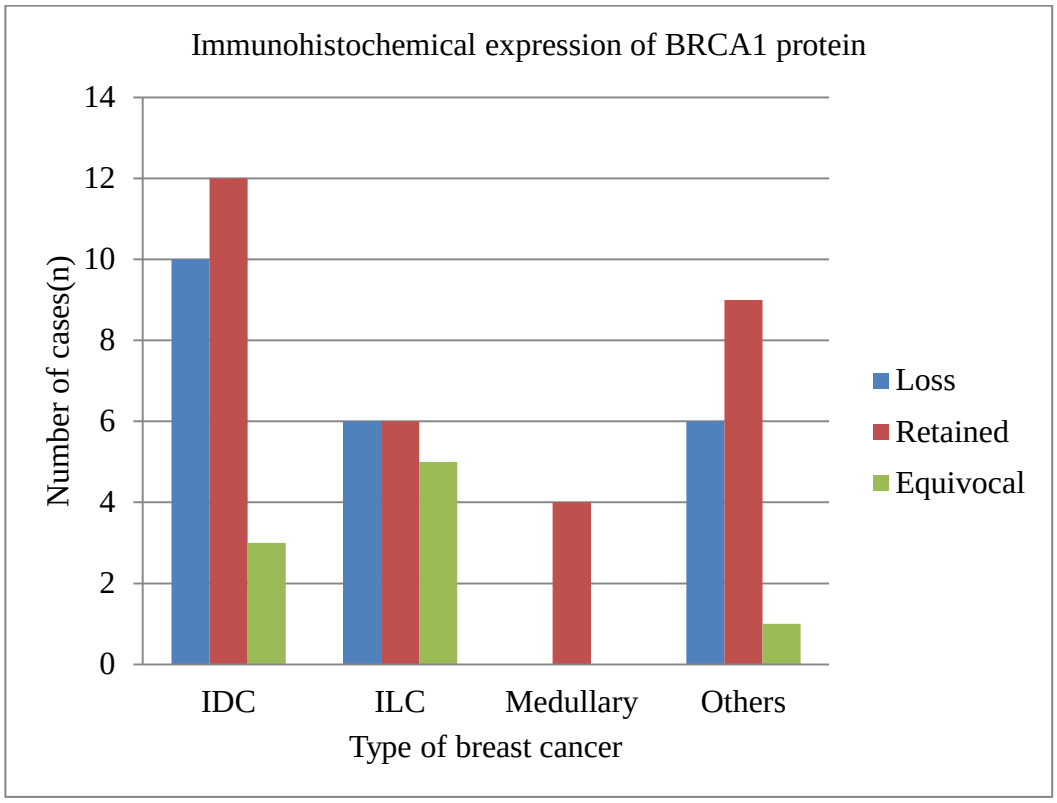

Figure 3: Immunohistochemical expression of BRCA1 protein

Key: IDC: Invasive ductal carcinoma, ILC: Invasive lobular carcinoma, BRCA1: Breast cancer gene 1.

4.5. Expression of BRCA2 protein

The expression of BRCA2 showed similar variability to that of BRCA1in terms of the immunohistochemical staining. Medullary carcinoma still showed the highest expression of BRCA 2 with 100% retention. The results are illustrated in the figure 4 below.

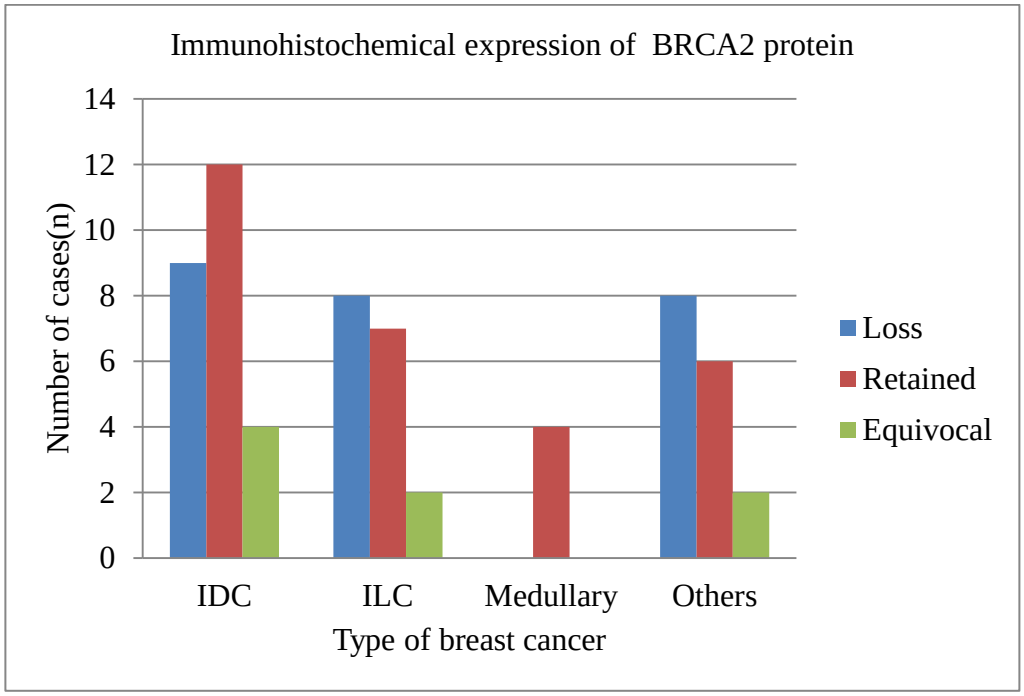

Figure 4: **Histological phenotypes expression of BRCA2 protein**
Key: IDC: Invasive ductal carcinoma, ILC: Invasive lobular carcinoma, BRCA: Breast cancer gene

4.6 Expression BRCA 1 and BRCA 2 in the other BC phenotypes

Papillary invasive carcinoma**,** Pagets Disease with squamous cell carcinoma, Solitary Myeloma, Malignant mesenchymal tumours, Non-keratinizing squamous cell carcinoma, invasive micropapillary carcinoma, Clear cell carcinoma**,** Oncocytic carcinoma**,** Signet ring variety mucinous carcinoma**,** and ductal carcinoma with ductal carcinoma in situ, comprised other breast cancer phenotypes in this study. They were 13 in total, BRCA1 was expressed in 9 of the cases, was lost in 3 of the cases and there was an equivocal reaction in 1 case. And BRCA2, was expressed in 8 cases, lost in 4 of the cases and had 1 equivocal reaction. Invasive micropapillary carcinoma was the most common among other breast cancer.

4.7. Histological grade with respect to BRCA1 and BRCA 2 protein expression

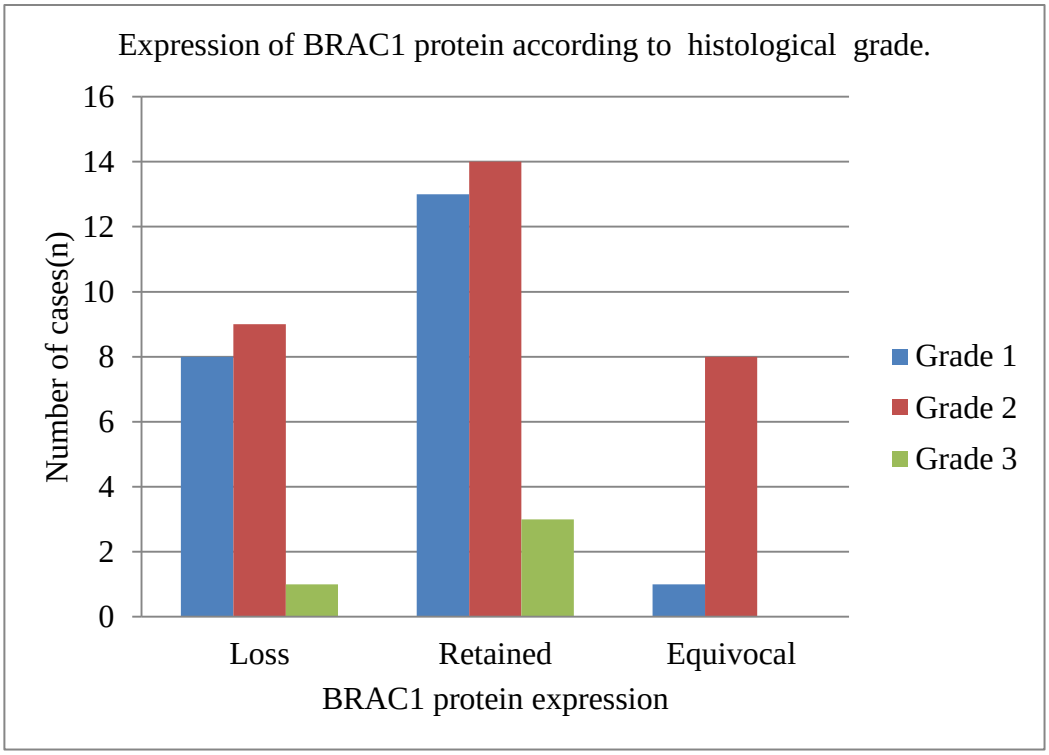

Figure 4: BRCA1 protein expression with respect to Nottingham grade.

The figure shows that grade 2 showed the highest retention of BRCA1 protein.

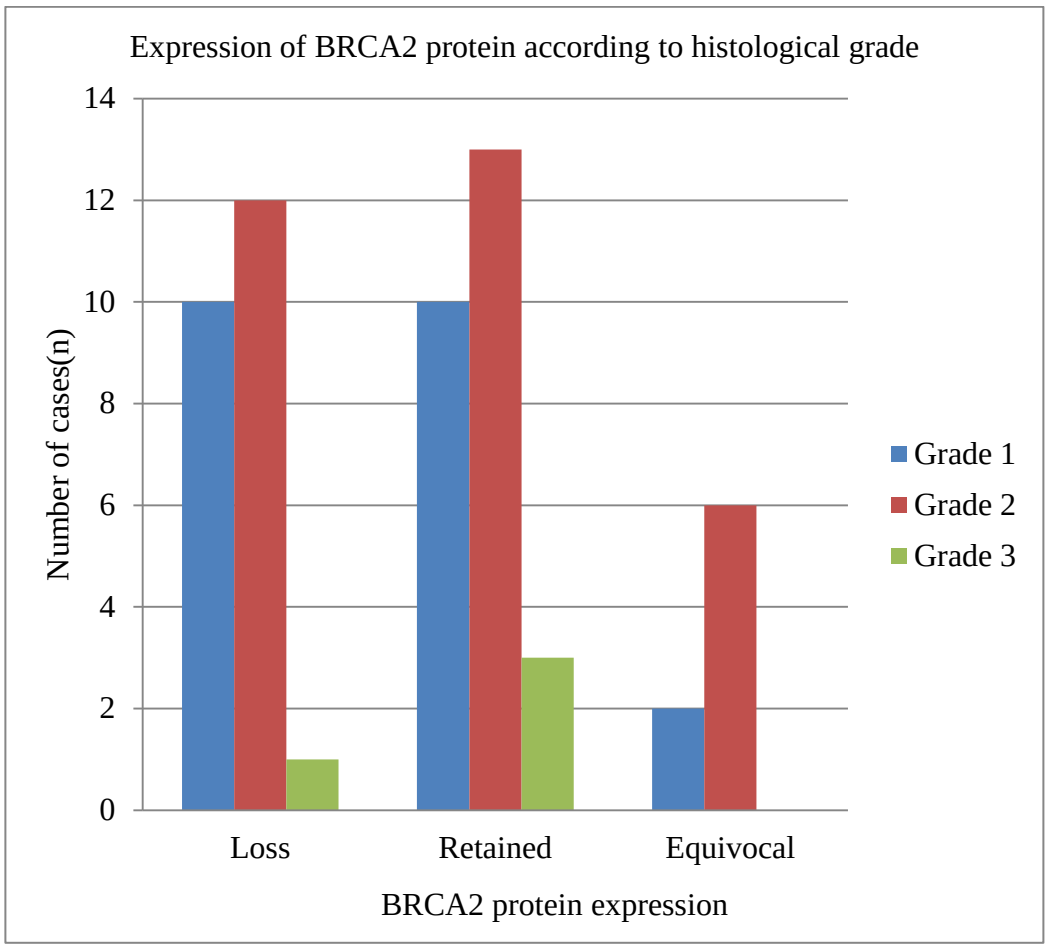

Figure 5: BRCA2 protein expression with respect to Nottingham grade.

Grade two expressed the highest retention of BRCA2 protein followed by grade one.

4.8. Clinical signs

The following were the clinical signs that cases in this study presented with; breast swelling or mass 38%, wound with palpable nodes 9%, invasive ductal carcinoma 3%, Paget's disease 2%, bloody nipple 1%, retracted nipple 2%, recurrent breast cancer 1%, suspected metastatic cancer 2%, previous mastectomy 3%. In 22% of the cases the records showed"? BC" while 13% of the cases' records had no any information and in 3% of the cases records showed "wanted to know excision margin". Over all swelling was the commonest presenting sign.

Chapter 5: Discussion.

In this study on histological phenotypes, invasive ductal carcinoma was the commonest histological phenotype and was presented by a mass or swelling in most of the cases, which were grade two at diagnosis. Young age at diagnosis was the common feature like in most African setting, majority of the cases were less than 50 years and the peak age was 41-50years.

BRCA1 and BRCA2 protein loss was noted in the below 50 years category while retention of both BRCA1 and BRCA2 protein was seen in the above 50 years category. Medullary carcinoma was the only phenotype that expressed 100% retention of both BRCA1 and BRCA2 protein, this means good prognosis for these breast cancers though they have aggressive characteristics. After chemotherapy these individuals have a longer over all survival and disease free survival than those who do not express BRCA1 and BRCA2 protein (Gachechiladzea, et al., 2013)

5. 1 Prevalence

Breast cancer is the most common cancer in women and a leading cause of cancer death worldwide (Rakha, et al., 2008) Breast cancer is one of the most common human neoplasms, accounting for approximately one-quarter of all cancers in females worldwide and 27% of cancers in developed countries with a Western lifestyle (Makki, 2015). Breast cancer occurs in ductal and lobular epithelial cells or myoepithelial cells of the mammary gland and portrays a wide scope of morphological features, different immunohistochemical profiles, and unique histopathological subtypes that have specific clinical course and outcome. Breast carcinoma is usually classified primarily by its histological appearance, originating from the inner lining epithelium of the ducts or the lobules that supply the ducts with milk (Makki, 2015). Cancers that affects the ducts are known as ductal carcinomas, while those affecting the lobules are known as lobular carcinomas.

The data for the period of three years (2012, 2013, and 2015)shown in table 3 showed an increase in the incidence of breast cancer at QECH, though these results cannot be generalized due to sample size. This agrees with findings from studies carried out in neighboring countries and by International Agency for Research on

Cancer (IARC) which observed that breast cancer incidence was on the rise in Africa (Msyamboza, et al., 2012; Mukupo, et al., 2007; Zyaambo, et al., 2013; Msyamboza, et al., 2014). In spite of this many African governments including Malawi are not ready to cope with this increase in terms of finance, infrastructure and human resource capacity.

5.2. Age distribution

Roy *et al* in her study observed that Ugandan women with breast cancer presented at a younger age when compared with American women of both European and African origins, 73% Ugandan women were 50 years or younger at diagnosis, compared to 25% of European American and 33% of African American women (Roy, et al., 2011). In this study 55.6% of the women were 50 years or below at diagnosis, which was higher than the American and European women. In the current study the mean age at diagnosis was 49.1 years; this agrees with the findings of Kendig *et al* whose mean age at diagnosis was 47 years and other African studies (Ebughe, et al., 2013; Gyorki, et al., 2012; Kendig, et al., 2013; Mbewe, et al., 2014). The biggest age group affected by breast cancer was between 41 and 50years which was 25.4% of all the age groups. IDC was the commonest histological type making 50% of all breast cancers in this age group. This was different from Kheirelseid *et al* 2011 who found that ductal carcinoma was evenly distributed across different age groups (Kheirelseid, et al., 2011). There was no statistical significant association between age and BRCA1 (p-value 0.133) and BRCA2 (p-value 0.129) protein expressions in this study may be due to the sample size used.

According to Elgali *et al*, a higher incidence of breast cancer in younger women has also been seen in women of African origin staying in United States, they correlated the short-term increase in breast cancer risk occurs in the postpartum period with premenopausal breast cancer risk and concluded that the higher prevalence of early childbearing among African-American when compared with Caucasian-American women may account for the increased incidence of early-

onset breast cancer (Elgaili, et al., 2010). In another study Palmer *et al* reported that multiparity increased breast cancer risk prior to age of 45 but offer a protective effect after age of 45 years (Palmer, et al., 2003). These factors may justify the age distribution of breast cancer in African females who normally have multiple children at a younger age.

5.3. Histological phenotypes

5.3.1. Invasive ductal carcinoma not otherwise specified (IDC-NOS) was the commonest phenotype 41.3% which was different from the study done by Kohler *et al* whose finding was 86% and other international studies were 85.2% and 82% (Kohler, et al., 2015; Ebughe, et al., 2013; Elgaili, et al., 2010).This difference might be due to the small sample size used in this study. Although IDC was still the most common BC in the Malawian women presenting at QECH. Invasive ductal Carcinoma (NOS) is known to be aggressive and heterogeneous which was reflected in this study by three cases of relapse. The other forms of breast cancer apart from invasive ductal carcinoma (NOS) are called special types; within this group others are quite rare but still well defined by the WHO classification (Yerushalmi, et al., 2009).

5.3.2. Invasive lobular carcinoma is one of the special types, which was the second most prevalent with 27% prevalence rate in this study. This finding was high compared to Yerushami *et al* finding of 15% (Yerushalmi, et al., 2009) and Kohler *et al* who got 6% (Kohler, et al., 2015). But in a study done in Zambia by Natalia *et al*, prevalence was 57% (Mbewe, et al., 2014). The different variations seen in the prevalence could be due to differences in sample sizes used in these studies, but it could point to diverse underlying risk factors that need to be investigated further.

5.3.3. Medullary carcinoma was 6.3 %, similar finding to Elgaili *et al* who found 7.3% prevalence rate in Sudanese women (Elgaili, et al., 2010). Despite its histopathologically highly malignant characteristics it has a favourable prognosis compared with the more common infiltrating ductal breast carcinoma. The

prognosis of medullary breast carcinoma is still not well known. Some studies have reported that medullary breast carcinoma survival rates do not differ from those of IDC (Santosh, et al., 2015) . There were four cases in this study they all expressed BRCA1 and BRCA2 protein expression which favours good prognosis.

5.3.4. Rare breast cancers

5.3.4.1. Malignant mesenchymal tumour comprised 1.6% of all breast cancers; overall, tumours in this group represent less than 1 % of all invasive breast tumours (Tse, et al., 2013).Malignant mesenchymal tumours are known to be aggressive in nature and refractory to all types of conventional chemotherapy and radiotherapy. Surgery is the only effective treatment (Gurleyika, et al., 2009; Cil, et al., 2008; Cleton-Jansen, et al., 2005).The case in this study presented at 48 years of age and had complete loss of both BRCA1 and BRCA2 protein expression. This finding may agree with the aggressive nature of this type of breast cancer.

5.3.4.2. Solitary myeloma is another rare cancer whose breast involvement as a solitary extra medullary myeloma is equally rare; it comprised 1.6% of all the cases .Only few cases have been reported in the literature (Kalyani, et al., 2010). Most of the time solitary myeloma may herald the recurrence of previously treated or quiescent multiple myeloma (Chiara, et al., 2001; Kalyani, et al., 2010; Roy, et al., 2011). Unfortunately there was no clinical data given in the study case to exclude a recurrence of previous multiple myeloma.

5.3.4.3. Clear cell carcinoma comprised 1.6% of all the breast cancers. This is a rare type and knowledge about the characteristics of this type is fragmentary (Yerushalmi, et al., 2009). Many authors have reported that this tumour has a poor prognosis; however this might vary depending on special characteristics such as low grade and strongly positive Estrogen Receptor expression (Yerushalmi, et al., 2009; Markopoulos, et al., 2008) . The case in this study presented at 25years with multiple breast masses and expressed complete loss of both BRCA1 and BRCA2 protein which suggested poor prognosis.

5.3.4.4. Invasive micropapillary carcinoma comprised 1.6% of all breast cancers. This is among the rare types of breast cancer with poor survival rate. Its outcome is not very much different from IDC-NOS with similar node status and it is reported that about 70% of cases have lymph node involvement (Yerushalmi, et al., 2009). In the current study no lymphnodes were submitted for analysis.

5.3.4.5. Non-keratinizing squamous cell carcinoma with DCIS was one of the breast cancers found in this study .These cancers are usually aggressive and high grade at presentation with a high metastatic potential via the hematogenous route rather than the lymphatic route (Tse, et al., 2013). The study case was grade 2 at presentation and had loss of BRCA2 protein expression which agrees with its aggressive nature. Though it retained BRCA1 expression, both BRCA1 and BRCA2 are required for normal function.

5.3.4.6. Paget's disease and squamous cell carcinoma of surface origin was one of the rare breast cancers in this study. This is an aggressive disease but has favourable prognosis, (Talei, et al., downloaded. http://dx.doi.org/10.5772/52105 ,on 09 may 2016) .This case presented at 42 years and expressed retention of BRCA1 protein and a complete loss of BRCA2 protein, both these proteins are needed for normal function.

5.3.4.7. Papillary intraductal carcinoma comprised 1.6% of the cases. It generally has good prognosis and usually occur in order women (Tse, et al., 2013; Kumar, et al., 2010). The case in this study was 67 years old and had expressed retention of both BRCA1 and protein BRCA2.The retention of both BRCA proteins suggested good prognosis.

5.3.4.8. Oncocytic carcinoma of the breast is a very rare histological phenotype of breast cancer with very scanty literature written that no significant conclusion can be made about it (Yerushalmi, et al., 2009; Makki, 2015). The case in this study expressed BRCA1 and BRCA2 protein retention which suggested good prognosis.

5.3.4.9. Signet ring variety of mucinous carcinoma commonly affects post-menopausal women (Makki, 2015), but there are conflicting literature results on its prognosis. Some studies indicate that it has good prognosis (Tse, et al., 2013) while others indicate poor prognosis (halls, 2016; Markopoulos, et al., 2008). The solitary case in this study was a 51 year old lacking clinical data. Most Malawian women attain menopause by age 50 years. This conflict might come about due to paucity of information.

5.3.5. BRCA1 and BRCA2

Breast cancer is one of the most frequent neoplasias and the second cause of mortality in women. It represents a multi-stage process, which involves alterations of the normal cellular functions (Maria, et al., 2009). Most cases of breast cancer are sporadic and do not result from a hereditary genetic predisposition. BRCA1 and BRCA2 are cancer susceptibility genes, the normal genes play a role in repairing breaks in DNA, but when mutated or supressed this repair function become disabled leading to more DNA-replication errors and cancerous growth. (Maria, et al., 2009) .

In the current study there was a complete loss of BRCA1 protein nuclear staining in breast cancer tissue specimens in 34.9% of samples; this was similar to the findings of Dinesh *et al* who found 30% loss of BRCA1 protein (Dinesh, et al., 2006). Other investigators have reported increased loss between 50-80% (Hedau, et al., 2015; Amirrad, et al., 2005). This study also found equivocal expression of the BRCA1 protein in 14.3% while Hedau *et al*, 2015 found equivocal expression in 57% and in contrast other investigators reported 34.3% reduction of BRCA1 protein (Hedau, et al., 2015; Yang, et al., 2001). These differences may be due to sample sizes used in these studies.

As the histological grade was getting poor there was reduced expression to complete loss of BRCA1 expression in the tumour cells, this was similar to Tulchin et.al who found reduced BRCA1 nuclear staining in breast tumour tissue compared

to normal tissue, and less BRCA1 staining with higher histological grade in the tumours (Tulchin, et al., 2013; Yang, et al., 2001) In many cases there was a reduced expression of BRCA1 and not changes in its subcellular location which may suggest a post–transcription or post translation suppression rather than affecting the upstream signalling of BRCA1 and functional inactivation which was also reported by Hedau *et al* (Hedau, et al., 2015). Lack of somatic mutations observed in sporadic breast carcinomas suggests that BRCA1 expression might be down regulated by mechanisms other than point mutations (Rakha, et al., 2008) . Hypermethylation of CpG rich areas located within the promoter region might be a common mechanism of silencing tumour suppressor genes (Hedau, et al., 2015; Chodosh, 1998). It was reported that mammary epithelial cells (MEC) that lack BRCA1 protein due to mutation or other epigenetic processes fail to differentiate to acinus structures but continue to proliferate (Furuta, et al., 2005).

BRCA2 protein expression was also analysed in the same breast cancer tissues and was completely lost (no expression in cancer tissues) in 39.7% of the cases. This was high compared to BRCA1 which was completely lost in 34.9 %, similar to Hedau et al who found a slight increase in BRCA2 tissue loss 52.5% when compared to BRCA1 tissue loss 50% (Hedau, et al., 2015). Most of the cases that showed loss(did not express) of BRCA1 and BRCA2 protein were in the age group of 31yrs to 50yrs; this concurs with Zakhartseva *et al* who observed that the *BRCA1*and *BRCA2* mutations or suppressions are very common in young breast cancer patients (Zakhartseva, et al., 2009). The retention of both BRCA1 and BRCA 2 proteins increased with age which agrees with finding of Malone *et al* where BRCA1 mutation frequency decreased fairly steadily with age and BRCA2 mutation frequency also decreased with age, although less dramatically (Malone, et al., 2006).

All medullary carcinoma cases expressed 100% retention of BRCA 1 and BRCA2 protein in cancer tissues in the current study, which was also reported by Tulchin *et al*, who observed that breast cancer without mutation in BRCA gene showed similar reaction in normal and cancerous tissues (Tulchin, et al., 2013) though they did not specify if it was in specific BC phenotypes. However it has been reported

that in sporadic breast cancer, BRCA1 and BRCA2 are not frequently mutated, but there is high frequencies of loss of heterozygosity on 17q12-21 and 13q12-13 respectively (Hedau, et al., 2015; Brozek, et al., 2009) which point to a significant role of these two genes in the pathogenesis of sporadic breast cancer.

Hypermethylation of cytosine residues in CpG islands within the promoter of many tumour suppressor genes is strongly correlated with the absence of gene expression. The epigenetic transcriptional silencing provides an alternative mechanism for the loss of function of tumour suppressor gene during cancer development (Hsu, et al., 2013; Baylin, et al., 1998). Hypermethylation of the BRCA1 and BRCA2 promoter has been reported in breast cancers which result to down-regulation of mRNA and protein level in tumours. In another study by Hsu *et al,* BRCA1 and BRCA2 protein expression was found to be absent or markedly reduced in most of the BRCA1 and BRCA2 methylated tumours, suggesting an epigenetic gene silencing aspect involvement (Hsu, et al., 2013; Birgisdottir, et al., 2006). This suggests that these proteins are associated with breast cancer though in the current study no significant association was observed between BRCA1 (p-value 0.388) and BRCA2 (p-value 0.647) protein expression and histological phenotypes.

5.4. Histological Grade

In this study grade 2 was the commonest grade which represented 50.8%, this finding is similar to what Hedau et al found 57.5% in India ((Hedau, et al., 2015).But different from another study done in Malawi by Kohler et al (Kohler, et al., 2015) in which they found grade 2 to be 22.3% and other studies in the region 23.5% (Ebughe, et al., 2013; Zyaambo, et al., 2013)**.** Grade 1 was 34.9% and grade 3 was 6.3%, these finding differ from most findings in African setting where grade 3 is the commonest grade due to the fact that many patients present late at the hospital (Ebughe, et al., 2013). In the current study histological grade was significantly associated with BRCA1 (p-value 0.000) and BRCA 2(p-value-0.000) protein loss, and was also significantly associated with histological phenotypes p-value 0.004. Grade was again significantly associated with young

age at diagnosis p-value 0.014. Histological grade is very important in the management and treatment of breast cancer.

Histological grade, vascular involvement, lymphnode involvement and tumor size are important parameters considered in the management and treatment of breast cancer patients. However this information was missing in most of the cases in this study.

5.5. Laterality

The right breast was the most affected side by invasive breast cancer among female Malawians. This was similar to study in Nigeria by Dauda *et al* who found that breast carcinomas occurred more on the right breast than the left breast. (Dauda, et al., 2011). It is not known whether laterality has any prognostic significance in breast cancer.

5.6. Signs and symptoms

There are different clinical presentations of breast cancer ranging from pain, palpable mass, limpness, and nipple discharge. Pain is a common symptom that may be cyclic with menses or non-cyclic. Diffuse periodic pain has no pathologic relevance and any treatment targets hormone levels. When the pain is non-cyclic normally it is localized to one area of the breast. Causes include ruptured cysts, physical injury, and infections. Literature shows that 95% of painful masses are benign though 10% of the breast cancers are painful (Kumar, et al., 2010). The common clinical sign in the current study was swelling or mass 38% which agreed with Kumar et al who found that invasive ductal carcinoma presents as palpable mass (Kumar, et al., 2010).And 22% of the cases came to the hospital in an advanced stage of breast cancer and the surgeons just wanted confirmation and to know breast cancer phenotypes and stage.

5.7. Limitations.

The sample size was small due to storage flaws at QECH. This was due to the fact that there was no SOP for receiving, processing, storage and retrieval of tissue samples.

Incomplete demographic details resulted in exclusion of cases hence a small sample size.

This study's results and findings are relevant to the local level and cannot be applied to the national level due to the small sample size.

Chapter 6: Conclusions and Recommendations.

6.1. Conclusions

The commonest histological phenotype of breast cancer in Malawian females presenting at QECH was invasive ductal carcinoma followed by invasive lobular carcinoma.

The commonest grade at diagnosis was grade two followed by grade one. The most affected age group was 41-50 years. Malawian women were more likely to suffer from breast cancer at an earlier age than their counterparts in the west. Breast cancer affected women less than 50 years.

BRCA1 and BRCA2 protein loss was noted in the below 50 years category while retention of both BRCA1 and BRCA2 protein was in above 50 years category. Medullary carcinoma expressed 100% of both BRCA1 and BRCA2 protein, this means good prognosis for these breast cancers. They respond very well to chemotherapy and have a long disease free and overall survival.

6.2. Recommendations.

6.2.1. Conduct a study with a large sample size to help determine the underlying cause of the increase in breast cancers in young female Malawian population.

6.2.2. Introduce and sustain immunohistochemistry diagnostic assays with more panels of antibodies to improve the diagnosis, treatment and management of breast cancer patients.

6.2.3. Strengthen breast cancer preventive programs at local and national levels in Malawi. Early detection is associated with decreased mortality therefore it is important to minimize delays in detection and diagnosis.

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Chapter 8: Appendices

8.1. (A) Microtomy procedure.

1. **Purpose**

To manually create sections of specimens of varying thickness for use in routine histopathology procedures

1. **Principle**

Microtomy is dependent on the rotating of the microtome thereby cutting the specimens blocks into sections. The desired thickness of the sections is achieved by manipulating the clearance angle, the adjusting knob and the scale.

1. **Equipment, Reagents, Supplies, Personal Protective Equipment (PPE)**

Equipment	Reagents	Supplies	PPE
Microtome Water bath	NA	Microtome blades Tissue forceps Frosted slides Cleaning brush Sharps box	Lab Coat Safety Goggles (where necessary)

1. **Specimen**

Formalin fixed, paraffin embedded blocks.

1. **Safety precautions**

Refer to the UTH Laboratory Safety Manual (QMS-SFT-v1) and the Histopathology Safety handbook (HIS-SFT-v1) for details.

Only experienced personnel should use the microtome
a) Always observe the correct resting angle of the instrument to the table to avoid pinching your fingers.
b) Make sure that all the necessary parts and tools are assembled before

	attempting to use the instrument.
c)	Always clamp the specimen block before clamping the knife or changing the specimen block and during all work breaks. The hand wheel must be locked prior to any manipulation of the knife.
d)	Be very careful when handling microtome blades. The cutting edge is extremely sharp and can cause severe injury.
e)	Always turn the hand wheel evenly in clockwise direction; otherwise the brake will not work properly. The rotation speed of the hand wheel must be adapted to sit the hardness of the specimen.
f)	Take care not to block the coarse driving wheel when turning the hand wheel otherwise there will be no feedback motion of the section thickness and thus no sectioning will occur.

1. Calibration procedures

Always ensure that the Microtome stage is well aligned and the desired thickness to be section is selected before use.

Step	TRIMMING
1.	Set the blocks on the cold plate. (Only large tissue can be trimmed first before sectioning)
2.	Insert a specimen block into the specimen clamp/holder. (Clean the Microtome prior to this as stipulated in the HIS-TECH-037v1)
3.	Carefully insert disposable blade into the knife holder and clamp making sure that the knife is clamped parallel to the upper edge of the pressure plate.
4.	Advance the block until it touches the knife block.
5.	Orient the position of the specimen surface (only in the case of specimen holders that can be oriented).
6.	Release the hand wheel lock or brake respectively
7.	Using the trimming lever selects the required trimming stage.

	Trimming is done at 10 -15 microns
8.	Begin the cutting process by turning the hand wheel.
9.	Trim the specimen block until the required specimen level has been reached.
10.	Let go of the trimming lever, clump the hand wheel lock and remove the specimen block from the specimen clamp and put it back on ice cold surface for further cooling. Tissue blocks are cooled for 1-3 minutes before they can be sectioned.
SECTIONING.	
11.	Mount a well-trimmed and cooled specimen block into the specimen holder.
12.	Install a new blade or proceed with an unused area of the blade for sectioning.
13.	Set the required section thickness or check the value setting on the display respectively. Section cutting is done at 3-5 microns
14.	For sectioning, turn the hand wheel evenly in a clock wise direction ensuring that evenly sized section are produced. Ideally successive sections will stick edge to edge due to local pressure with each stroke forming a ribbon.
15.	Lock the hand wheel, pick up the sections using a forcep and place the sections in the water bath ensuring that the shiny surface faces down.
16.	Float the sections in the water bath.

1. **Procedure Step-by step**

2. **Quality Control**

    a. Standardize all the steps involved e.g. the temperature of the block when it is cut and the mode of cooling should be standardized to give a similar consistency

b. Never use a blunt bade for sectioning as this may injure the tissue.

c. Always refer to the document EXT-DOC-002v1 for troubleshooting on Microtomy for paraffin sectioning to solve problems encountered during Microtomy.

d. Tissue specimen is examined after staining for the quality of section and thickness.

2. Calculation of results

N/A

3. Reference range/Test Interpretation

N/A

4. Alert/critical values, where appropriate

N/A

5. Notes, Limitations and Anything Else

a) Practical experience necessary to manipulate the microtome is gained under the guidance of the skilled tutor.

b) It is possible to damage the tissue by gouging or scoring when trimming the block.

c) Blocks should be arranged in a numerical order for smart work.

d) Over cooling of the block may cause tissue distortion and expansion.

e) Always cut the sections at the required thickness.

f) Cutting speed and length of ribbon must also be standardized.

6. **Reference**

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8.2. (B) Hematoxylin and Eosin (H&E) Staining Protocol.

Principle

The oxidation product of haematoxylin is haematin, and is the active ingredient in the staining solution. Haematoxylin is not classified as a dye since the molecule possesses no chromophore. The *in situ* oxidation of haematoxylin is effected by the addition of a strong oxidant to the stain, in this case sodium iodate.

Haematin exhibits indicator-like properties, being blue and less soluble in aqueous alkaline conditions and red and more soluble in alcoholic acidic conditions. In acidic conditions, haematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant, in this case aluminium. To ensure saturation of chemical binding sites, the stain is applied longer than necessary, resulting in the over staining of the tissues with much non-specific background colouration. This undesirable colouration is selectively removed by controlled leaching in an alcoholic acidic solution, (acid alcohol), the process being termed "differentiation". Differentiation is arrested by returning to an alkaline environment, whereupon the haematin takes on a blue hue, the process of "blueing-up". The haematin demonstrates cell nuclei.

Full cellular detail is obtained by counterstaining with the eosin mixture. Colour enhancement is achieved by fortifying the stain with phloxine, a chemical member of the same family as eosin (halogenated fluorosceins). The mechanism of their staining is not fully understood, but is believed to be of an electrostatic nature. Visualisations most acceptable to the histologist are obtained by applying the dyes in acidic conditions, whereby more intense specific colourations are obtained, the more acidic tissue components taking up the dye to a greater intensity, hence the addition of acetic acid.

Technical Points

1. (step 2) - The length of time necessary to over-stain the tissues will depend upon fixation and the type of alum haematoxylin employed. Lillie Mayer's alum haematoxylin-formalin fixed tissues should take 5 minutes.

Tissue Type	Haematoxylin	Acid alcohol 0.3%	Eosin	Comment
Routine tissues	4 minutes	See technical point 2	2 minutes	
Renal biopsies	10 minutes	1-2 seconds	2-4 minutes	Check staining
Decals	10 minutes	1-2 seconds	30 seconds	Check staining after blueing. Hx step may need to be repeated if prolonged decal.

2. (Step 4) - Differentiation with acid alcohol requires some practical experience to ascertain the correct end-point, since the acid solution alters the colour of the tissue to red. The correct end-point is when, after blueing up, the background is almost colourless. For renal biopsy sections, two quick dips in 0.3% acid alcohol are all that is required

3. (Step 6) - If Scott's tap water substitute is employed; blueing up is achieved in a much shorter time.

4. (Step 8) - Eosin is highly soluble in water. Over-staining is removed by washing in running water.

5. Fixation - Not critical. Acidic fixatives will give a more eosinophilic result. Picric acid containing fixatives give an overall enhanced result. Acidic decalcifying fluids give poor nuclear staining.

6. Renal biopsies - 10% buffered formalin. Sections cut at 2 micrometers

Method

1. Bring sections to distilled water
2. Stain nuclei with the alum haematoxylin (see note)
3. Rinse in running tap water
4. Differentiate with 0.3% acid alcohol (see note)
5. Rinse in running tap water
6. Rinse in Scott's tap water substitute (see note)
7. Rinse in tap water
8. Stain with eosin 2 mins
9. dehydrate, clear and mount.

Results

Collagen.....pale pink

Muscle.....deep pink

Acidophilic cytoplasm.....red

Basophilic cytoplasm.....purple

Nuclei.....blue

Erythrocytes.....cherry red

Reagent Formulae

Lillie Mayer alum haematoxylin

aluminum ammonium sulphate ----- 200 g

hematoxylin (CI 75290) ---------- 20 g
ethanol ---------- 40 ml
sodium iodate ---------- 4 g
acetic acid ---------- 80 ml
glycerol ---------- 1200 ml
distilled water ---------- 2800 ml

In a 4L Ehrlenmeyer flask, to 1000 mls of the distilled water add the aluminium ammonium sulphate. Place the flask on a heater/stirrer, turn on the heater and allow mixing until the alum dissolves - this takes about 15 minutes. Remove the flask from the heater/mixer, allow cooling, and then adding the remaining 1800 mls distilled water - this will further cool the solution. Add the haematoxylin powder to the alcohol and dissolve as much of the powder as possible by shaking for a few minutes. Pour the strong alcoholic solution of haematoxylin into the cooled alum solution and stir to ensure all the powder is dissolved, preferably overnight. Add the sodium iodate, acetic acid, and finally the glycerol. Mix well, plug loosely and store.
It is appropriate to make up a batch of the required amount, dependent upon the usage rate.

2. Acid alcohol 0.3% Acid Alcohol

commercial grade ethanol ----- 2800 ml
distilled water ---------- 1200 ml
conc. hydrochloric acid --------- 12 ml

In a sufficiently large container, add the acid to the water, then add the alcohol and mix thoroughly. The generation of fine bubbles is an indication that mixing is thorough.

3. Scott's tap water substitute

sodium hydrogen carbonates --- 10 gm.
magnesium sulphate ---------- 100 gm.
distilled water ---------- 5 L

Dissolve the salts in the water.

Store stock solutions at room temperature.

4. alc. acetified eosin/phloxine TQEH

1% eosin Y (CI 45380) ---------- 400 ml

1% aqphloxine (CI 45405) ----- 40 ml

95% alcohol ---------- 3100 ml

gl acetic acid ---------- 16 ml

Mix the above reagents together, and stir well. The solution keeps well.

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8.3. (C) Immunohistochemistry staining procedure

Slide preparation

1. Cut 3-4µm tissues sections and pick up on positively charged adhesive slides.

2. Heat-fix sections to the slides for 1hr at 56 - 60°C on a hot plate or in a heat incubator.

3. Store slides at room temperature away from dust if not staining immediately, or in the fridge if storing for longer than a week (to preserve labile antigens).

Reagent Preparation

1. **Target Retrieval Solution, High pH (50x) or Low pH (x50)**

Diluted 1:50 with dH2O

Each 30ml bottle makes up 1.5L

Diluted retrieval solution can be kept in the fridge for up to 1 month

The retrieval solution can be used up to 3 times before discarding

1. **Wash Buffer (20x)**

Diluted 1:20 with dH2O

Each 1L bottle makes up 20L

Diluted wash buffer can be kept in the fridge for up to 1 month

Use fresh wash buffer for each rinsing step

1. **DAB+ Chromogen and Substrate Buffer**

Chromogen is diluted 1:20 with Substrate Buffer

1 drop (50ul) of DAB+ Chromogen per 1ml of Substrate Buffer

Make up as much as is needed for the day preferably

Diluted DAB+ can be kept in the fridge and used within 5 days

Discard if solution turns brown or forms a precipitate

1. **Primary antibodies**

Dilute primary antibodies with citrate buffer

BRCA1Antibody (MS110) 1:50 dilution

BRCA2 Antibody 1:200 dilution

Target Retrieval Procedure – in waterbath/heat incubator/steamer:

1. Deparaffinize and hydrate sections to water

2. Preheat Target Retrieval Solution (working solution) to 95 - 99 °C in a suitable glass, metal or plastic container with loosely fitted lid or lid with a small hole in to stabilize the temperature and avoid evaporation, but prevent too much pressure from building up.

Note: It may take at least an hour or more for the Target Retrieval Solution to reach this temperature, but it is very important that it does reach a temperature just below

boiling point. The temperature of the water bath or incubator usually would need to be set a few degrees higher than the preheat temperature needed. In may be necessary to weigh down the container if using a water bath as smaller or lighter containers may float or tip. Ensure no water from the water bath enters the container being used.

3. Immerse the room temperature sections in the preheated Target Retrieval Solution.

4. Incubate for 20 minutes at 95-99 °C

5. Remove the container of slides from the water bath / incubator and allow slides to cool in the Target Retrieval Solution for 20 minutes at room temperature.

6. Remove slide rack from container (or decant the Target Retrieval Solution if in a coplin jar) and rinse sections in a container with cold running tap water immediately to prevent drying out of tissue.

7. Place the rack with rinsed sections in Wash Buffer working solution at room temperature for 1–5 minutes.

8. Step 1 of the staining procedure will begin hereafter

NB: At no point after retrieval can slides be allowed to dry out, staining will be severely affected!

Staining Preparation

1. Prepare a humidified staining chamber for the staining procedure steps to prevent drying out of slides, as a minimum, stain in an area that avoids drafts from outside or from air-conditioning from reaching the slides.

2. Handle slides one by one: remove slides from the buffer, flick to disperse buffer without letting the tissue dry out, and outline tissue with a hydrophobic IHC pen, then place the slide on a staining rack (or where it will be stained) and immediately cover tissue with wash buffer

Staining Procedure:

For each step below, tip off wash buffer, flick slide to disperse buffer and add sufficient reagent to cover the tissue section

Reagent	Time
1. Peroxidase blocking solution	5 minutes
2. Wash buffer – working solution	Rinse well
3. Primary Antibody	45 minutes
4. Wash buffer solution	Rinse
5. Horse Radish Peroxidase(HRP)	20 minutes
6. Wash buffer	Rinse well
7. Diaminobenzidine(DAB)+ working solution	5minutes
8. Tap water	Rinse well
9. Counter stain with Heamatoxylin	Determined by strength ~1 minute(light counter stain only)
10. Blue Heamatoxylin: running tap water	Until blue, 5 minutes
11. Dehydrate ,Clear and Mount	

Quality Control

Each staining run should include a known positive control specimen to ascertain a proper performance of all the applied reagents. If the positive control specimen fails to demonstrate positive staining, labelling of test specimens should be considered invalid.

A negative control reagent should be used with each specimen to identify any non-specific staining. If non-specific staining cannot be clearly differentiated from the specific staining, the labelling of the test specimen should be considered invalid.

Interpretation of Results

The diaminobenzidine-containing Substrate Working Solution gives a brown colour at the site of the target antigen recognized by the primary antibody. The brown colour should be present on the positive control specimen at the expected localization of the target antigen. If non-specific staining is present, this will be recognized as a rather diffuse, brown staining on the slides treated with the negative control reagent. Nuclei will be stained blue by the hematoxylin counterstain.