

**Alterations in Immunohistochemical Biologic Markers Following Neoadjuvant Chemotherapy
in Breast Carcinoma**

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partial fulfilment of the degree of Masters of Medicine in Anatomical Pathology

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DEDICATION:

For my family

DECLARATION:

I, Dr Michael Jared Bromfield (0300696e) am a student registered for the degree of Masters of Medicine (Anatomical Pathology) in the academic years 2014 to 2020.

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ABSTRACT:

BACKGROUND:

Neoadjuvant chemotherapy is an alternate treatment modality in which patients receive treatment before surgery. The use thereof in patients with breast carcinoma is not without practical and therapeutic implications. Neoadjuvant chemotherapy may allow for the in vivo selection of resistant tumour cells. In addition, neoadjuvant chemotherapy may cause an alteration in the status of hormone receptors and human epidermal growth factor receptor 2 (HER2). Although the impact of primary chemotherapy has generated considerable literature, studies continue to report conflicting results. This report is the first from South Africa and provides valuable insight into the need for repeat testing in a resource constrained setting.

OBJECTIVES:

The aim of this study was to determine the effect of neoadjuvant chemotherapy on biologic marker status in patients with primary invasive breast carcinoma.

METHOD:

A non-interventional, cross-sectional, retrospective and comparative analysis was performed. Data was collected over an 18-month period between 1 January 2014 and 30 June 2015 from the databases of the department of Anatomical Pathology and the Breast Unit in the department of Surgery at Chris Hani Baragwanath Academic Hospital. Biopsies and the corresponding post-primary chemotherapy surgical excision specimens from seventy-four patients were investigated for any change in hormone receptor status, HER2 receptor status and Ki67.

RESULTS:

Three hundred and seventy-three patients were newly diagnosed with an invasive mammary carcinoma. Invasive carcinoma of no special type was the most frequent diagnosis (86%) with 320 cases identified. Seventy-four patients (20%) underwent neoadjuvant chemotherapy followed by

excision biopsy. Pathological complete response was identified in 7 patients (9%). In patients with residual post-primary chemotherapy tumours, significant reductions in progesterone receptor expression ($p=0.003$) and Ki67 proliferation index ($p<0.001$) were found when compared with the needle core biopsies. No significant alterations were identified for oestrogen receptor ($p=0.08$) and HER2 receptor ($p=0.655$) expression.

CONCLUSION:

Hormone receptor status should be re-evaluated in the surgical excision specimen following neoadjuvant chemotherapy, as there can be an alteration in biomarker status that may impact treatment strategy. In current practice, with available resources limited, the repeat testing of HER2 may be of little value in the absence of neoadjuvant trastuzumab.

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LIST OF ACRONYMS / ABBREVIATIONS:

CA – Cancer

AJCC – American Joint Committee on Cancer

HER2 – Human Epidermal Growth Factor Receptor 2

ER – Oestrogen receptor

PR – Progesterone receptor

WHO – World Health Organisation

NHLS – National Health Laboratory Services

CHBAH – Chris Hani Baragwanath Academic Hospital

SANAS – South African National Accreditation System

IGF – Insulin-like Growth Factor

ERBB2 – v-erb-b2 avian erythroblastic leukaemia viral oncogene homolog 2 gene

MAPK – Mitogen-Activated Protein Kinase

PI3K – Phosphoinositide 3-kinase

PKC – Protein Kinase C

DCIS – Ductal carcinoma in situ

STAT1 – Signal Transducer and Activator of Transcription 1

EGFR – Epidermal Growth Factor Receptor

CAP – College of American Pathologists

ASCO – American Society of Clinical Oncology

UICC – Union for International Cancer Control

PCR – Pathologic complete response

FISH – Fluorescence in situ hybridisation

NCCN – National Comprehensive Cancer Network

RCPA – Royal College of Pathologists of Australasia

CHAPTER 1: INTRODUCTION

Breast carcinoma (CA) is the most common malignancy in women worldwide with over 1.6 million cases diagnosed in 2012 (Ferlay et al. 2015).

The outcome for women with breast CA varies considerably. A number will have a normal life expectancy, while those with metastatic disease may only expect a 5-year survival of 24%. Prognosis is dependent upon tumour extent (tumour burden), the underlying tumour biology and the age and health of the patient (American Cancer Society 2014).

Various combinations of factors related to the tumour (tumour burden) are used by the American Joint Committee on Cancer (AJCC) to separate breast CA into five stages (0 to IV) that correlate with overall patient survival. These factors include: “invasive carcinoma versus in situ disease, distant metastasis, lymph node metastasis, tumour size, locally advanced disease, inflammatory carcinoma and lymphovascular invasion” (Egner 2010).

Clinically, it is assumed that the response of a tumorous lesion to chemotherapy is a reflection of all the tumour cells and that tumour cells are clonal. Breast CA is however, characterised by its cellular heterogeneity (Sørli et al. 2001). Therefore, of late, knowledge gained by studying the molecular biology of breast CA has improved outcome prediction. Factors related to tumour biology include: molecular subtype, histologic subtype, histologic grade, proliferative rate, hormone receptor status, Human Epidermal growth factor Receptor 2 (HER2) status and reaction to neoadjuvant chemotherapy.

Neoadjuvant chemotherapy is an alternative approach in which patients are treated with cytotoxic agents before surgery. It is used to reduce tumour size and improve surgical prospects. In addition, it adds valuable information regarding tumour response and long-term disease free survival (Kaufmann et al. 2006). The use of neoadjuvant chemotherapy however, has raised many concerns. Its use may

allow for in vivo selection of resistant tumour cells (van de Ven et al. 2011). Histologic changes may influence the tumour grade. In addition, neoadjuvant chemotherapy may alter hormone or HER2 receptor status.

In a local case series, McCormack and colleagues showed that breast CA in all racial groups within Soweto, Johannesburg were predominantly oestrogen receptor (ER) positive (McCormack et al. 2013). Although the demographics have been described, to our knowledge, an alteration in biologic markers following neoadjuvant chemotherapy has not yet been reported within South Africa.

The prevalence of biomarker alteration will be assessed with this study. If this study shows no modification in biomarker status, then this may obviate the need to repeat investigations with a potential financial implication. Alternatively, a negative-to-positive alteration in hormonal status may influence treatment strategy and identify those who may benefit from endocrine therapy.

CHAPTER 2: AIMS AND OBJECTIVES

The aim of this study was to determine the effect of neoadjuvant chemotherapy on hormonal and HER2 receptor status in patients with primary invasive breast CA.

The objectives of this study were to:

- Identify any change in hormonal and HER2 receptor status following neoadjuvant chemotherapy.
- Compare pre- and post- chemotherapy morphology and assess for any change in histopathologic grade and proliferative index (Ki67).
- Assess tumour response to neoadjuvant chemotherapy.

CHAPTER 3: LITERATURE REVIEW

3.1 The Tumour Biology of Breast Carcinoma:

3.1.1) Hormone receptor status:

Guidelines recommend routine measurement of both ER and progesterone receptor (PR) status in patients diagnosed with breast CA. Knowledge of the biomarker status is critical for breast CA management as endocrine therapy offers considerable benefit to patients with a positive receptor status (Fisher et al. 1983).

3.1.1.1) Oestrogen receptor:

Oestrogen is a steroid hormone that influences the regulation of transcriptional processes involved in growth, differentiation and function of various tissue within the body, including the breast. Depending on menopausal status, oestrogen is produced primarily by the ovaries in the form of 17 β -oestradiol, oestrone and oestriol (Hammond et al. 2010). These hormones facilitate their biological effects through binding of nuclear receptors ER- α and ER- β via the classical steroid receptor mechanism (Björnström and Sjöberg 2005). Although studies have shown ER- β to be expressed in breast CA, its physiologic and pathophysiologic role remains obscure (Roger et al. 2001).

ER- α was the first receptor subtype identified in the breast and its biological role has been best studied. Once stimulated, transcription is enhanced once the receptors dimerise and fix to “oestrogen response elements” located in promotor regions of target genes (Nilsson et al. 2001).

ER- α promotes the proliferation of breast CA through directed expression of the “insulin-like growth factor” (IGF) system. Oestrogens are believed to sensitise breast CA cells to the proliferative effects of IGF. Thus, exposing the modulatory role steroids have in determining the response of tumour cells to growth factors (Stewart et al. 1990).

3.1.1.2) Progesterone receptor:

The biological effects of progesterone are appreciated primarily within the breast and uterus. During gestation, progesterone causes widespread epithelial proliferation and differentiation of alveolar lobules within the breast. The effects of progesterone are interceded by receptors PR-A and PR-B (Conneely, Jericevic, and Lydon 2003). Similarly, these receptors facilitate their biological effects through the classical steroid receptor mechanism and once stimulated, adhere to “progesterone response elements” located on promoter regions of target genes.

The functional role of PR in carcinogenesis is complex. The activation of PR by progesterone in patients who have their first pregnancy at an early age, has a vital role in protection against mammary CA. When stimulated by synthetic progestins in hormone replacement therapy; however, PR activation contributes towards breast tumour formation (Chlebowski et al. 2010).

The expression of PR is oestrogen dependant and suggestive of an intact ER signalling pathway (Horwitz and McGuire 1978). As such, it is frequently absent in the majority of ER-negative tumours (Dowsett et al. 2005). In patients with ER-positive CA, PR is valuable for prognostication of disease recurrence and outcome. A worse prognosis is documented in patients with ER-positive / PR-negative CA when compared to patients with PR-positive CA (Clark et al. 1983). Metastasis is 3.6 times less probable in patients with PR-positive lesions (Pichon et al. 1980). In addition, patients with ER-positive / PR-positive CA show greater improvement from endocrine therapy (Bardou et al. 2003). A similar benefit from endocrine therapy is documented in patients with metastatic breast CA (Ravdin et al. 1992).

3.1.1.3) Quantitative assessment of hormone receptor status:

The application of immunohistochemical stains to detect hormone receptor and HER2 expression has become standard histopathological practice in breast CA patients. ER and PR status is assessed through quantitative measurement of receptor expression using the Allred score (Allred et al. 1998; Harvey et al. 1999).

Table 1 - Quantitative assessment of receptor status:

The Allred Score (Allred et al. 1998; Harvey et al. 1999):

Proportion Score:	Positive nuclear staining (%):	Intensity:	Intensity Score:
0	0	None	0
1	<1	Weak	1
2	1 - 10	Intermediate	2
3	11 - 33	Strong	3
4	34 - 66		
5	67 – 100		

The sum of the intensity score and proportion score give a total score with eight possible values.

“Scores of 0 and 2 are considered as negative (See Image 4 and 6). Positive staining (See Image 3 and 5) is defined as a score between 3 and 8” (Harvey et al. 1999).

3.1.2) Human Epidermal Growth Factor Receptor 2 (HER2) status:

HER2 is an oncoprotein belonging to the human “epidermal growth factor receptor” (EGFR) family.

This family of receptors has a significant role in embryonic development, tissue renewal and carcinogenesis. Within normal breast development, the major contributions of the human EGFR transpire through puberty, pregnancy and lactation. In this period, signalling stimulates growth of mammary epithelium and ductal differentiation (Eccles 2011). Similarly, the human EGFR regulates cell growth, survival and differentiation in the pathogenesis of multiple malignancies (Iqbal 2014).

There are four receptors within the family: HER-1, HER-2, HER-3 and HER-4. Each receptor is composed of a “cysteine-rich extracellular domain, a single transmembrane domain and a large cytoplasmic domain” (Riese and Stern 1998).

HER2 has no recognised triggering molecule and as such may exist in an innate stimulated state. Alternatively, HER2 may be activated by dimerization with other members of the EGFR family. On activation, tyrosine kinase domains and tyrosine residues within the cytoplasmic domain become phosphorylated. These activate second messengers including “mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5 bisphosphate 3-kinase (PI3K), and protein kinase C (PKC)” (Riese and Stern 1998; Iqbal 2014).

The HER2 gene was first identified in human breast CA as a v-erbB-related gene (King, Kraus, and Aaronson 1985). It has subsequently been recognised by several names including “NEU; NGL; TKR1; CD340; MLN 19 and HER2/neu” however, “ERBB2” and “erb-b2 receptor tyrosine kinase 2” are the official symbol and name given by the HUGO Gene Nomenclature Committee. The HER2 oncoprotein is encoded by the v-erb-b2 gene on chromosome 17 (NCBI 2019).

ERBB2 gene amplification is hypothesized to occur early in breast carcinogenesis. It is identified more frequently in ductal carcinoma in situ (DCIS) when compared with invasive ductal carcinoma (Park et al. 2006). In addition, overexpression of the HER2 oncoprotein in benign breast ductal epithelial cells can cause acinar proliferation and loss of polarized organisation, characteristic of the premalignant phase of breast CA (Muthuswamy et al. 2001). HER2 status is maintained with disease progression, invasion and metastasis (Park et al. 2006).

Between fifteen and twenty percent of invasive breast CA overexpress the HER2 oncoprotein (Burstin 2005). Overexpression of HER2 is an adverse prognostic factor and is related to a shorter disease free and overall survival (Slamon et al. 1987).

Agents that target HER2 (e.g. Trastuzumab - Herceptin®) have been shown to be particularly effective. Trastuzumab is a “humanized monoclonal antibody”. When used in isolation or in combination with chemotherapy, HER2 directed treatment with trastuzumab improves response rates, duration of response, time to progression and survival in patients with metastatic breast CA (Slamon

et al. 2001). In addition, trastuzumab significantly improves disease-free survival and reduces mortality in patients with HER2 positive breast CA (Piccart-Gebhart et al. 2005; Romond et al. 2005). It is essential therefore, that all patients diagnosed with invasive breast CA have HER2 status assessed.

In order to ensure accuracy, the College of American Pathologists (CAP) and American Society of Clinical Oncology (ASCO) have recommended guidelines for HER2 testing and interpretation.

Table 2 - Reporting results of HER2 receptor testing by immunohistochemistry and in situ hybridization (Wolff et al. 2007, 2013):

Negative HER2 status:	
- <i>Immunohistochemistry:</i>	- Score of 0 or 1 for cellular membrane expression. - Score 0 is no staining observed or incomplete, faint/barely perceptible membrane staining in $\leq 10\%$ of invasive tumour cells (Image 8). - Score 1 is incomplete, faint/barely perceptible membrane staining in $>10\%$ of invasive tumour cells.
- <i>In situ hybridization:</i>	- Single-probe assay average HER2 copy number < 4.0 signals/cell. - HER2/CEP17 ratio < 2.0 AND average HER2 copy number < 4.0 signals/cell
Equivocal HER2 status:	
- <i>Immunohistochemistry:</i>	- 2+ score is defined as incomplete membrane staining that is weak/moderate, circumferential in $>10\%$ of tumour cells or intense complete circumferential membrane staining $\leq 10\%$ of tumour cells.

Positive HER2 status:	
- <i>Immunohistochemistry:</i>	- Score of 3+ is intense, complete circumferential membranous staining that occurs in >10% of invasive tumour cells (Image 7).
- <i>In situ hybridization:</i>	- Single-probe average HER2 copy number ≥ 6.0 signals/cell - Dual-probe HER2/CEP17 ratio ≥ 2.0 with an average HER2 copy number ≥ 4.0 signals per cell.

3.1.3) Pre-analytical factors affecting biomarker assessment:

In addition to the guidelines used for interpretation of results, many pre-analytical factors influence immunohistochemistry predictability for an accurate result.

3.1.3.1) Specimen choice:

Several institutions have adopted the core biopsy as the preferred technique for investigating breast lesions. With significance assigned to the use of neoadjuvant chemotherapy, a needle core biopsy affords valuable information with the potential to influence patient management. It is pertinent, therefore, that concordance between core biopsy and excision specimen be addressed.

By evaluating the reliability of ER expression on needle core biopsy in invasive CA, Hodi et al. (2007) have shown concordance of 98.8% with surgically excised specimens (Hodi et al. 2007). Similarly, Dekker et al. have reported 99.1% concordance of ER results (Dekker et al. 2013). When investigating PR and HER2 status in breast core biopsies and excision specimens, studies have shown concordance of over 86% (Wood et al. 2007). Similarly, a meta-analysis of twenty-seven studies concluded that the core biopsy had high diagnostic accuracy in evaluating hormone and HER2 status

(Chen et al. 2012). Thus, assessment of hormone and HER2 receptor status on diagnostic needle core biopsy has become the standard practice within our institution.

Potential reasons for the difference observed in staining between core and excision specimen may be due to tumour heterogeneity and sampling error, with the limited needle core biopsy failing to reflect the expression of the entire tumour. In addition, differences in staining may expose a fixation artefact. The delay in penetration of the fixative to the centre of the specimen will delay fixation and may produce a false-negative result (Mann et al. 2005). Furthermore, needle core biopsies are prone to crush artefacts which may lead to false-positive results (Asogan, Hong, and Arni Prabhakaran 2017).

3.1.3.2) Delayed fixation:

“Cold ischaemic time” is defined as the interval between tissue removal and initiation of fixation. Although data is limited, a cold ischaemic time of one hour or less is recommended by the ASCO/CAP guidelines. A noteworthy decline in immunohistochemical staining for hormone receptor and HER2 staining can occur when cold ischaemic time exceeds two hours in non-refrigerated specimens. This false-negative result occurs due to antigenic degradation. Degradation can be delayed up to four hours by refrigerating specimens (Yildiz-Aktas, Dabbs, and Bhargava 2012).

3.1.3.3) Fixation time:

To ensure rapid diagnosis, clinicians submit some specimens marked as “urgent”. A prompt diagnosis ensures that patients can receive treatment early in the course of their disease. Cost of medical care is reduced by decreasing unnecessary days spent in hospital. Nevertheless, the effect of processing biopsy specimens too rapidly is that tissues are exposed to a fixative for only a few hours. This has a serious impact on the quality of fixation. Tissue fixatives prevent temporal changes of autolysis, microorganism-induced modifications and soluble molecule diffusion. To produce constant and reproducible ER staining, breast tissue requires at least 6-8 hours of fixation before being processed in a tissue processor. If fixed for less than the prescribed time, as may occur when specimens received at

the end of the day are processed shortly thereafter, pathologists risk false-negative results (Fox et al. 1985; Goldstein et al. 2003; Gown 2008).

In addition, over-fixation of breast tissue can lead to reduced immunoreactivity for breast prognostic markers. However, this has only been shown to occur after extensive fixation (over 57 days) and may not be clinically relevant (Arber 2002).

3.1.3.4) The fixative solution used:

Fixative solutions with a low pH such as “bouin picroformol-acetic fixative” and “B-5 sodium acetate-sublimate” can degrade ER. Buffering formalin ensures a pH between 7.0 and 7.4 (Fitzgibbons et al. 2014).

3.1.3.5) Other factors:

False-negative results may also occur from decalcification, causing a loss in immunoreactivity (Arber et al. 1996). Immunoreactivity of tumour cells may be influenced by excessive heat as a consequence of thermo-cauterisation during surgery. A dark haematoxylin counterstain may obscure faintly positive diaminobenzidine staining. Furthermore, non-optimised antigen retrieval and antibody type may result in a false-negative result (Fitzgibbons et al. 2014).

False-positive results are less commonly encountered (Viale et al. 2007). This occurs when an antibody cross-reacts with an antigen not being sought (Fitzgibbons et al. 2014).

3.1.4) Molecular classification of breast carcinoma:

Through gene expression profiling by microarray analysis, Perou and Sørli were the first to describe the major molecular subtypes of breast CA. Their findings suggest that breast CA may be separable into ER-positive (luminal A and luminal B) and ER-negative (HER2-expressing, basal-like and normal breast-like) groups (Sørli et al. 2001; Perou et al. 2000).

Immunohistochemical receptor analysis offers a surrogate technique for molecular subgroup recognition. These molecular subgroups have important clinical implications as each are associated with different therapeutic responses and different outcomes (Rastogi et al. 2008; Carey et al. 2006).

3.1.4.1) Luminal breast carcinoma:

Hormone receptor positive (luminal) breast CA is defined by the overexpression of genes normally expressed by luminal epithelium. It is a subtype that is known with a favourable prognosis despite lower sensitivity to chemotherapy (Tan et al. 2014). The luminal subgroup is further subcategorised into luminal A and luminal B. In addition to being ER-positive, luminal A CA is negative for HER2 and has a Ki67 of less than 14%. Luminal B CA is ER-positive, negative for HER2 and has a higher proliferation index with Ki67 greater or equal to 14% (Feeley et al. 2014). Notably, there is a significant proportion of ER-positive tumours that do overexpress HER2 and are still classified in the luminal B category rather than the HER2 molecular subtype (Parker et al. 2009; Rouzier et al. 2005). The significance associated with the greater proliferation in luminal B CA is a higher histologic grade and worse prognosis (Sorlie et al. 2003; Perou et al. 2000; Sørli et al. 2001; Geyer et al. 2009).

3.1.4.2) Basal-like breast carcinoma:

Basal-like breast CA has a gene expression profile that is similar to the basal/myoepithelial cells of normal breast tissue. These tumours characteristically express basal-type cytokeratins and are typically “triple-negative” lacking immunohistochemical staining for ER, PR and HER2 (Nielsen et al. 2004; Bhargava and Dabbs 2007; Perou et al. 2000). Patients with basal-like CA have been shown to have a poor prognosis (Sørli et al. 2001; van de Rijn et al. 2002). Tumours have high histologic grades and elevated mitotic rates. Other significant morphological features include “geographic tumour necrosis, pushing border of invasion, stromal lymphocytic response and atypical medullary features”. Metaplastic components have also been described (Livasy et al. 2006).

3.1.4.3) HER2 molecular subtype of breast carcinoma:

The HER2 molecular subtype of breast CA has an aggressive clinical course (Weigelt, Baehner, and Reis-Filho 2010). These tumours are defined by overexpression of the ERBB2 gene (Perou et al. 2000; Sørli et al. 2001). They show strong (3+) staining for HER2 on immunohistochemistry. In addition, they are hormone receptor negative (Rouzier et al. 2005).

3.1.4.4) Normal breast-like breast carcinoma:

The normal breast-like molecular collection shares a grouping with fibroadenomas and normal breast tissue. This molecular form of breast CA is controversial as it has not been reproducibly defined (Allison 2012). Authors have argued that the normal breast-like molecular subtype could represent a technical artefact from contamination with normal tissues in microarrays. It is defined by gene expression typical of basal epithelial cells and adipose tissue. The expression of luminal epithelial genes is low. The normal breast-like molecular subtype of breast CA has an intermediate prognosis. These tumours are associated with the absence of receptor markers and consequently, like basal-like, can be classified as a subtype of triple negative breast CA (Perou et al. 2000; Sørli et al. 2001; Eroles et al. 2012).

3.1.4.5) Emerging molecular subtypes:

Recently, three new molecular subgroups have been defined. These include the “claudin-low, molecular apocrine and interferon-related subtypes” (Alizart et al. 2012).

The “claudin-low” subgroup is characterised by augmentation of “epithelial-to-mesenchymal transition markers and immune response genes”. This subgroup demonstrates CA stem cell-like features. In addition, there is low-to-absent expression of luminal differentiation markers. The majority have a poor prognosis and are of triple negative immunohistochemical phenotype. Morphologically, a high frequency of metaplastic and medullary differentiation is observed. These tumours have a neoadjuvant chemotherapy response rate that is intermediate between that of basal-like and luminal tumours (Prat et al. 2010; Lu et al. 2013).

The “molecular apocrine” subtype is estimated to account for over ten percent of invasive breast CA. These tumours show prominent apocrine features on histological examination and are androgen receptor positive. This group includes ER-negative tumours outside the basal group (Farmer et al. 2005; Alizart et al. 2012).

The “interferon-related” molecular subtype is curious considering that interferons are mediators of the anti-tumour response. This subgroup is defined by high expression of gene clusters regulated by interferon. This cluster contains signal transducer and activator of transcription 1 (STAT1) which facilitates interferon regulation of gene expression (Hu et al. 2006). Research suggests that interferon regulated gene clusters have been linked to lymph node metastasis and poor prognosis. Tumours of the interferon-related molecular subtype therefore, may produce an ineffective anti-tumour response or may reflect a deviation in the healthy anti-tumour response (Huang et al. 2003).

Table 3 - Characteristics of molecular subtypes of breast cancer - Adapted from Alizart et al., 2012:

ER Expression:	Molecular Classification:	HER2:	Ki67:	Histologic Grade:
Positive:	Luminal A	Negative	Low	1-2
	Luminal B	Negative / Positive	High	2-3
Negative:	HER2	Positive	High	2-3
	Basal-like	Negative	High	3
	Molecular apocrine	Negative	High	2-3
	Claudin-low	Negative	High	3
	Interferon-related	Negative	High	3

3.1.5) Histologic type and grade:

Assessment of histologic type and histologic grade allow invasive breast CA to be subdivided morphologically, based on cell morphology, growth pattern and degree of differentiation. There are many histologic types described by the World Health Organization (WHO). Sixty to seventy-five percent of breast CA are of no special type and lack peculiar features to merit classification into one special type. Certain special tumour types provide useful prognostic information (Northridge et al. 1997). Tubular CA is associated with an excellent prognosis (Rakha et al. 2010). In contrast, metaplastic CA has an unfavourable outcome (Luini et al. 2007). Nevertheless, special type carcinomas (e.g. Mucinous carcinoma – Image 1) are uncommon and therefore, the role of histologic typing in the clinical evaluation and management of patients is currently limited (Pereira et al. 1995).

Various professional international bodies including the WHO and AJCC recommend the Nottingham Grading System (Elston-Ellis modification of the Scarff-Bloom-Richardson grading system). The Nottingham grading system takes into consideration the degree of tubular differentiation, mitotic count and nuclear pleomorphism. Each component is given a score from 1 to 3. “The sum score corresponds to a grade. Grade 1 tumours have a score from 3 to 5. Grade 2 tumours have a score of 6 or 7. Grade 3 tumours (Image 2) have a score of 8 or 9” (Elston and Ellis 1991).

Table 4 - The Nottingham Grading System for breast tumours (Elston and Ellis 1991):

Feature:	Score:
Tubular differentiation: ■ Majority of tumour (>75%) ■ Moderate (10-75%) ■ Minimal (<10%)	1 2 3
Nuclear pleomorphism: ■ Small regular nuclei	1

■ Moderate increase in size	2
■ Marked variation in size, nucleoli, chromatin clumping	3
Mitotic count (25x objective with field diameter of 0.59mm):	
■ Less than 10/10 HPF	1
■ 10-20/10 HPF	2
■ >20/10 HPF	3
Mitotic count (40x objective with field diameter of 0.44mm):	
■ <5/10 HPF	1
■ 6-10/10 HPF	2
■ >10/10 HPF	3

The Nottingham Grading system provides significant prognostic information comparable to that of lymph node status (Walker 2003). Henson et al. have demonstrated equivalent survival rates in patients with histologic grade 1, stage II disease to those with grade 3, stage I disease (Henson et al. 1991).

The histologic grade and histologic type of breast CA has an appreciable influence on ER status. In a study of 5993 patients, Nadji et al. showed that virtually all grade 1 tumours are ER-positive. In addition, pure tubular, colloid and classic lobular carcinomas are ER-positive (Nadji et al. 2005).

3.1.6) Proliferative rate:

Limitless replicative potential and proliferation is a hallmark of malignancy (Hanahan and Weinberg 2000). There are several methods that can be adopted to assess proliferation index including “the manual counting of cells in visible mitosis, incorporation of labelled nucleotides into DNA and flow cytometric evaluation of the fraction of cells in the S-phase of the cell cycle” (Tubiana et al. 1984; Dressler et al. 1987). The most widely used method however, is the measurement of Ki67 antigen using immunohistochemistry (Dowsett et al. 2011).

Demonstrable in all stages of the cell cycle except the resting phase, G0, Ki67 is a robust nuclear biomarker that can be evaluated by immunohistochemistry in a variety of circumstances used in routine fixation and tissue processing (Gerdes et al. 1984; Dowsett et al. 2011).

In addition to separating tumours into luminal A and luminal B phenotypes, the pre-therapy assessment of Ki67 is valuable to oncologists for its predictive and prognostic significance. Studies report a better response and significant reduction in Ki67 following neoadjuvant chemotherapy in tumours with a higher pre-therapy proliferation index (Archer et al. 2003; Ellis et al. 1998). Similarly, improved pathological complete response rates (PCR) have been reported following anthracycline and taxane-containing neoadjuvant chemotherapy (Yoshioka et al. 2015).

Literature regarding post-therapy prognostic value shows conflicting results. In a series of 61 enrolled patients with locally advanced breast CA, post-therapy Ki67 was the only meaningful prognostic factor for better survival (Lee et al. 2008). A high Ki67 in post-treatment tumours was shown to strongly correlate with poor disease free and overall survival (Yoshioka et al. 2015). In contrast, other studies have not shown a relationship between post-neoadjuvant chemotherapy Ki67 and survival (Colleoni et al. 2004; Bottini et al. 2001; Penault-Llorca et al. 2003; Faneyte et al. 2003).

Regarding its predictive role, a higher Ki67 proliferation index is predictive of benefit from the addition of docetaxel to adjuvant treatment in ER-positive tumours (Penault-Llorca et al. 2009).

Proliferation can be assessed by other means. A promising and clinically useful application of microarray technology is the prediction of response to treatment. There is an increase in chemotherapy sensitivity in ER-positive tumours expressing proliferation-related gene sets (Iwamoto et al. 2011).

3.2 Neoadjuvant therapy:

3.2.1) Neoadjuvant endocrine therapy:

The use of reasonably tolerable and non-toxic hormonal therapy before surgery may allow for breast conservation to elderly postmenopausal patients who are unfit for chemotherapy and have hormone receptor positive tumours that are initially considered to be overly large or not suitable for surgery. Using traditional endocrine manipulation such as tamoxifen, studies have shown an objective response approaching 70% within approximately three months of treatment. Although newer endocrine agents such as letrozole have been found to be superior when compared with tamoxifen in overall objective response rate and rate of breast conserving surgery, the PCR rate for all primary endocrine therapies is low. Long-term survival data has shown no difference in survival in patients that received endocrine and cytotoxic therapies (Kaufmann et al. 2006; Eiermann et al. 2001; Cheung, Howell, and Robertson 2000; Cameron et al. 1997).

3.2.2) The role of neoadjuvant chemotherapy:

Neoadjuvant chemotherapy is an established alternative treatment modality in which patients with high-risk, large ($\geq 2\text{cm}$) or locally advanced CA are administered chemotherapy prior to surgery. When first introduced in the 1980's, the goal of neoadjuvant chemotherapy was to convert inoperable tumours to operable CA by reducing tumour size. Today however, many additional clinical advantages are noted (Thompson and Moulder-Thompson 2012; Mieog, van der Hage, and van de Velde 2007).

A significant benefit of neoadjuvant chemotherapy is that it allows for monitoring of treatment response. Neoadjuvant chemotherapy is an in-vivo assay of drug sensitivity as the primary tumour remains intact. Response-guided modifications to the neoadjuvant chemotherapy regimen will subsequently afford early non-responders the chance to receive alternative agents with the potential to improve survival (von Minckwitz et al. 2013).

Neoadjuvant chemotherapy has influenced standard surgical practice. Studies assessing pre- and postoperative chemotherapy in women with surgically treatable breast tumours have found equivalent overall survival and an increased breast conservation rate. Neoadjuvant chemotherapy therefore, is no longer reserved for merely down-staging of a primary tumour. It is appropriate for women with small operable tumours who would prefer breast conservation (Mieog, van der Hage, and van de Velde 2007). Crucially, no significant difference in surgical complication rate is identified in patients receiving either neoadjuvant or adjuvant therapy (Hu et al. 2011).

From a research perspective, neoadjuvant chemotherapy provides opportunities to analyse the biological modifications that occur within the microenvironment of tumours undergoing treatment. Through improved comprehension of key biologic alterations achieved in such research, histopathologists may assist in identifying patients who are most likely to benefit from neoadjuvant chemotherapy (Pinder et al. 2015).

3.2.3) Mechanism of action of cytotoxic agents:

Anthracyclines and taxanes are the two key groups of cytotoxic agents that exhibit most activity towards breast CA. They are often used in combination for enhanced efficacy. As tumours represent a heterogenous population of cells, combination treatment offers a broader range of coverage (Cleator et al. 2002).

Anthracyclines function predominantly through their “interaction with the nuclear enzyme topoisomerase II” (Smith and Souès 1994). With the “formation of covalent topoisomerase II α -DNA complexes, anthracyclines prevent topoisomerase from completing the re-ligation part of the ligation-re-ligation reaction” (Chen and Liu 1994).

Taxanes act as microtubule inhibitory agents. Microtubules are long filamentous protein polymers that have many cellular functions. They have key roles in preserving cell shape, movement, signalling

and division. It is these key roles that make them effective targets for chemotherapy (McGrogan et al. 2008; Jordan and Wilson 2004).

3.2.4) Gross effects of neoadjuvant chemotherapy on breast cancer:

Various combinations of chemotherapeutic agents are used for the treatment of primary breast CA. Nonetheless, typical changes occur in most breast CAs with any treatment. The changes seen macroscopically vary depending on the response to treatment. In patients with a poor response, the remaining tumour may not appear dissimilar to that seen in patients who had not received neoadjuvant chemotherapy. In cases with a more clear response however, the tumour bed may be seen as an area of fibrosis with a pale and oedematous cut surface. Neoadjuvant therapy often reduces the size of the tumour bed which can often be assessed on palpation. However, in patients who have had a significant response, delineation on gross palpation may be challenging because of softening of the tumour stroma, alterations in stromal desmoplastic response and reduced tumour cellularity (Sahoo and Lester 2009; Pinder et al. 2015).

3.2.5) Microscopic effects of neoadjuvant chemotherapy on breast cancer:

As previously mentioned, alterations caused by neoadjuvant chemotherapy will vary depending on response to treatment.

The histological appearance of a breast CA may remain relatively unchanged when compared to the diagnostic core biopsy. An alteration in cellularity and cytomorphology may be expected in patients who do demonstrate a response. Characteristically, breast CA will become less cellular. This often occurs even when features of tumour regression are not pronounced and the overall tumour size remains stable. When residual CA is not present, the tumour bed is characterised by a hyaline vascular stroma with oedema and fibroelastosis (Image 10). Normal glandular breast ducts and lobules are often absent. There may be an infiltrate of mononuclear inflammatory cells in which foamy histiocytes and aggregates of lymphocytes are identified (Sahoo and Lester 2009; Pinder et al. 2015).

Within the residual CA, an increase in nuclear grade is the most commonly appreciated histologic effect. There is an increase in nuclear size, multinucleation, prominent nucleoli and nuclear vacuolization. There is a decrease in mitotic activity. Tumour cells become enlarged due to an increase in cytoplasm. Cytoplasmic vacuolization and eosinophilic change may occur (Moll and Chumas 1997; Pinder et al. 2015). The observed changes are favoured to represent degeneration rather than dedifferentiation (Close, Tiltman, and Hacking 1987).

Data regarding the influence of primary chemotherapy on histologic CA type is limited, but studies have shown that tumours may demonstrate a lobular morphology post treatment. This occurs due to a combination of fibrosis and decreased cellularity (Carder 1999).

3.2.6) Effects of neoadjuvant chemotherapy on normal breast parenchyma:

Non-carcinomatous breast tissue consistently demonstrates histologic effects of neoadjuvant chemotherapy. There is lobular atrophy often accompanied by intralobular fibrosis. The fibrosis may vary from basement membrane thickening to hyalinization. Normal breast epithelial structures show focal or widespread atypia in the form of enlarged and pleomorphic nuclei. Although the stromal compartment demonstrates the lowest frequency of drug-induced changes, elastosis and interstitial calcification have been reported (Moll and Chumas 1997).

3.2.7) Effects of neoadjuvant chemotherapy on lymph nodes:

Following neoadjuvant chemotherapy, lymph nodes may be difficult to appreciate macroscopically because of atrophy and fibrosis. As a result, fewer lymph nodes are retrieved. Microscopically, a complete response to treatment is characterised by hyaline stromal scars, aggregates of histiocytes or mucin pools (Image 9). The fibrotic scars have a wedge shape with the broad margin observed at the capsular edge. In addition, metastases may resolve without scar formation. In these cases, no residual viable tumour cells are identified and prior metastatic involvement cannot be determined. In lymph nodes showing a partial response, tumour cells are surrounded by hyaline stromal fibrosis (Sahoo and Lester 2009; Pinder et al. 2015).

3.2.8) Classification systems used to evaluate tumour response to chemotherapy:

Numerous methods have been described to assess and classify tumour response to chemotherapy.

These systems differ in their definitions of PCR and permittance of DCIS (Table 5) (Baker, King, and Schnitt 2019).

Table 5 – Classification systems evaluating response to neoadjuvant chemotherapy – Adapted from

Baker, King, and Schnitt 2019:

Classification system:	Pathological complete response (PCR):	Is residual DCIS permitted in classification as PCR:
Regression of Sinn (Sinn et al. 1994)	No remaining CA in both breast and lymph nodes	No
Sataloff method (Sataloff et al. 1995)	No remaining CA in both breast and lymph nodes	Yes
Chevallier method (Chevallier et al. 1995)	No remaining CA in both breast and lymph nodes	No
NSABP B-18 (Wolmark et al. 2001)	No remaining CA in breast only	Yes
Miller-Payne system (Ogston et al. 2003)	No remaining CA in breast only	Yes
Pinder et al. 2007	No remaining CA in both breast and lymph nodes	Yes
Residual cancer burden – RCB (Symmans et al. 2007)	No remaining CA in both breast and lymph nodes	Yes

Residual disease in breast and nodes – RDBN (Chollet et al. 2008)	No remaining CA in both breast and lymph nodes	Yes
AJCC ypTNM (Hortobagyi et al. 2017)	No remaining CA in both breast and lymph nodes	Yes

The significance of residual DCIS post primary chemotherapy in the absence of invasive CA is controversial. Research from the United States of America concluded that residual DCIS does not adversely affect survival or local recurrence rates (Mazouni et al. 2007). In contrast, research from the German breast group showed significantly superior disease free survival in patients with no invasive CA and no DCIS when compared to patients with residual DCIS only (von Minckwitz et al. 2012). For this reason, pathology reports should clearly state the presence of residual DCIS, especially if no invasive CA is present (Baker, King, and Schnitt 2019).

Of significance, certain systems require that the pre-treatment specimen be re-evaluated in the assessment of treatment response. These particular systems emphasise change in cellularity when determining response to primary chemotherapy (Sataloff et al. 1995; Sinn et al. 1994; Ogston et al. 2003; Pinder et al. 2007).

3.2.9) The effect of neoadjuvant chemotherapy on hormone and HER2 receptor status:

The use of neoadjuvant chemotherapy has raised many concerns. Its use may allow for in vivo selection of resistant tumour cells with altered expression (Neubauer et al. 2008). Histologic changes may influence tumour grade. In addition, neoadjuvant chemotherapy may change hormone or HER2 receptor status (Adams et al. 2008).

When reviewing previous studies that have considered the potential for hormone receptor status to change following neoadjuvant chemotherapy, conflicting results are observed (Table 6 and 7).

Nevertheless, majority of identified studies have concluded that neoadjuvant chemotherapy does alter hormone receptor status.

Table 6 – Synopsis of studies that concluded neoadjuvant chemotherapy DOES alter hormone receptor status:

Author:	Study Design:	Number of Patients:	Receptor Status:	
			ER:	PR:
1. Jain, Landry and Levine, (1996)	Retrospective No control group	18	17% alteration in status. Predominantly changed from (-) to (+).	22% alteration. All changed from (+) to (-).
2. MacGrogan et al. (1996)	Retrospective No control group	86	No statistically significant alteration	Decrease PR expression (p=0.007)
3. Frassoldati et al. (1997)	Prospective No control group	29	Decrease expression (p=0.015)	
4. Makris et al. (1999)	Prospective Control group used	31	46% change in expression. Predominantly decreased expression.	58% change in expression. Predominantly increased expression.
5. Taucher et al. (2003)	Prospective Control group used	191	(+) to (-) (p=0.02)	(+) to (-) (p=0.0005)
6. Colleoni et al. (2004)	Retrospective No control group	255	Premenopausal: 7% change in status, predominantly (+) to (-). Postmenopausal:	Premenopausal: 24% change in status, Predominantly (+) to (-) Postmenopausal: 23% alteration in status,

			8% alteration in status, Equally distributed	predominantly (+) to (-)
7. Piper et al. (2004)	Retrospective Control group	35	33% alteration in expression	42% alteration in expression
8. Burcombe et al. (2005)	Prospective No control group	118	2.5% alteration in status, Equally distributed	5.9% alteration in status, (-) to (+)
9. Shet et al. (2007)	Prospective No control group	73	13% alteration in status. Predominantly (-) to (+)	22% alteration in status. Predominantly (-) to (+)
10. Tacca et al. (2007)	Retrospective Control group	420	23% change in hormone receptor status. Predominantly (-) to (+)	
11. Kasami et al. (2008)	Prospective Control group	173	11% change in status, (-) to (+)	15.6% change in status, (+) to (-)
12. Neubauer et al. (2008)	Retrospective No control group	87	8% change in status, Predominantly (+) to (-)	18% change in status, Predominantly (+) to (-)
13. Hirata et al. (2009)	Retrospective No control group	368	14.9% change in status	29.1% change in status
14. Mittendorf et al. (2009)	Prospective No control group	25	20% change from (-) to (+)	Not tested
15. Chen et al. (2012)	Retrospective No control group	224	Positive to Negative (p=0.010)	Positive to Negative (p=0.019)
16. Dede et al. (2013)	Retrospective No control group	63	5.7% change (+) to (-) (p<0.001)	30.1% change in PR status. (p=0.160)
17. Yang et al. (2013)	Retrospective Control group	113	12.4% ER status modulation when compared with	15.9% PR status alteration compared to 6.9% alteration

			3.9% in control (p=0.025)	in control (p=0.038)
18. Lee et al. (2013)	Retrospective No control group	345	ER expression did not change significantly (p=0.1185)	42.2% change from PR- positive to PR- negative (p=0.0009)
19. Cockburn et al. (2014)	Retrospective Control group	133	9.02% change in status (p=0.0148)	18.4% change in status (p=0.011)
20. Parinyanitikul et al. (2015)	Retrospective No control group	398	10.9% change in status from (+) to (-) after treatment. 20.8% status change from (-) to (+) after treatment	35.2% status change from (+) to (-) after treatment. 11.9% status change from (-) to (+) after treatment
21. Jin et al. (2015)	Prospective No control group	423	18.4% of patients showed a change in hormone receptor status. 13% showed a change from (+) to (-).	
22. Ozmen et al. (2015)	Retrospective No control group	128	11% change from (+) to (-) (p>0.05)	13% change from (+) to (-)
23. Zhou et al. (2015)	Retrospective No control group	107	14% change, predominantly (+) to (-)	Significant decrease in expression (p=0.013)
24. Gahlaut et al. (2016)	Retrospective Control group	133	12% change, predominantly (-) to (+)	14.5% change, predominantly (+) to (-) (p=0.023)
25. Xian et al. (2017)	Retrospective No control group	83	30% change in biomarker status	
26. Wu et al. (2017)	Retrospective	525	Percentage of (+) cases decreased	Percentage of (+) cases

	No control group		from 65.3 to 63.2% (p>0.05)	decreased from 51.0 to 42.6% (p>0.05)
27. Subhendu and Shekhar (2018)	Prospective No control group	100	Percentage of (+) cases dropped from 80 to 78% (p=0.671)	Percentage of (-) cases increased from 34 to 38% (p=0.002)
28. Xie et al. (2018)	Retrospective Control group	143	75.4% concordance rate (p=0.158)	Significant decrease expression (p=0.003)
29. Peng et al. (2019)	Retrospective No control group	112	ER (+) rate decrease from 66.1% to 56.2% (p=0.04)	No significant change in PR
30. Sarıtaş et al. (2019)	Prospective No control group	69	Statistically significant reduction in ER and PR expression following neoadjuvant chemotherapy (p=0.0001)	

Key: (+) Positive, (-) Negative

Table 7 – Synopsis of studies that concluded neoadjuvant chemotherapy does NOT alter hormone receptor status:

Author:	Study Design:	Number of Patients:	Receptor Status:	
			ER:	PR:
1. Hawkins et al. (1990)	Prospective No control group	62	No change in expression	
2. Morris, Edwards, and Gelder (1991)	Prospective	20	No substantial change in hormone receptor levels	
3. Lo et al. (1994)	Prospective No control group	10	No correlation between neoadjuvant chemotherapy on hormone receptor status	

4. Bottini et al. (1996)	Prospective No control group	44	0.6% alteration in status	4.5% alteration in status
5. Makris et al. (1997)	Prospective No control group	128	91.5% concordance	75.5% concordance
6. Schneider et al. (2000)	Prospective No control group	37	No significant alteration in expression (p=0.8394)	No significant alteration in expression (p=0.1881)
7. Faneyte et al. (2003)	Prospective No control group	49	29% alteration in status (p=non-significant)	
8. Lee et al. (2003)	Prospective Control group	56	18% alteration in status vs 21% alteration in the control group	
9. Penault-Llorca et al. (2003)	Retrospective No control group	91	No significant alteration in expression	
10. Arens, Bleyl, and Hildenbrand (2005)	Prospective Control group	25	No change in status	No change in status
11. Varga, Caduff, and Pestalozzi (2005)	Prospective No control group	23	9% change in status (p=0.5)	21% change in status
12. Adams et al. (2008)	Retrospective No control group	26	7.7% change in status (p=non-significant)	19.2% change in status, Positive to Negative
13. Miglietta et al. (2009)	Retrospective No control group	52	No alteration is status	
14. Pedrini et al. (2011)	Retrospective No control group	90	No alteration in steroid receptor status (p=0.83)	
15. Kinsella et al. (2012)	Retrospective No control group	38	ER status unchanged (p=1.00)	37% positivity for PR pre-neoadjuvant therapy and 21% positivity post-

			treatment (p=0.03)
16. Avci et al. (2015)	Prospective No control group	100	No significant impact on ER or PR expression

Similarly, conflicting results have been reported when reviewing articles investigating the effect of preoperative chemotherapy on HER2 status (See Table 8 and 9 below). It is only when primary chemotherapy is used in conjunction with trastuzumab that a significant reduction in HER2 receptor expression becomes noted (Mittendorf et al. 2009; Hurley et al. 2006).

Table 8 – Studies demonstrating that preoperative chemotherapy does influence HER2 receptor status:

Author:	Study design:	Number of patients:
1. Rasbridge et al. (1994)	Prospective No control group	30
2. MacGrogan et al. (1996)	Retrospective No control group	125
3. Piper et al. (2004)	Retrospective Control group	35
4. Burcombe et al. (2005)	Prospective No control group	118
5. Shet et al. (2007)	Prospective No control group	73
6. Neubauer et al. (2008)	Retrospective No control group	87
7. Adams et al. (2008)	Retrospective No control group	26
8. Hirata et al. (2009)	Retrospective No control group	368
9. Avci et al. (2015)	Prospective No control group	100
10. Parinyanitikul et al. (2015)	Retrospective No control group	398
11. Xian et al. (2017)	Retrospective No control group	83

12. Subhendu and Shekhar (2018)	Prospective No control group	100
13. Peng et al. (2019)	Retrospective No control group	112
14. Sarıtaş et al. (2019)	Prospective No control group	69

Table 9 – Studies reporting preoperative chemotherapy does NOT influence HER2 receptor status:

Author:	Study design:	Number of patients:
1. Bottini et al. (1996)	Prospective No control group	77
2. Vargas-Roig et al. (1999)	Prospective No control group	37
3. Vincent-Salomon et al. (2002)	Retrospective No control group	42
4. Faneyte et al. (2003)	Prospective No control group	50
5. Penault-Llorca et al. (2003)	Retrospective No control group	105
6. Taucher et al. (2003)	Prospective No control group	85
7. Arens, Bleyl, and Hildenbrand (2005)	Prospective Control group	25
8. Quddus et al. (2005)	Prospective Control group	39
9. Varga, Caduff, and Pestalozzi (2005)	Prospective No control group	23
10. Kasami et al. (2008)	Prospective Control group	173
11. Pedrini et al. (2011)	Retrospective No control group	90
12. Kinsella et al. (2012)	Retrospective No control group	38
13. Lee et al. (2013)	Retrospective No control group	345

14. Xie et al. (2018)	Retrospective Control group	143
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In a meta-analysis, Zhang et al. reviewed fourteen studies exploring the influence of primary chemotherapy on receptor status change in breast CA. They found that both ER and PR receptor status were altered significantly by neoadjuvant chemotherapy. Significant alterations in HER2 and Ki67 were not identified and were attributed to limited case numbers (Zhang et al. 2011).

Jabbour et al. reviewed eight studies revealing an average of 12.9%, 32.0% and 8.9% discordant rates for ER, PR and HER2 expression respectively post-neoadjuvant chemotherapy. They concluded that primary chemotherapy reduces the expression of PR while the effect on ER and HER2 is not prominent (Jabbour, Massad, and Boulos 2012).

Similarly, van de Ven et al. reviewed thirty-two studies (including nine trials evaluated in the meta-analysis by Zhang et al.) investigating the concordance of hormone receptors and HER2 after neoadjuvant chemotherapy with or without trastuzumab. There was a change of between 8–33% in hormone receptor status after neoadjuvant chemotherapy. A change in PR receptor status was more common when compared to ER. Although results varied, most studies found the PR receptor changed from positive to negative. Studies found HER2 to be more constant, with discordance only reported in one of the seven trials that tested HER2 using fluorescence in situ hybridization (FISH). A positive-to-negative conversion of the HER2 receptor status was more likely when neoadjuvant chemotherapy was combined with trastuzumab (van de Ven et al. 2011).

3.2.10) Implications of neoadjuvant chemotherapy induced alteration in hormone receptor status:

Differing conclusions have been reported with regards the prognostic impact of receptor status alteration. Nevertheless, data does exist and a change in biologic markers may be of prognostic value.

Kaplan-Meier survival analysis by Ozmen et al. showed a significant relationship between positive-to-negative hormone receptor alteration and survival with a worse prognosis noted in terms of overall and disease free survival (Ozmen et al. 2015). Patients with a negative-to-positive conversion in hormone receptor status may benefit from adjuvant endocrine therapy. A positive switch may indicate a better prognosis with studies reporting a better overall and disease free survival in patients that were treated with adjuvant endocrine therapy to those with a positive switch who were not (Hirata et al. 2009; Tacca et al. 2007).

Other studies have shown that a specific reduction or loss in PR expression following neoadjuvant chemotherapy may have a positive impact on outcome for breast CA patients (Montagna et al. 2015). However, metastatic tumours with an ER-positive / PR-negative phenotype have a more aggressive course and are associated with a reduced overall survival when compared to those retaining PR (Gross et al. 1984; Balleine et al. 1999).

Identifying any alteration in biologic marker status will be of therapeutic and financial value for both the patient and health care providers. By re-evaluating receptor status, patient tailored regimens can be chosen that could improve treatment outcomes.

3.2.11) Discordance in hormone receptor status in the absence of neoadjuvant chemotherapy:

It is significant to note that studies have reported an alteration in hormone receptor status in the absence of confounding factors and neoadjuvant chemotherapy (Piper et al. 2004; Lee et al. 2003). Such discordant results are to be attributed to technical artefacts as previously mentioned.

Intra- and interobserver variability should also be considered in these cases. A false-positive result may occur when entrapped normal cells are incorrectly interpreted as malignant. In addition, the misinterpretation of in situ carcinoma as invasive carcinoma can cause a false-positive result. Image analysis devices can generate false-positive results by counting overstained nuclei. Although not

proven by clinical trial, “highly sensitive assays may detect very low levels of ER in CA that will not respond to hormone therapy” (Fitzgibbons et al. 2014).

3.2.12) Radiotherapy:

With regards neoadjuvant treatment of breast CA, radiotherapy is not usually considered. Unlike anal, rectal and oesophageal CA, neoadjuvant radiotherapy has been little studied nor used in breast tumours. Nevertheless, dramatic effects have been achieved on large locally advanced disease. In addition, radiotherapy still has a central role in palliation and haemostasis of inoperable bleeding tumours (Thompson and Moulder-Thompson 2012).

CHAPTER 4: METHODOLOGY

4.1 Study design and setting:

4.1.1) Study Design:

A non-interventional, cross-sectional, retrospective and comparative analysis was performed. The study was performed within a laboratory setting.

4.1.2) Study setting:

Research was conducted within the department of Anatomical Pathology at the National Health Laboratory Service (NHLS) laboratory at Chris Hani Baragwanath Academic Hospital (CHBAH). CHBAH is situated in Soweto within the southern part of Johannesburg, South Africa. It is the third largest hospital in the world and occupies 173 acres with 3200 beds and 6760 staff members (“Chris Hani Baragwanath Hospital” 2020). The Batho Pele Breast Clinic run at CHBAH is an open access walk-in clinic. Approximately 1100 patients are treated at the clinic per month with an estimated 350 new cases of CA diagnosed in a year.

4.2 Study population and sampling technique:

4.2.1) Study population:

The study population included all cases of histologically diagnosed invasive (ductal and lobular) breast CA that were treated with primary neoadjuvant chemotherapy followed by surgery at CHBAH from 01 January 2014 to 30 June 2015.

4.2.2) Sampling technique:

The sampling technique was purposive. All cases of invasive breast CA who received neoadjuvant chemotherapy and then underwent surgery were identified.

4.3 Ethics:

The Division of Anatomical Pathology has been granted blanket approval by the Committee for Research on Human Subjects (Medical) of the University of the Witwatersrand (M150555) to conduct retrospective studies using archived material. A formal project specific ethics clearance was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (M151048) (ANNEXURE A).

The patients' names were not recorded on the data capture sheet. Each case was identified by laboratory number and recorded successively. The identity of the patient was known only to the principal investigator and was not divulged.

Permission to utilise the TrakCare system was obtained from the National Health Laboratory Services (NHLS) CHBAH Business Manager (Violet Gabashane – ANNEXURE B).

Permission for use of the CHBAH breast unit's database was obtained from the head of the Breast Unit (Dr H. Cubasch – ANNEXURE C).

4.4 Material and data collection:

4.4.1) Case selection:

All patients diagnosed with invasive (ductal and lobular) breast CA and treated with primary neoadjuvant chemotherapy followed by surgery at CHBAH from 01 January 2014 to 30 June 2015 were identified by searching the laboratory's electronic database (TrakCare).

The following identifying words were used to identify cases using the TrakCare search function:

- Breast
- Malignant neoplasm

- Carcinoma
- Infiltrating duct carcinoma
- Infiltrating lobular carcinoma

4.4.2) Exclusion criteria:

Once all cases of invasive breast CA were identified, the following cases were excluded:

- Metastatic CA to the breast.
- Patients who had not received neoadjuvant chemotherapy.
- No available surgical pathology report.
- Absent histology slides for either the pre- or post-neoadjuvant chemotherapy biopsies or if they were performed and interpreted at a separate laboratory.
- Tumours without complete immunohistochemical expression profiles corresponding to both pre- and post-neoadjuvant chemotherapy.
- All male patients with breast CA.

4.4.3) Data collection:

The formal histopathological reports of the diagnostic core biopsy and corresponding post chemotherapy excision were retrieved and the following were recorded:

- Patient age, breast laterality, tumour location and time from diagnosis to excision was gathered from the formal histopathological reports at the NHLS at CHBAH.
- Diagnosis and histopathologic tumour type pre- and post- neoadjuvant chemotherapy.
- Hormone receptor status (As detailed in ANNEXURE D) pre- and post- neoadjuvant chemotherapy.
- Histologic grade (As detailed in ANNEXURE E) pre- and post- neoadjuvant chemotherapy.
- HER2 receptor status (As detailed in ANNEXURE F) pre- and post- neoadjuvant chemotherapy. In the event of an equivocal HER2 immunohistochemical stain (i.e. score of 2+) the results of FISH were obtained from TrakCare.

- Ki67 proliferative index (As detailed in ANNEXURE G) pre- and post- neoadjuvant chemotherapy.
- Tumour regression (i.e. evidence of chemotherapy effect – As detailed in ANNEXURE H)
- Cases with residual tumour following neoadjuvant chemotherapy were classified as either having no change in grade or biomarker status OR as having any change in grade, hormone or HER2 status.
- The hormone and HER2 receptor statuses were assessed as positive or negative as defined by the Allred score and ASCO/CAP guidelines (As detailed in ANNEXURES D & F respectively).
- Concordance was defined as biomarker receptor status that remained positive or remained negative. Discordance was defined as change in hormone or HER2 receptor status from positive-to-negative or from negative-to-positive.
- All relevant clinical data and information regarding chemotherapy cytostatic agents, number of cycles, hormonal therapy and use of Trastuzumab were retrieved from the CHBAH Breast Unit's database.
- Relevant information was entered into a data capture sheet (ANNEXURE I) and then transferred onto an Microsoft Excel® spreadsheet for statistical analysis.

4.5 Reliability of data

4.5.1) Pilot study

A re-evaluation pilot study (ANNEXURE J) was performed to assess consistency in histopathological reporting:

- a. Of the cases identified on the TrakCare database, ten percent (quantity determined with the aid of a statistician) were randomly selected using online research randomization software.

- b. Relevant slides from both the diagnostic core biopsy and corresponding post chemotherapy excision were examined and the abovementioned variables were re-evaluated in a blinded fashion.
- c. The principal investigator assessed all relevant slides for the pilot study. Thereafter, these slides were reviewed by the supervisor. In all cases, the principal investigator and the supervisor were blinded to reported results and previous findings.
- d. Findings on review of the slides included in the pilot study were compared with the findings recorded on the formal histopathological report at time of sign out.
 - i. No significant differences in the findings on review obviated the need for continued re-evaluation of all relevant slides. Therefore, only histopathological reports were used to gather the aforementioned information for the remainder of the study.
 - ii. Should there have been a statistically significant difference in reporting, continued re-evaluation of all relevant slides for the remainder of the study would have been performed.

4.5.2) Immunohistochemistry:

All stains during this period were performed on the same fully automated Ventana Benchmark XT immunostainer. All samples had been processed in the presence of adequate positive and negative controls and checked by a qualified pathologist within the department daily. The NHLS CHBAH laboratory is accredited by the South African National Accreditation System (SANAS), which performs annual quality-control checks. In addition, the Department of Anatomical Pathology at CHBAH participates in external quality assurance tests run by the Royal College of Pathologists of Australasia (RCPA).

4.5.3) Accuracy of demographic data and clinical information:

Demographic data entered onto the TrakCare system were obtained from the requisition slip completed by the submitting clinical doctor. The accuracy of data obtained from TrakCare and the

Breast Unit's database are dependent on the accuracy of data capturers and on clinicians who submit information on requisition slips.

4.6 Data analysis:

The data entered on a Microsoft Excel® spreadsheet were analysed using Stata® (version 14.2). This was done in consultation with a statistician from the University of the Witwatersrand.

4.6.1) Descriptive demographic data were analysed as follows:

- Mean/median age of patients and age distribution of patients with CA.

4.6.2) Descriptive data regarding the tumour were analysed as follows:

- Range, mean/median number and length of diagnostic tissue cores.
- Range and mean/median size of tumour before and following neoadjuvant chemotherapy.
- Frequency of each histopathologic type (ductal or lobular).
- Frequency of biomarker positive and negative receptor status (As defined in ANNEXURES D, F & G respectively) before and after neoadjuvant chemotherapy.
- Frequency of each histologic grade (As defined in ANNEXURE E) before and after neoadjuvant chemotherapy.
- Chemotherapy response rate - Frequency of response and non-response following neoadjuvant chemotherapy (As defined in ANNEXURE H).
- Frequency of positive lymph nodes and nodes that show chemotherapy effect.

4.6.3) Descriptive data regarding treatment were analysed as follows:

- Frequency of each agent used and the range and mean number of cycles received.

4.6.4) Statistical calculations:

- The difference between biomarker status in pre- and post- neoadjuvant chemotherapy specimens were compared using the McNemar's test. P-values less than 0.05 were considered significant.
- The difference between histologic grade in pre- and post- neoadjuvant chemotherapy specimens were compared using a Wilcoxon signed-rank test. P-values less than 0.05 were considered significant.
- A Wilcoxon signed-rank test was used in the analysis of Ki67.
- The pilot study (ANNEXURE J) compared findings from the reviewed random cases with the results of the formal histopathological reports at time of sign out. The data was gathered from the same participants and was therefore paired. Any reported difference in biomarker status was accordingly evaluated using the McNemar's test. Any reported difference in histologic grade was evaluated using Cohen's kappa coefficient. This test measures inter-rater agreement. P-values less than 0.05 were considered significant.

CHAPTER FIVE: RESULTS

5.1 Overview:

On review of the NHLS TrakCare database, three hundred and seventy-three patients were newly diagnosed with an invasive mammary CA in the department of Anatomical Pathology at CHBAH between 01 January 2014 and 30 June 2015. Chapter 5.1 briefly summarises the findings of all core biopsies in this period.

5.1.1) Patient Demographics:

Three-hundred and sixty-seven of the identified patients were female (98%). Six males (2%) were diagnosed with CA. The female-to-male ratio was 62.2:1.

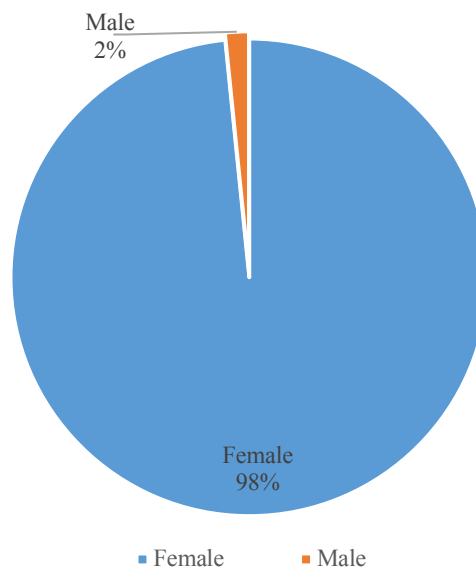


Figure 1 – Graphical Representation of Gender Distribution:

Age was available for 355 (95%) of the total 373 patients. Patients ages ranged from 25-to-94-years with mean age of 54.5-years (SD = 15.12). The median age was 53-years (IQR = 44-65). Forty-five percent of patients were between 41-years and 60-years of age (See Figure 2). No information regarding family history was available for any patients.

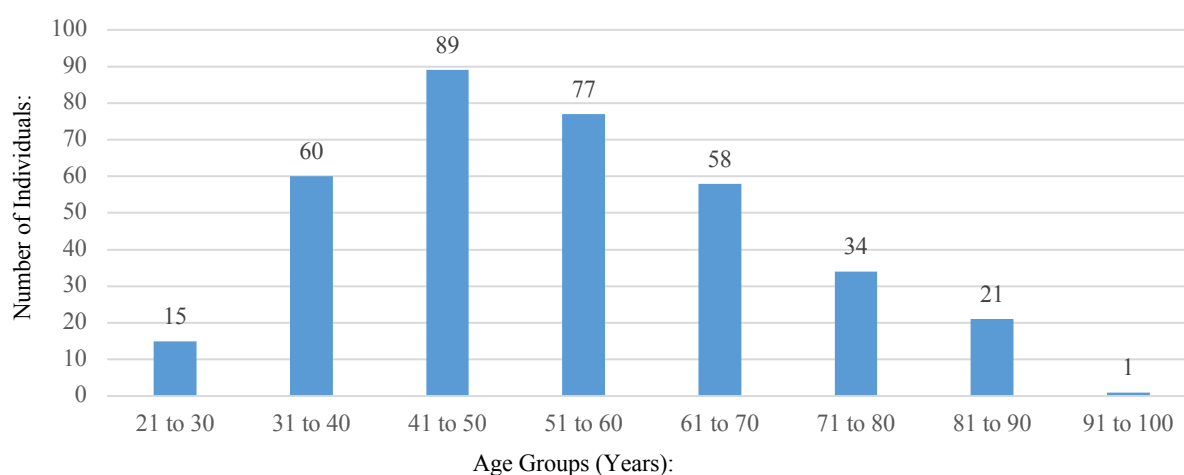


Figure 2 – Graphical Representation of Age Distribution:

5.1.2) Tumour Characteristics:

5.1.2.1) Histological type:

Invasive CA of no special type was the most frequent diagnosis (86%) with 320 cases identified.

There were 15 patients (4%) who were diagnosed with an invasive lobular CA. Thirteen cases of mucinous CA (3%) were noted. A mixed-type CA was detected in 8 patients (2%). As illustrated in Figure 3 below, micropapillary CA, metaplastic CA, CA with medullary features, tubular CA, secretory CA and CA with apocrine and neuroendocrine differentiation encompassed less than 5% of diagnoses made in the 18-month period.

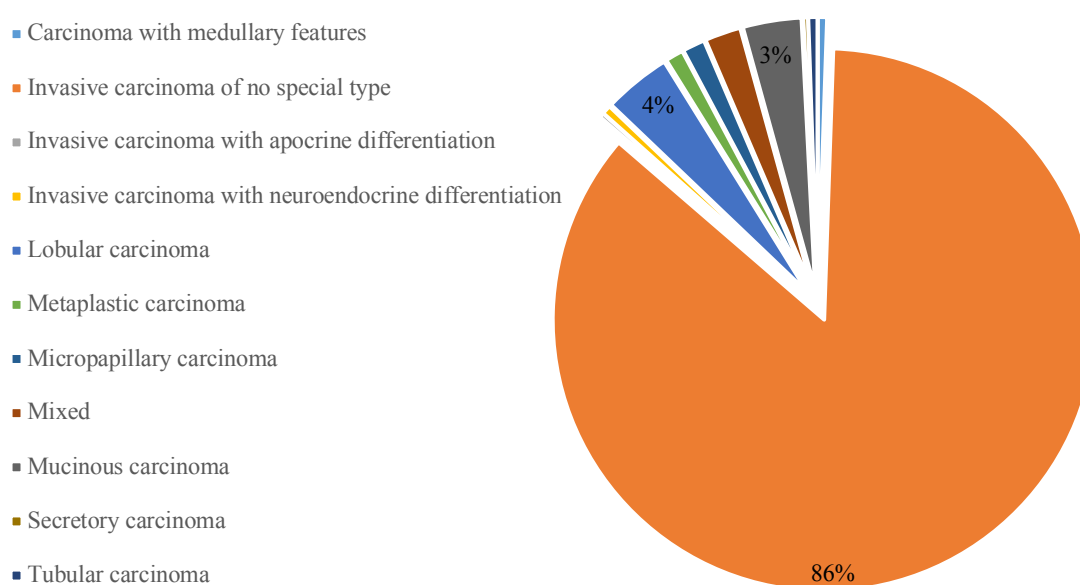


Figure 3 – Graphical Representation of Diagnosis Distribution:

5.1.2.1.) Tumour grade:

Using the Nottingham Histologic Score, 51% of tumours were classified as Grade 2, 38% as Grade 3 and 9% as Grade 1 (See figure 4). Four cases (2%) were not graded as submitted tissue was too sparse to make an accurate assessment.

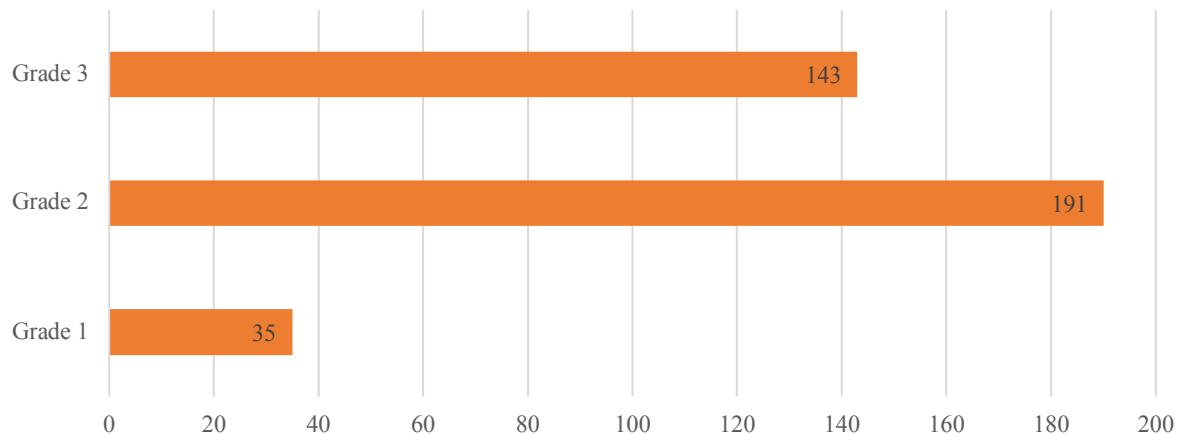


Figure 4 – Graphical Representation of Histologic grade:

ER was positive in 229 patients (62%), PR was positive in 211 patients (57%) and HER2 was positive in 86 patients (23%) on core biopsy.

Of the 373 diagnostic biopsies found, 74 patients (20%) underwent post-primary chemotherapy excision by the Breast Unit in the Department of Surgery at CHBAH. These 74 patients and their corresponding histology formed the study population for evaluation of alterations in immunohistochemical biologic markers.

Ninety (24%) of the total 373 patients underwent primary surgical excision and further treatment and follow-up data was not found for the remaining 209 patients (56%).

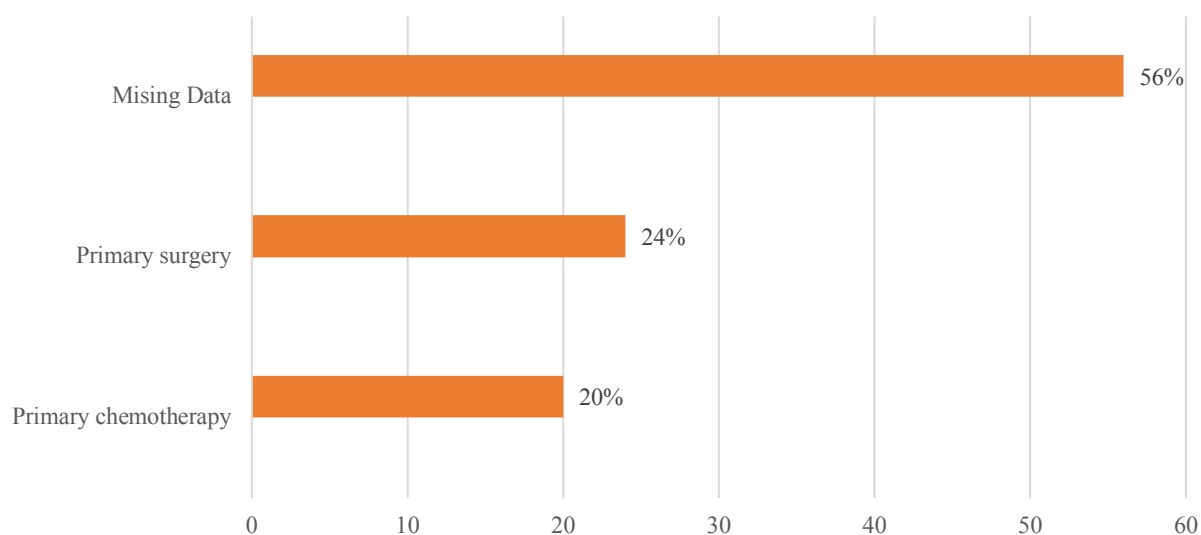


Figure 5 – Graphical Representation of Primary Patient Treatment:

5.2 Individuals Who Received Neoadjuvant Chemotherapy:

5.2.1) Pre-treatment clinicopathological characteristics of patients:

The pre-treatment clinicopathological characteristics of patients who received neoadjuvant chemotherapy are presented in Table 10 below:

Table 10 - Pre-treatment clinicopathological characteristics of patients:

	N (%)
Total	74 (100)
Age	
< 50 years	35 (47)
≥ 50 years	39 (53)
Ethnicity	
Asian / Indian	1 (1)
Black African	64 (87)
Coloured	6 (8)

White / Caucasian	1 (1)
Missing	2 (3)
HIV status	
Positive	14 (18)
Negative	56 (76)
Unknown	2 (3)
Missing	2 (3)
Laterality	
Left	36 (49)
Right	38 (51)
Histology	
Invasive carcinoma of no special type	61 (82)
Invasive lobular carcinoma	2 (3)
Mucinous carcinoma	4 (6)
Other	7 (9)
Grade	
1	4 (5)
2	43 (58)
3	27 (37)
ER status	
Positive	54 (73)
Negative	20 (27)
PR status	
Positive	46 (62)
Negative	28 (38)
HER2 status	
0/1+	40 (54)

2+	10 (14)
3+	24 (32)
Ki67:	
≥14%	73 (99)
<14%	1 (1)

Age within the study population was normally distributed (Shapiro-Wilk test $p=0.091$). The mean patient age for individuals who received neoadjuvant chemotherapy treatment was 51.8 years (SD = 12.56). Using 50 years of age as a surrogate for menopause, 47% of patients were premenopausal and 53% of patients were menopausal / post-menopausal.

A total of 251 needle cores of the breast from 74 patients were submitted for histopathological assessment. The core length ranged from 1mm to 32mm in length. The median core length was 15mm (IQR = 12-20).

The predominant histologic tumour classification was invasive CA of no special type (82%). Invasive mucinous CA was identified in four patients (5%). Two cases (3%) of invasive lobular CA underwent neoadjuvant chemotherapy (See figure 6).

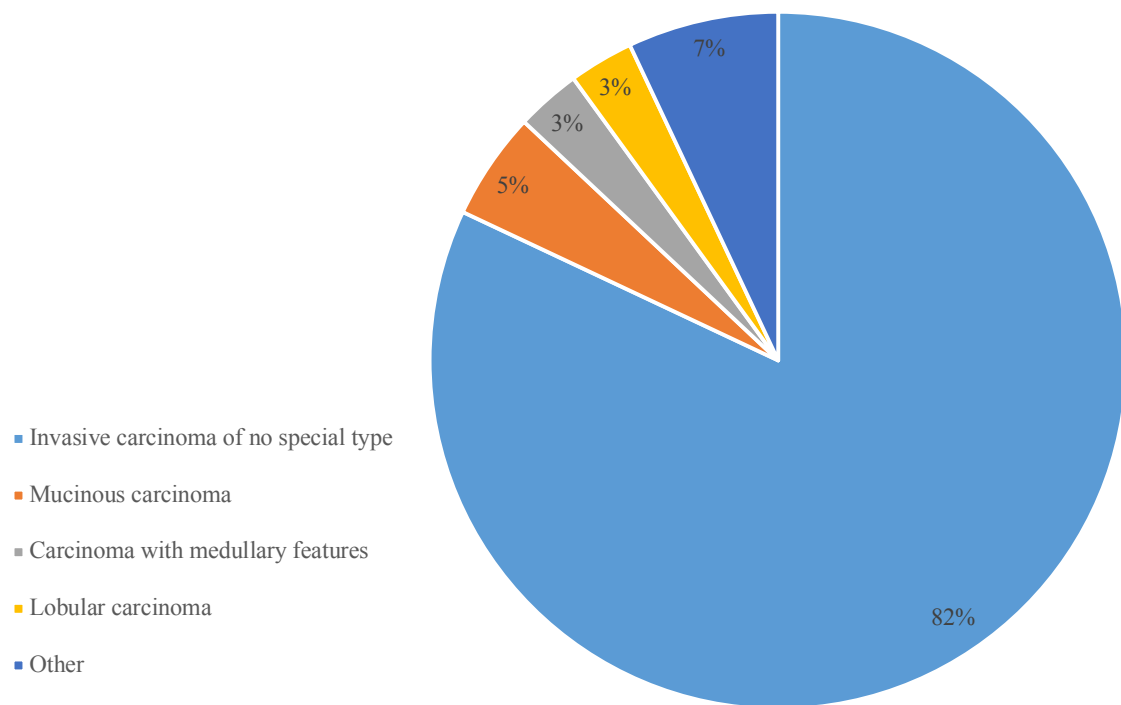


Figure 6 – Diagnosis in Patients Who Received Neoadjuvant Chemotherapy:

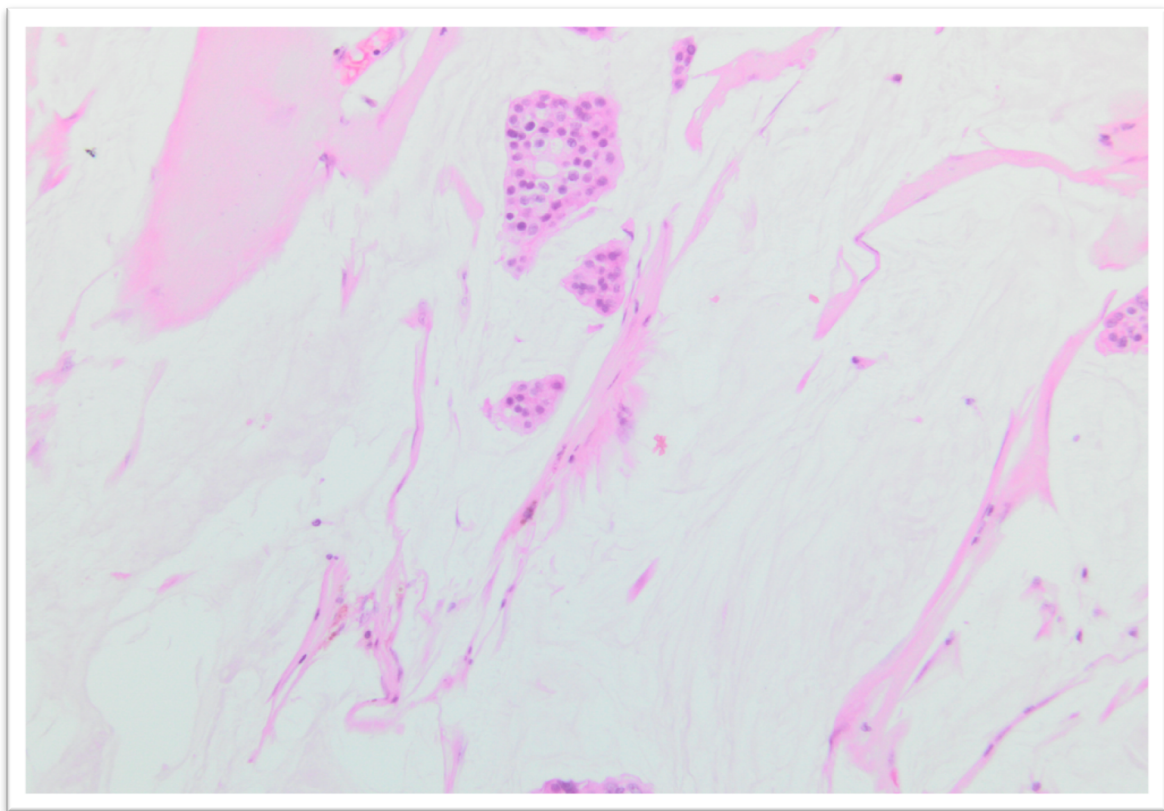


Image 1 - Micrograph at the original magnification of x200 showing an invasive mucinous carcinoma:

Of the 74 patients who underwent neoadjuvant chemotherapy, 43 patients had tumours classified as a Grade 2 using the Nottingham grading system. In 4 patients, carcinoma was categorised as a Grade 1. Twenty-seven patients had tumours categorised as a Grade 3 (See figure 7).

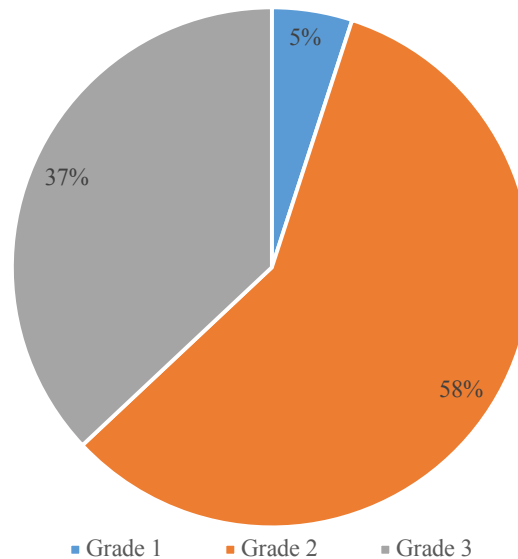


Figure 7 – Graphical Representation of Histologic Grade Before Neoadjuvant Chemotherapy:

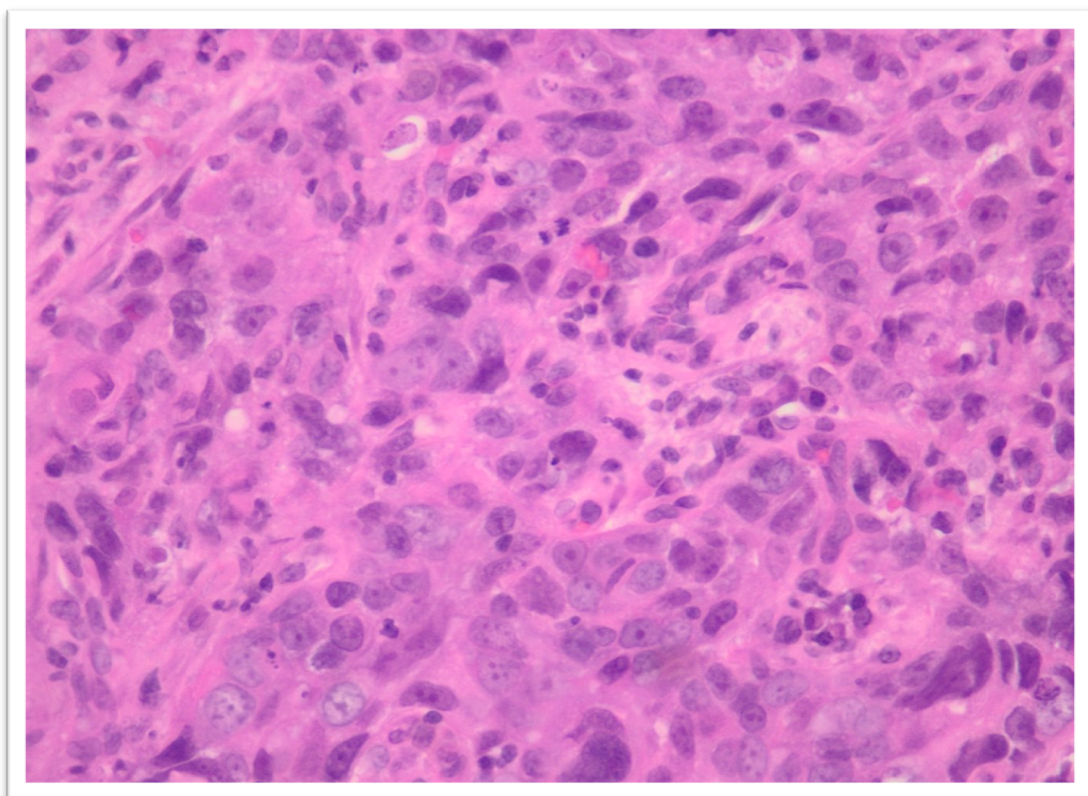


Image 2 - Micrograph at the original magnification of x400 showing a Grade 3 invasive carcinoma with lack of tubular differentiation and high-grade nuclear features:

From the needle core biopsy, lymphovascular invasion was documented in 17 patients (23%). Lymphovascular invasion was not identified in 54 patients (73%) and further data was missing for 3 patients (4%).

On immunohistochemistry, tumours were positive for ER in 54 patients (73%). Twenty patients (27%) had tumours that were ER-negative. Tumours were positive for PR in 46 patients (62%) and negative for PR in 28 patients (38%). In eight patients (11%), tumours were ER-positive, PR-negative.

HER2 (using immunohistochemistry and, if indicated, FISH) was negative in 50 patients (67%). HER2 was positive in 24 patients (33%). In nine patients (12%), HER2 was equivocal on immunohistochemistry and HER2 status was determined using FISH.

Expression of Ki67 was high ($\geq 14\%$) in 73 patients (99%). One patient (1%) had a low Ki67 proliferative index ($< 14\%$).

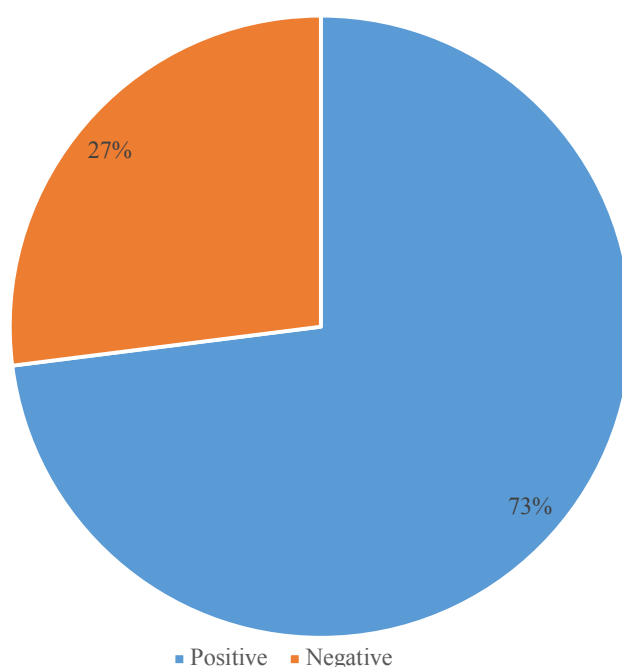


Figure 8 – Graphical Representation of Oestrogen Receptor Status Before Neoadjuvant Chemotherapy:

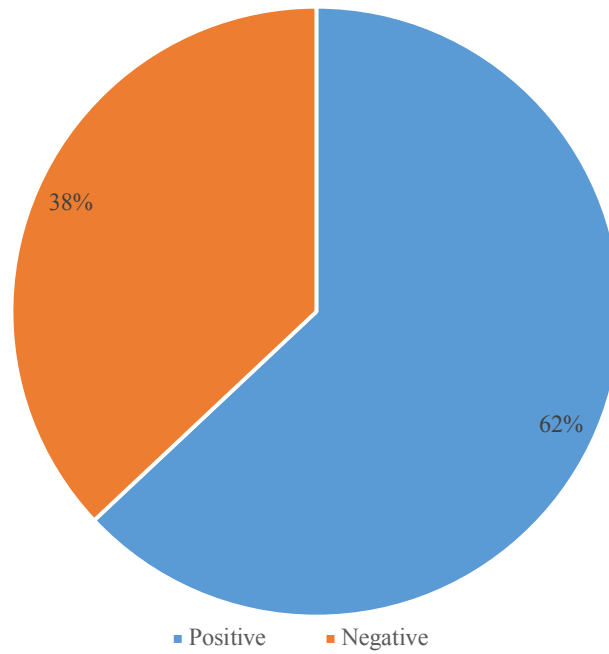


Figure 9 – Graphical Representation of Progesterone Receptor Status Before Neoadjuvant Chemotherapy:

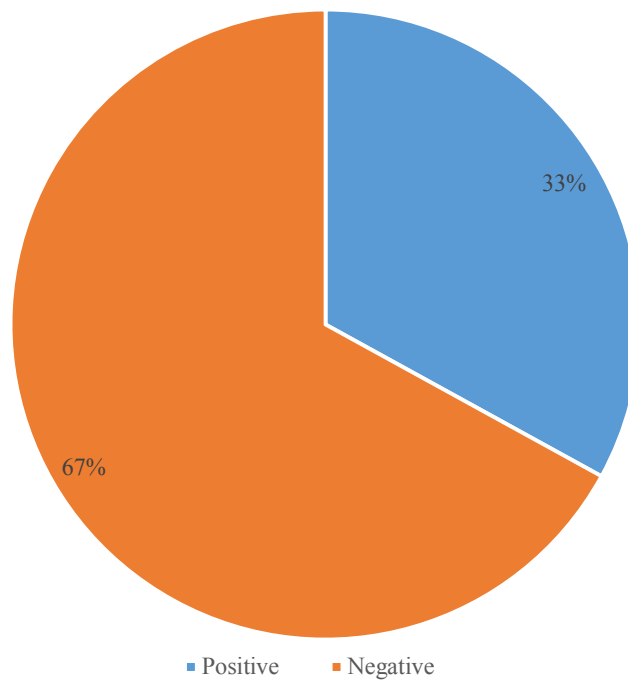


Figure 10 – Graphical Representation of HER2 Receptor Status Before Neoadjuvant Chemotherapy:

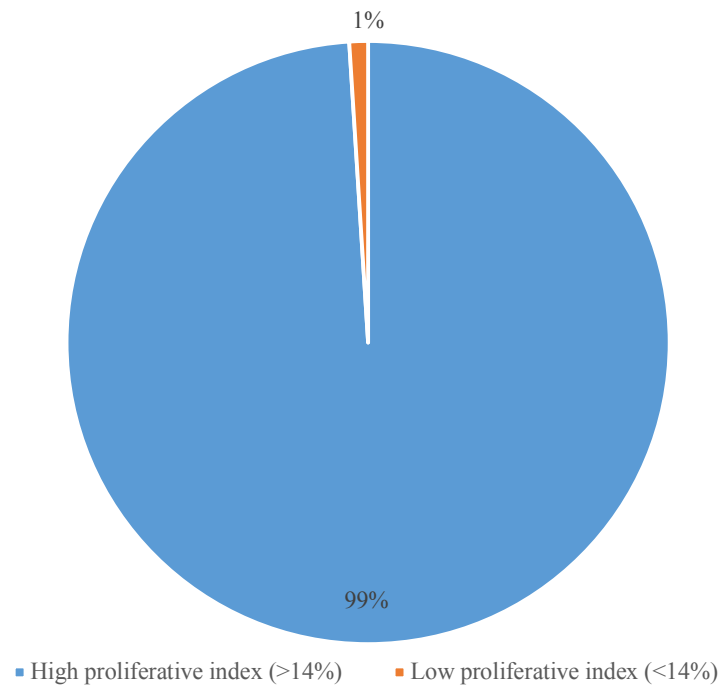


Figure 11 – Graphical Representation of Ki67 Before Neoadjuvant Chemotherapy:

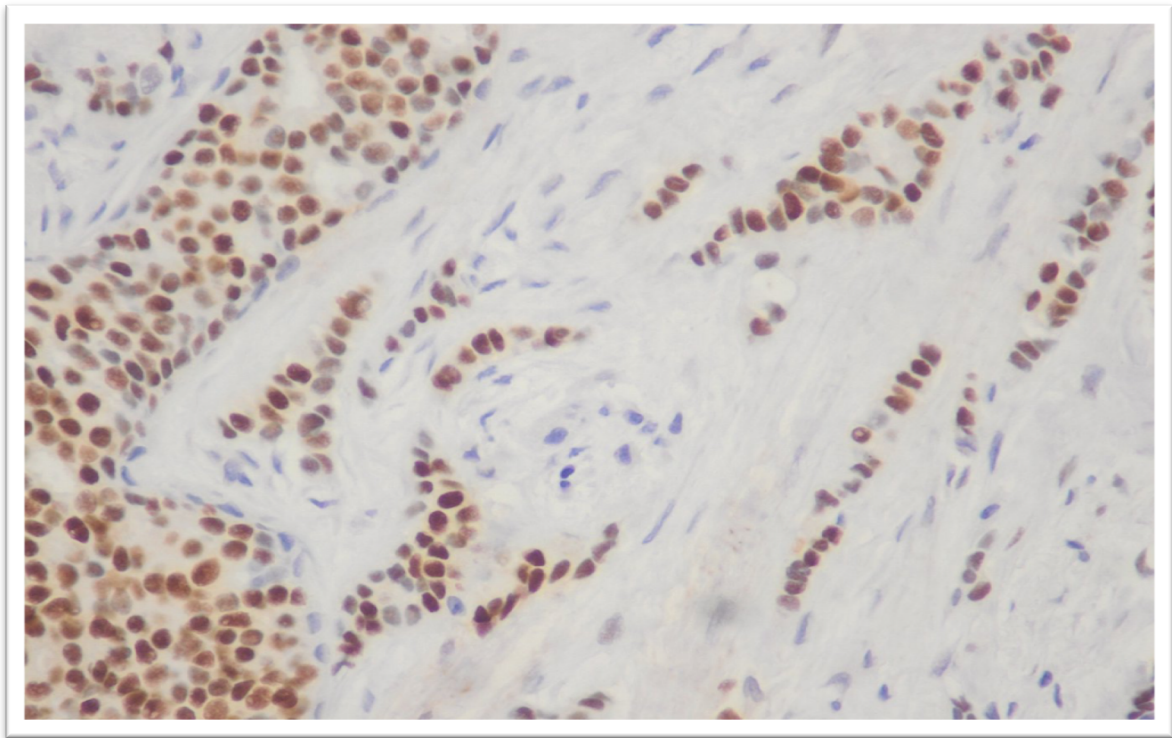


Image 3 - Micrograph at the original magnification of x400 showing a positive ER immunohistochemical stain:

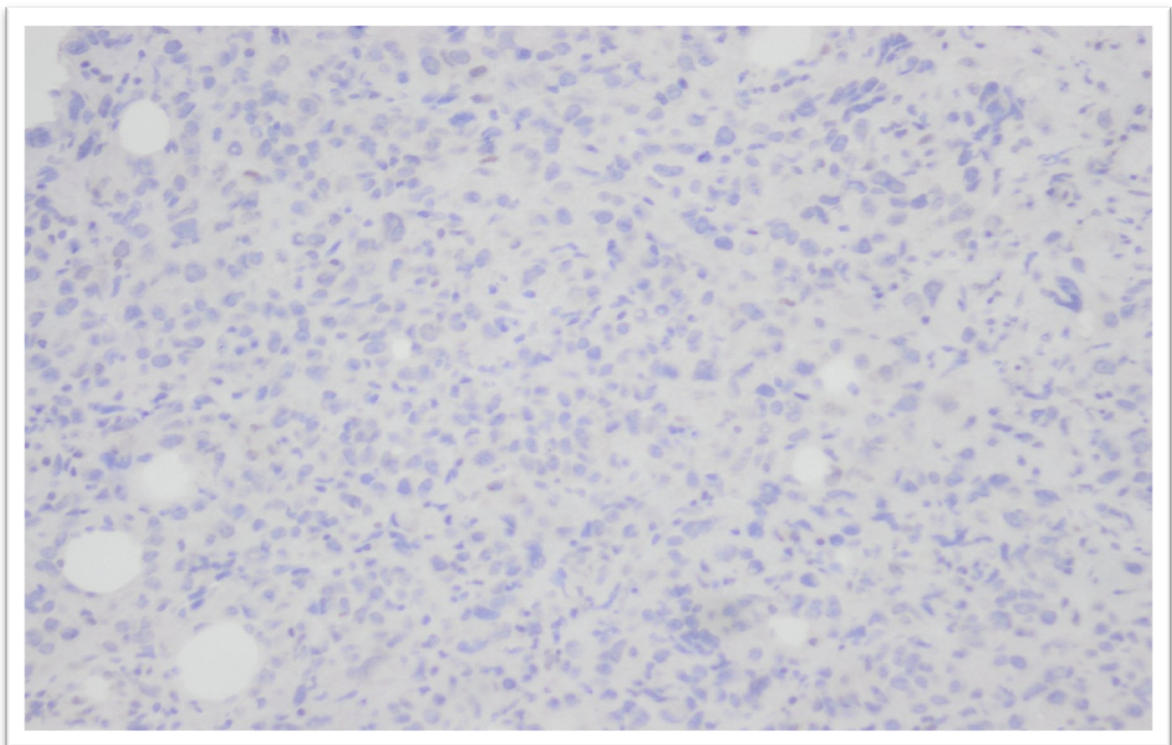


Image 4 - Micrograph at the original magnification of x200 showing a negative ER immunohistochemical stain:

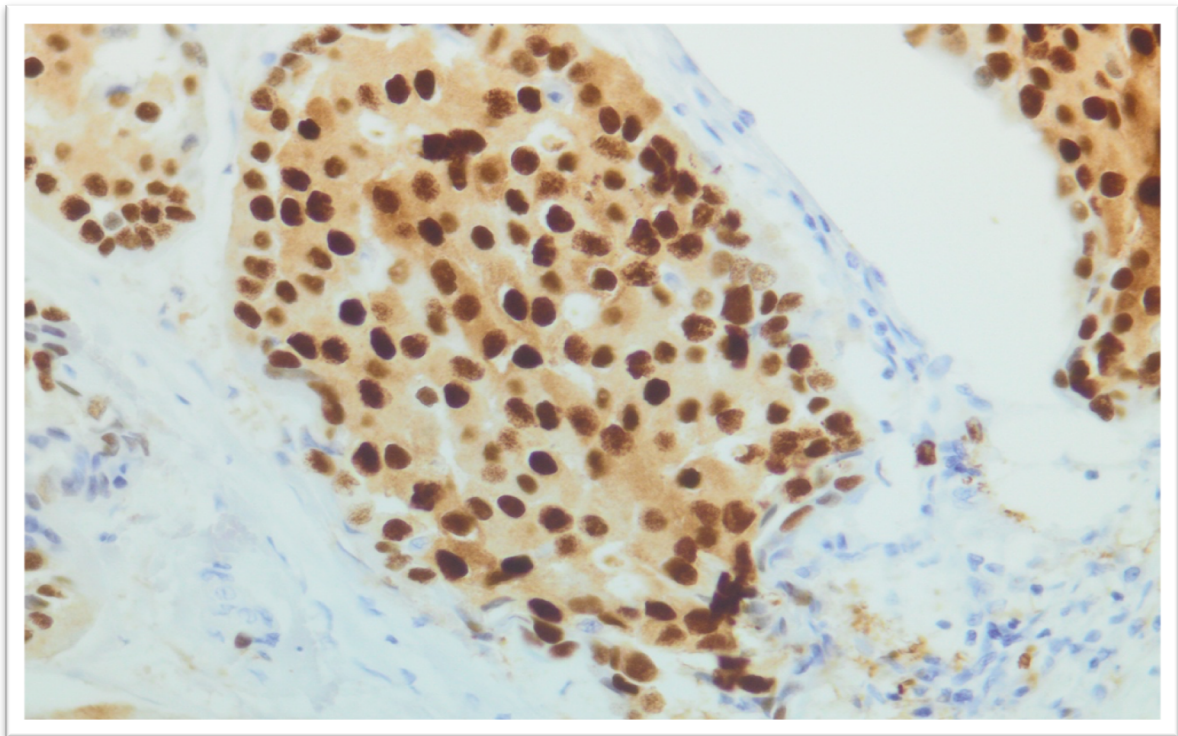


Image 5 - Micrograph at the original magnification of x400 showing a positive PR
immunohistochemical stain:

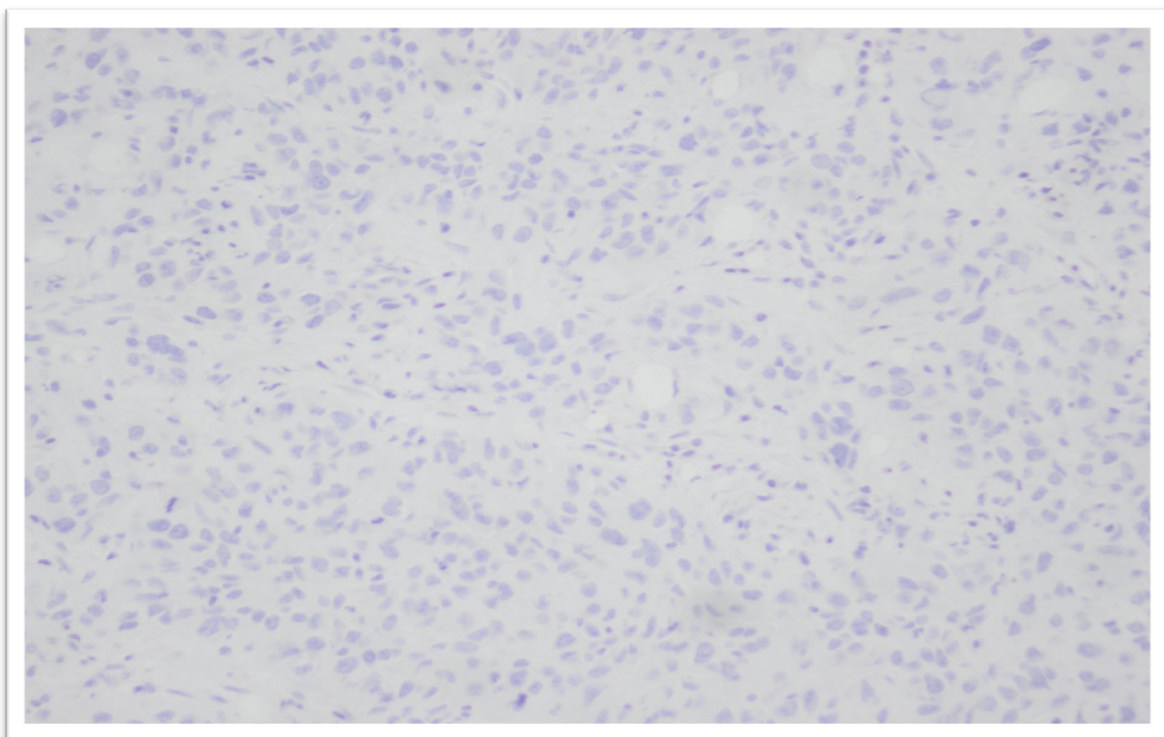


Image 6 - Micrograph at the original magnification of x200 showing a negative PR
immunohistochemical stain:

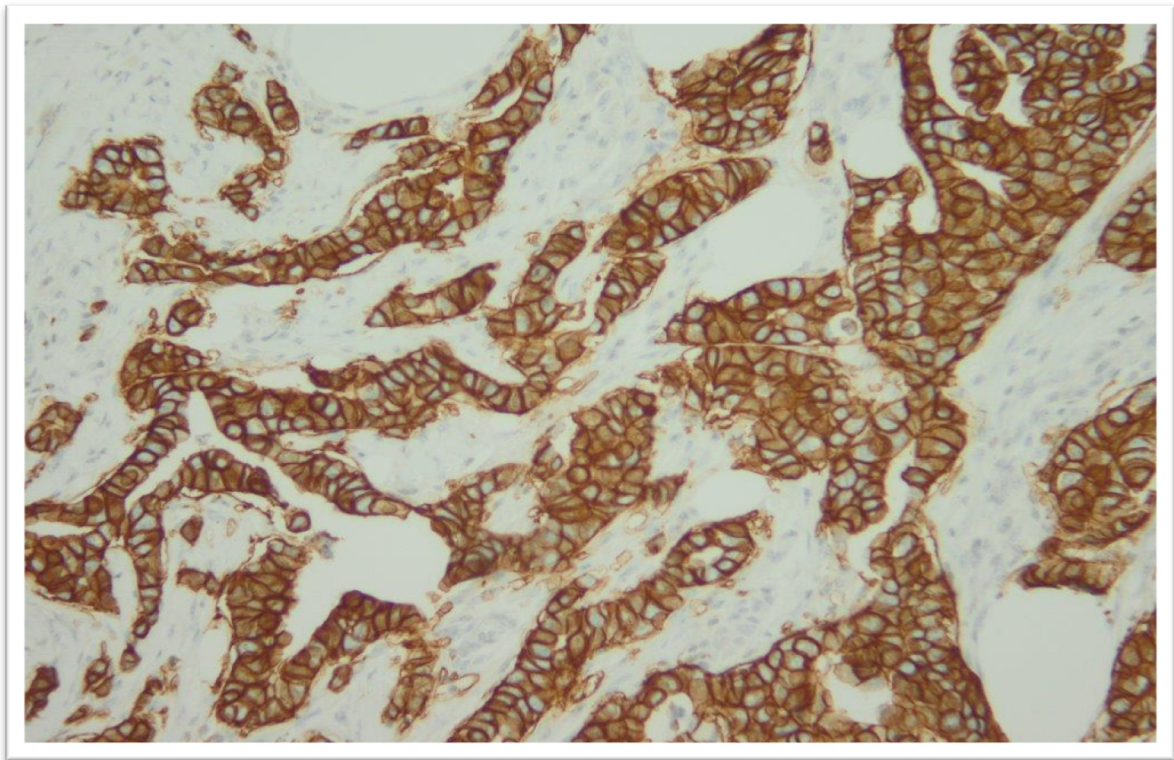


Image 7 - Micrograph showing a positive HER2 immunohistochemical stain (Score 3+) at the original magnification x200:

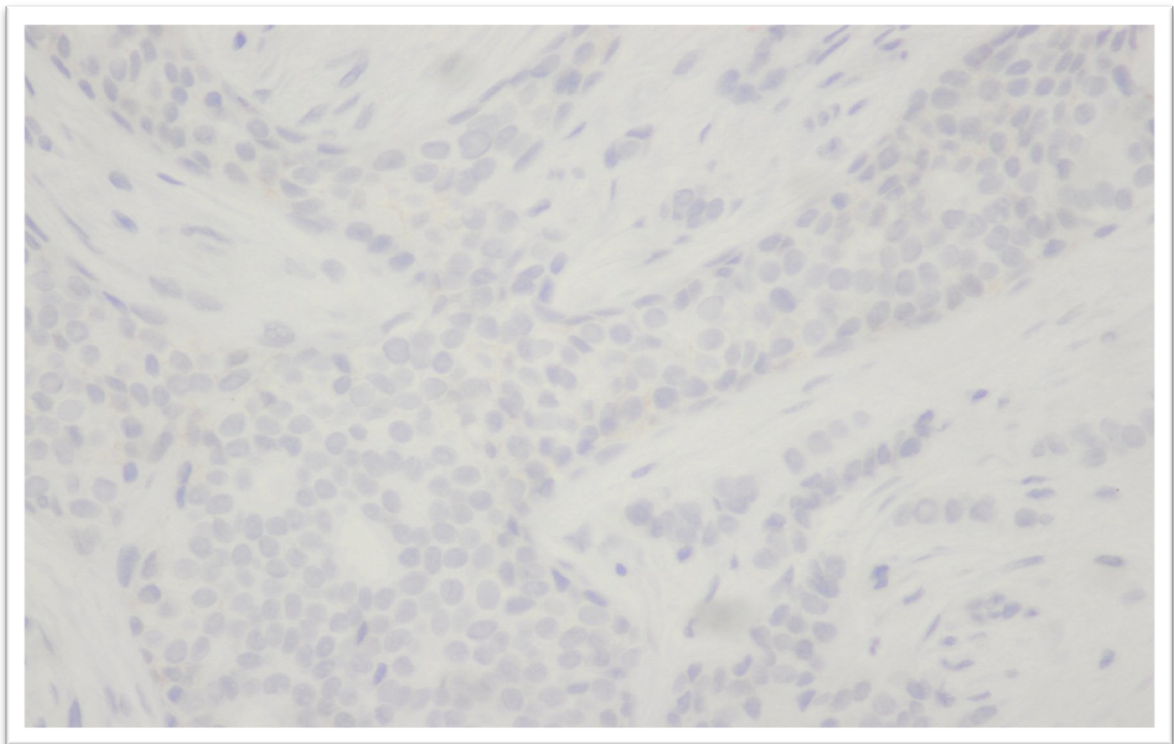


Image 8 - Micrograph at the original magnification of x200 showing a negative HER2 (Score 0) immunohistochemical stain:

With data regarding biomarker status, the tumours were further classified into molecular subtypes including luminal A, luminal B, HER2 amplified, triple negative and luminal-HER2 groups (See figure 12).

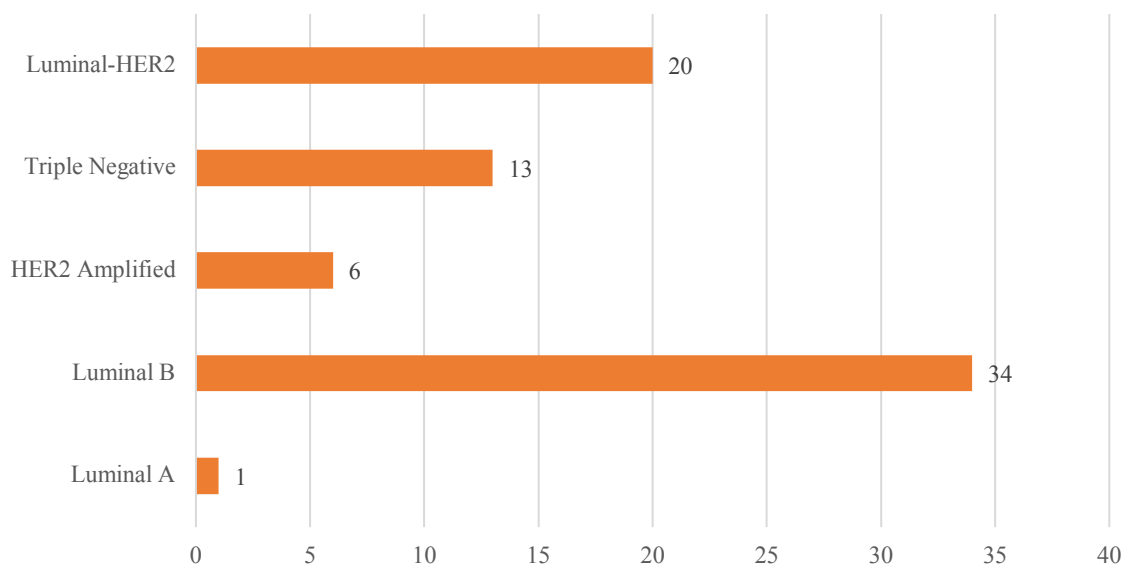


Figure 12 – Graphical Representation of Molecular Subtypes:

The duration from the core biopsy to excision varied from four to twenty-three months (See table 11 below). The average duration was 7.68 months (SD = 2.48). Median duration was 7 months (IQR = 6-9).

Table 11 – Duration between core biopsy and excision biopsy:

Duration (Months):	N (%):
≥ 9	18 (24)
$8 \leq \text{time} < 9$	16 (22)
$7 \leq \text{time} < 8$	16 (22)
$6 \leq \text{time} < 7$	17 (23)
$4 \leq \text{time} < 6$	7 (9)
Total:	74 (100)

5.2.2) Pre-treatment clinicopathological characteristics of patients with a complete pathological response to neoadjuvant chemotherapy:

Of the 74 patients who received neoadjuvant chemotherapy, 7 (9%) had a PCR. The remaining 91% of patients had no response or a partial response to preoperative treatment (See figure 13).

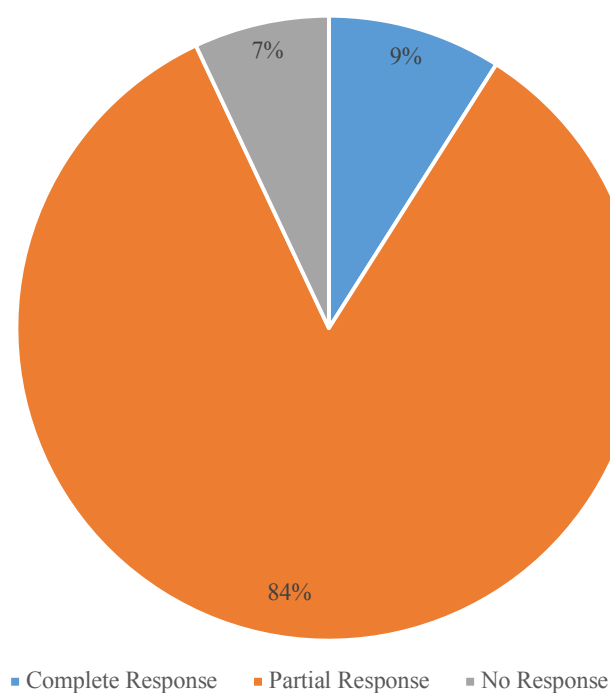


Figure 13 – Graphical Representation of Chemotherapeutic Response:

The pre-treatment clinicopathological characteristics of patients who showed a PCR are presented in Table 12 below:

Table 12 - Clinicopathological features of patients who showed a complete pathological response:

	N (%)
Total	7 (100)
Age	
< 50 years	6 (86)
≥ 50 years	1 (14)

Ethnicity	
Asian / Indian	0 (0)
Black African	6 (86)
Coloured	0 (0)
White / Caucasian	0 (0)
Missing	1 (14)
HIV	
Positive	1 (14)
Negative	5 (72)
Unknown	0 (0)
Missing	1 (14)
Laterality	
Left	6 (86)
Right	1 (14)
Histology	
Invasive carcinoma of no special type	6 (86)
Invasive lobular carcinoma	0 (0)
Mucinous carcinoma	0 (0)
Other	1 (14)
Grade	
1	0 (0)
2	4 (57)
3	3 (43)
ER status	
Positive	3 (43)
Negative	4 (57)
PR status	

Positive	3 (43)
Negative	4 (57)
HER2 status	
0/1+	5 (71)
2+	0 (0)
3+	2 (29)
Ki67	
≥14%	7 (100)
<14%	0 (0)

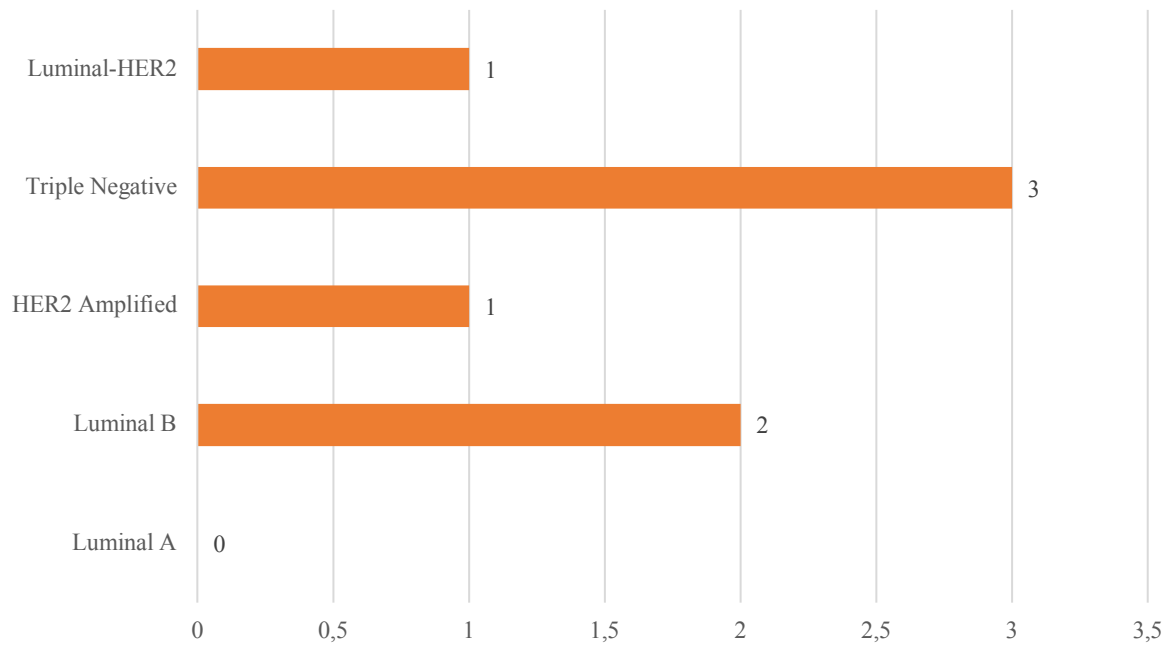


Figure 14 – Graphical Representation of Molecular Subtypes with a Complete Pathological Response to Neoadjuvant Chemotherapy:

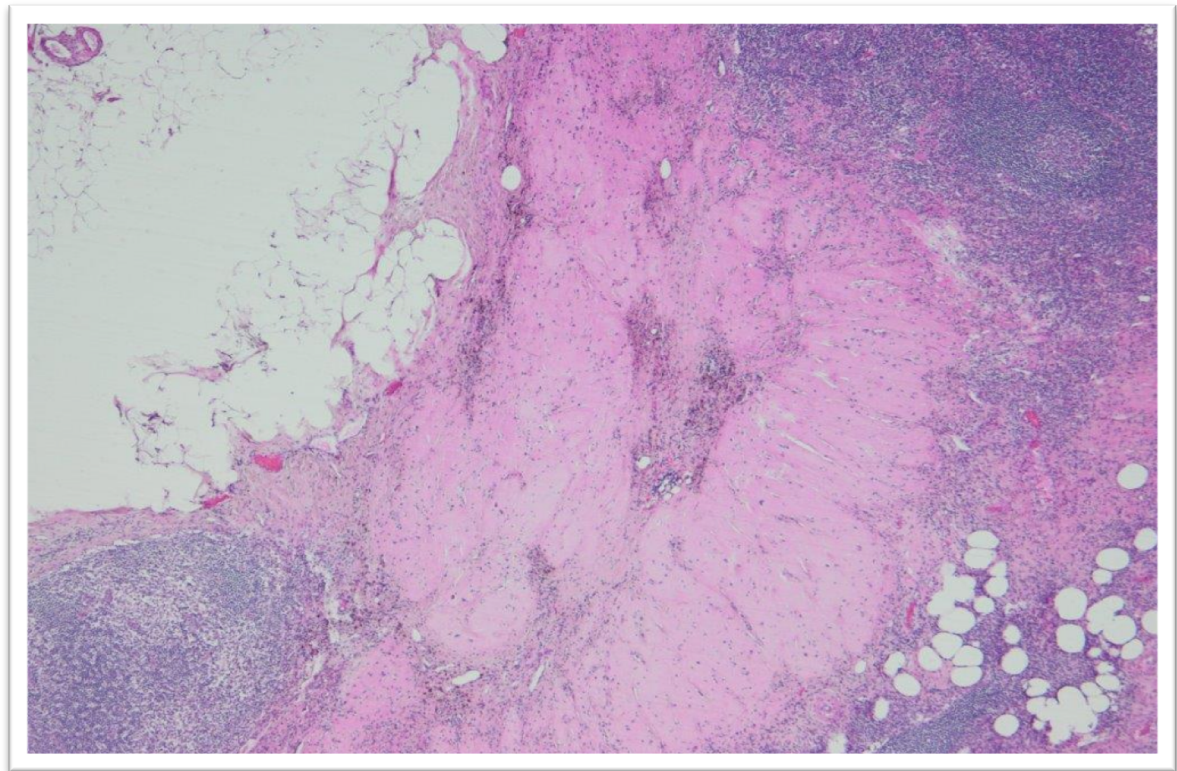


Image 9 - Micrograph at the original magnification of x40 showing hyaline stromal scarring within a lymph node showing complete response to chemotherapy:

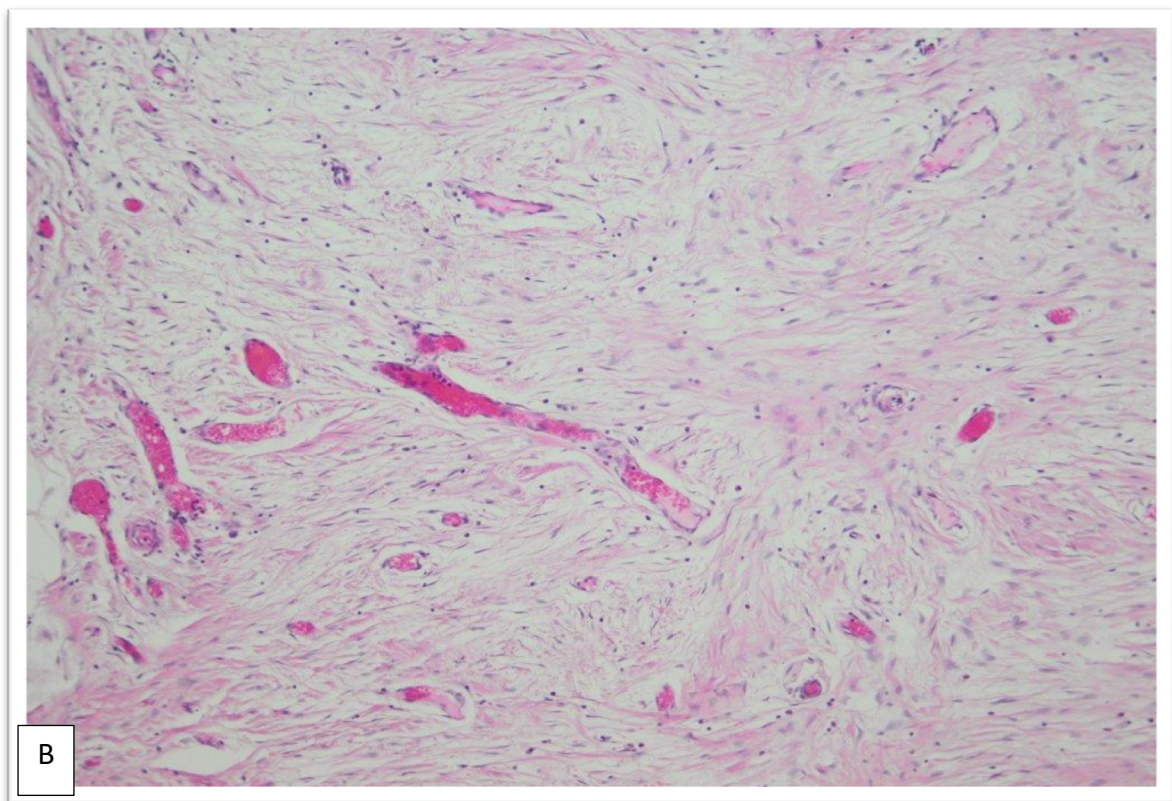
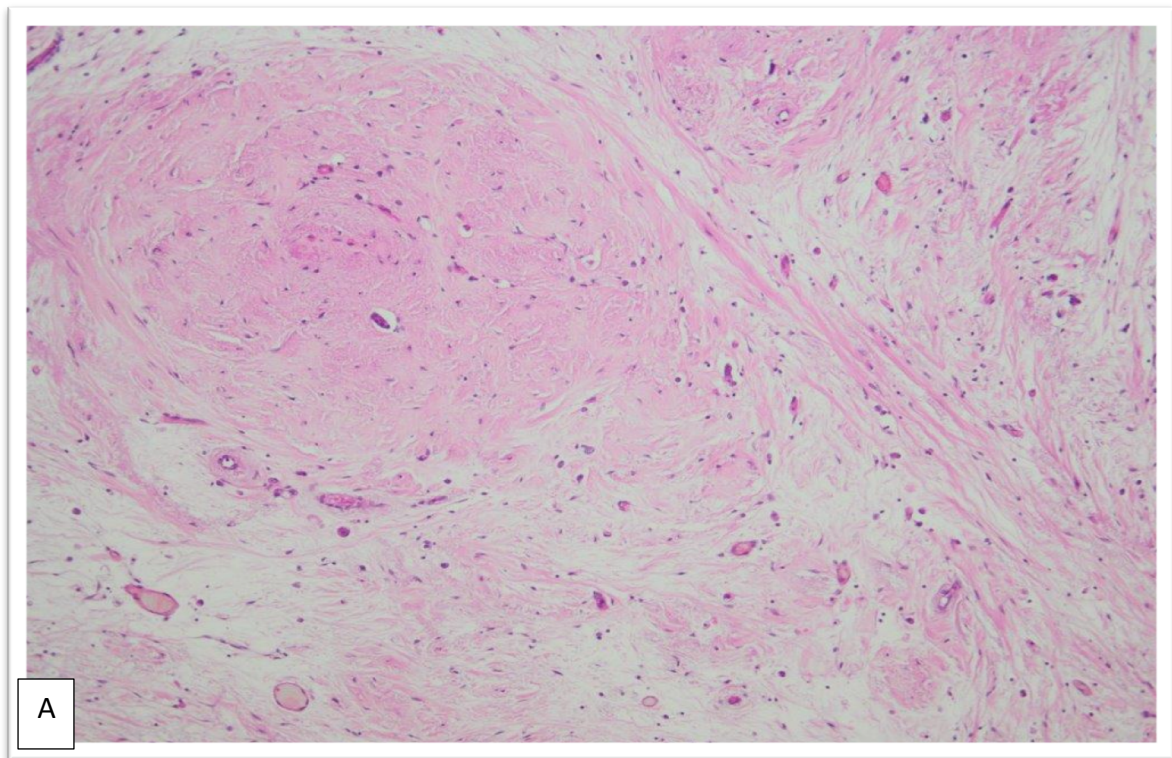


Image 10 - Micrographs at the original magnification of x100 showing a loose, oedematous, vascular fibroelastotic area of connective tissue representing tumour bed in the same lesion having undergone a complete pathological response (A & B).

PCR to chemotherapy was found to be dependent on patient age (Pearson's chi-squared test, $p=0.024$). Six of the seven patients (86%) who showed a complete response were under 50-years of age.

The relation between laterality and PCR was statistically significant (Pearson's chi-squared test, $p=0.037$) with 86% of these tumours being detected in the left breast.

No statistically significant relation exists between histologic tumour type and PCR to chemotherapy (Pearson's chi-squared test, $p=0.858$).

PCR to chemotherapy was found to be independent of hormone receptor immunohistochemical status. The relation between ER (Pearson's chi-squared test, $p=0.061$), PR (Pearson's chi-squared test, $p=0.309$) and chemotherapeutic response was insignificant. Similarly, pathological response to chemotherapy was found to be independent of HER2 receptor status (Pearson's chi-squared test, $p=0.823$). A statistically significant correlation did not exist between Ki67 and tumour response to chemotherapy (Spearman's rank correlation $p=0.845$).

Pathological response to chemotherapy was independent of HIV status. The relation between these variables was insignificant (Pearson's chi-squared test, $p=0.932$). A logistic regression analysis was conducted to predict response to chemotherapy in HIV positive patients. Although the model suggests that HIV positive patients are 34% less likely to have a PCR to chemotherapy, the result was statistically insignificant ($p=0.707$).

5.2.3) Alterations in tumour biology following neoadjuvant chemotherapy:

As immunohistochemical expression profiles could not be repeated, patients who showed a PCR were omitted from further assessment. In addition, tissue for immunohistochemistry from a post-chemotherapy mastectomy specimen repeatedly washed away in one case. After applying exclusion criteria, complete data sets were available for 66 patients.

5.2.3.1) Tumour grade post chemotherapy:

A change in overall histologic grade was identified in 18 patients (27%) (See table 13). An alteration in Grades 1, 2 and 3 was observed in 1 (2%), 9 (14%) and 8 (12%) patients respectively. A reduction in histologic grade from Grade 3 to Grade 2 was observed in 14 (21%) patients following neoadjuvant chemotherapy. There was a reduction in histologic grade from Grade 2 to Grade 1 in 2 (3%) patients.

A total of 9 (14%) patients had an increase in histologic grade following neoadjuvant chemotherapy. An increase in histologic grade from Grade 1 to Grade 2 and Grade 2 to Grade 3 was observed in 3 (4%) and 6 (9%) patients respectively.

Table 13 – Histologic grade before and after neoadjuvant chemotherapy:

Histologic Grade:	Before treatment N (%):	After treatment N (%):
1	4 (6)	3 (5)
2	38 (58)	47 (71)
3	24 (36)	16 (24)

Table 14 – Histologic grade on core and excision biopsy specimens:

Grade Core:	Grade Excision Biopsy:			Total:
	1	2	3	
1	1	3	0	4

2	2	30	6	38
3	0	14	10	24
Total:	3	47	16	66

A statistically significant overall change in histologic grade was not demonstrated (Wilcoxon signed-rank test, $p=0.230$). No statistically significant difference in the scores of the pre- and post-treatment groups was observed for the individual parameters of tubular differentiation, nuclear pleomorphism or mitoses (Stuart-Maxwell test, $p=0.321$, 0.846 & 0.160 respectively) . In addition, no significant change in histologic grade was demonstrated after neoadjuvant chemotherapy when separating patients into those <50 years (Wilcoxon signed-rank test, $p=0.06$) and ≥ 50 years (Wilcoxon signed-rank test, $p=0.796$) of age.

Table 15 – Effect of neoadjuvant chemotherapy on histologic grade in various tumour subtypes:

Histologic tumour type:	Median Grade Post-chemotherapy:	p-value:
Invasive carcinoma of no special type:	2	0.179
Carcinoma other than no special type:	2	0.655

No significant change in histologic grade was demonstrated in various histologic tumour types (See Table 15 above, Wilcoxon signed-rank test).

5.2.3.2) Hormone and HER2 receptor expression before and after chemotherapy:

Twelve patients had a change in ER receptor status. ER receptor status changed from positive-to-negative in 9 (14%) patients. ER receptor status changed from negative-to-positive in 3 (4%) patients.

16 patients had a change in PR receptor status. PR receptor status changed from positive-to-negative in 14 (21%) patients. PR receptor status changed from negative-to-positive in 2 (3%) patients.

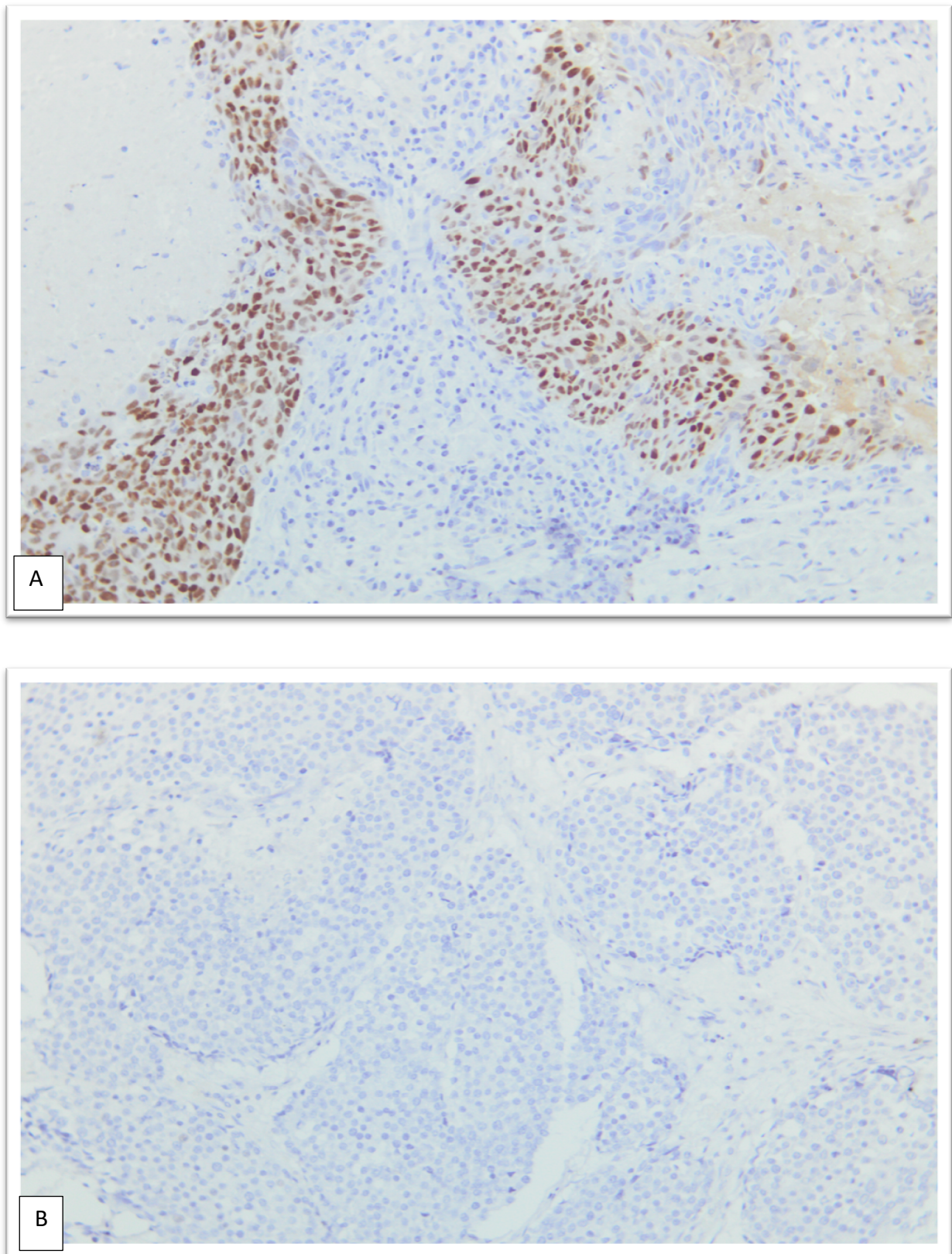


Image 11 - Micrographs showing a change in progesterone receptor status from positive (A) to negative (B) in the same tumour following neoadjuvant chemotherapy at the original magnification.

x200

Table 16 – Hormone and HER2 receptor expression before and after neoadjuvant chemotherapy:

Receptor:		Before treatment	After treatment
		N (%):	N (%):
ER:	Positive	50 (76)	44 (67)
	Negative	16 (24)	22 (33)
PR:	Positive	42 (64)	30 (45)
	Negative	24 (36)	36 (55)
HER2:	Positive	23 (35)	24 (36)
	Negative	43 (65)	42 (64)

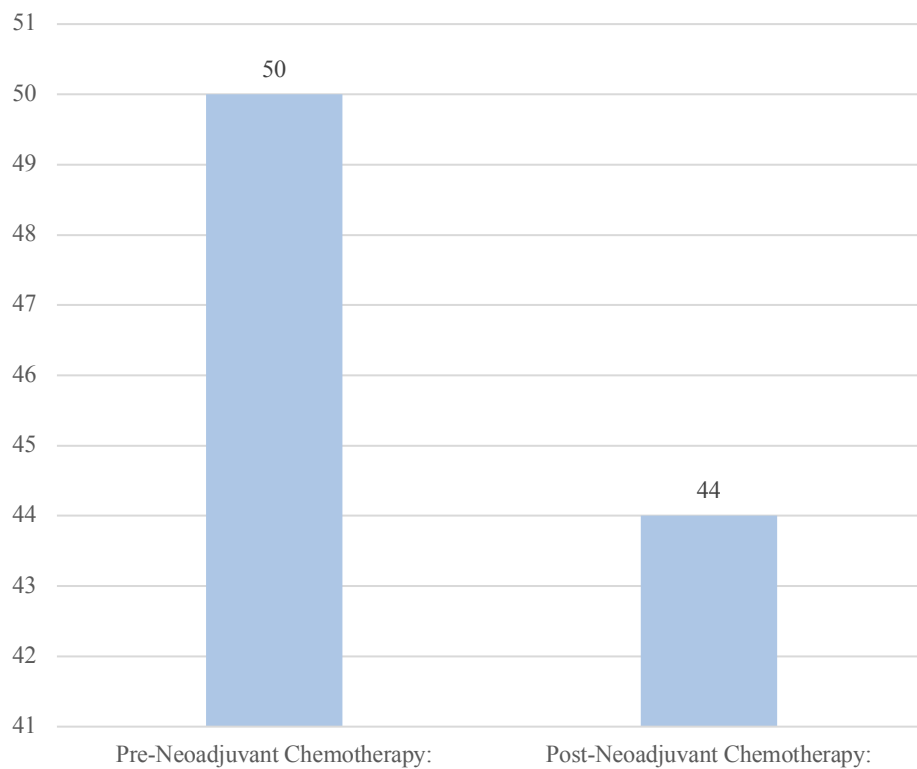


Figure 15 – Graphical Representation Showing Paired Expression of ER:

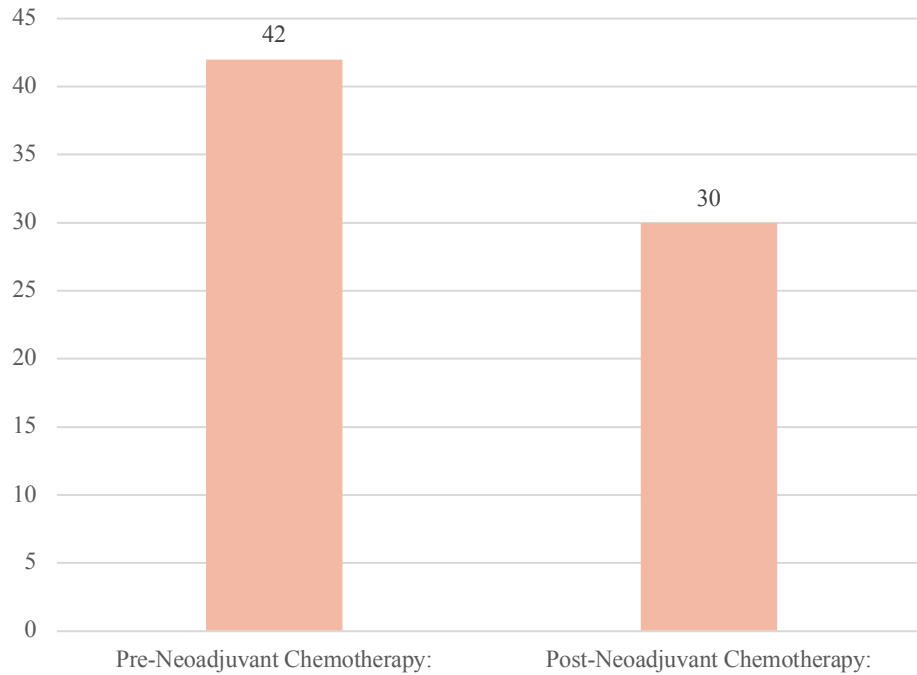


Figure 16 – Graphical Representation Showing Paired Expression of PR:

Table 17 – Treatment related changes of ER and PR:

ER Status:	PR Status:				Total N (%):
	Neg-Neg	Neg-Pos	Pos-Neg	Pos-Pos	
Neg-Neg*	13	0	0	0	13 (20)
Neg-Pos	3	0	0	0	3 (4)
Pos-Neg	5	0	4	0	9 (14)
Pos-Pos	1	2	10	28	41 (62)
Total N (%):	22 (33)	2 (3)	14 (21)	28 (43)	66 (100)

*the first represents the status on the core and the second the status in the excision post neoadjuvant therapy

Hormone and HER2 receptor expression between core biopsy and excision specimen was further assessed using a McNemar's test. A significant reduction in PR expression ($p=0.003$) was found in post-neoadjuvant chemotherapy tumours when compared with the needle core biopsy.

No significant change in receptor status was found for ER ($p=0.08$). A change in HER2 receptor status did not reach statistical significance ($p=0.655$).

5.2.3.3) Ki67 pre and post chemotherapy:

A significant decrease in Ki67 (See figure 17) expression was identified following neoadjuvant chemotherapy (Wilcoxon signed-rank test, $p<0.001$). A reduction in Ki67 from high (≥ 14) to low (<14) was observed in 22 (33%) patients (McNemar's test $p<0.001$).

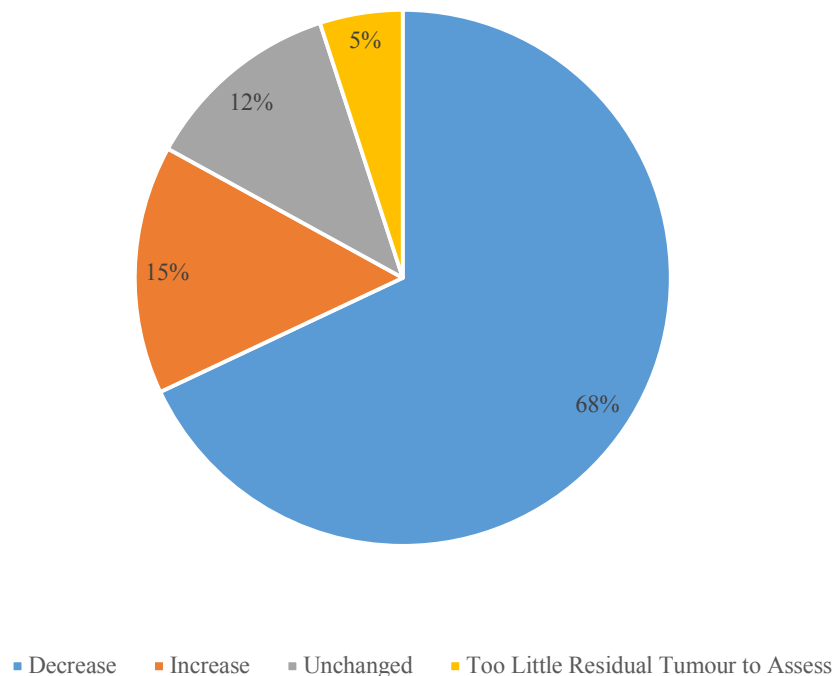


Figure 17 - Graphical Representation Showing Treatment Effect on Ki67:

5.2.3.4) Size of tumour bed:

Data regarding the size of the tumour bed post-neoadjuvant chemotherapy was not uniformly (Kolmogorov-Smirnov, $p < 0.001$) and not normally (Shapiro-Wilk test, $p<0.001$) distributed. The tumour bed had a median size of 45mm (IQR = 23-65) and mean size of 46.4mm (SD = 33.97).

Table 18 – Size range of the tumour bed following neoadjuvant chemotherapy:

Tumour Size Range (mm):	N (%)
$2 \leq \text{tumour size} < 10$	11 (17)
$10 \leq \text{tumour size} < 25$	6 (9)
$25 \leq \text{tumour size} < 45$	15 (22)
$45 \leq \text{tumour size} < 60$	12 (18)
$60 \leq \text{tumour size} < 75$	11 (17)
Tumour size ≤ 75	11 (17)
Total:	66 (100)

Size of the tumour bed was independent of hormone receptor status. The hormone receptor status, as assessed before and after neoadjuvant chemotherapy, showed no statistically significant correlation with the size of the tumour bed (Chi Square test, Table 19 below).

Table 19 – The influence of hormone receptor expression on size of the tumour bed:

Expressed Receptor:	Chi-Square test, p-value:	
	Before Chemotherapy	After Chemotherapy
ER:	0.336	0.485
PR:	0.673	0.074

5.2.3.5) Chemotherapy regimen:

Information regarding chemotherapy regimen was available for 26 patients (35%). Data was missing for the remaining 48 patients (65%). Of the 26 patients for whom information regarding chemotherapy regimen was available, 18 (69%) received sequential doxorubicin/cyclophosphamide followed by paclitaxel (AC-T). Five patients (19%) received cyclophosphamide, doxorubicin and 5-fluorouracil (FAC). Two patients (8%) were involved in a clinical trial. One patient (4%) received a

platinum containing regimen. Due to the limited available data, further stratification of tumour response to specific chemotherapy regimen was not possible.

CHAPTER SIX: DISCUSSION

6.1 Patient demographics

6.1.1) Patient gender:

Majority of CA types often occur more frequently in males relative to females (Cook et al. 2009). With only two percent of newly diagnosed patients seen at CHBAH being of male gender, breast CA is unmistakably the exception. This corresponds with findings from international data sets (Ly et al. 2013). Considered as a disease that occurs predominantly in females, there may be a lack of breast CA awareness in men which may lead to diagnosis at a later age and more advanced stage (Miao et al. 2011).

6.1.2) Patient age:

Patients newly diagnosed with breast CA ranged from 25-years to 94-years in age, highlighting the broad age range in which breast CA can occur. The incidence of breast CA increases with age (See Figure 2). The median age of diagnosis in the present study is 53-years-old. This is similar to the results reported by McCormack et al. and Ruff et al. in their studies of breast CA patients in Johannesburg, South Africa (McCormack et al. 2013; Ruff et al. 2018). This is younger when compared with age reports from the American National Cancer Institute who describe a median age of 62-years (Howlader et al. 2017). A younger age at diagnosis is a finding South Africa shares with other sub-Saharan countries and developing countries in Asia (Hisham and Yip 2003; Son et al. 2006; Fregene and Newman 2005).

Breast CA incidence peaked between the ages of 41-years and 50-years. Consistent with studies from other African countries, breast CA incidence decreases following menopause. This lower postmenopausal incidence is postulated to be as a result of demographics, especially population age and overall life expectancy. In comparison with American women with a life expectancy of 81-years,

the life expectancy of South African women is only 67-years (Fregene and Newman 2005; Adebamowo and Ajayi 2000; WHO 2018).

Although rare, breast CA does occur in very young patients. It has a significant effect on a young patient's life and future which extends to her family and society in general (Kruger and Apffelstaedt 2007). At CHBAH, fifteen patients (4%) were between the age of 25 and 30-years (See Figure 2). Tumours in young patients have more aggressive biologic features. They are more advanced at diagnosis and have a poorer prognosis when compared with tumours that occur in older premenopausal patients (Gonzalez-Angulo et al. 2005; Shannon and Smith 2003).

6.2 Tumour characteristics:

6.2.1) Histologic type:

Consistent with findings from other studies in sub-Saharan Africa, invasive CA of no special type was the most frequently made histopathological diagnosis (86%) (See Figure 3). This is comparable with the histopathologic predominance reported in western populations (Amir, Kitinya, and Parkin 1994; Fregene and Newman 2005; Amir et al. 1997).

As the second most frequent diagnosis (4%), invasive lobular CA is comparable with reports as the most common "special" morphologic subtype of breast CA (Reed et al. 2015). Nonetheless, assessing prevalence of distinct subtypes in Africa is challenging because of the frequency in which advanced-stage tumours are detected. These tumours are often poorly differentiated, having lost distinguishing features (Bjerregaard and Kung'u 1992).

6.2.2) Histologic grade:

Using the Nottingham histologic score, the majority (51%) of breast CA seen at CHBAH was assessed as grade 2 (See Figure 4 and 7). Grade 2 is the most frequent grade reported by the ONCOPOOL European database which includes 16,944 breast CA cases (Blamey et al. 2010). Grade

2 tumours are considered to be comparable to other parameters of intermediate risk significance including tumour dimension between 20mm and 50mm, one-to-three involved lymph nodes and intermediate scores on multigene assays (Goldhirsch et al. 2009).

Assigning histologic grade 2 to a breast tumour, however, is less informative for a clinical assessment of adjuvant chemotherapy than either grade 1 or grade 3. Distinct genetic differences between grade 1 and grade 3 tumours have been identified by applying comparative genomic hybridisation. This suggests that grade 1 and grade 3 tumours are two distinct diseases that have distinct molecular origins, pathogenesis and behaviour (Royslance et al. 1999). Similarly, when applying a gene expression grade index to grade 2 breast CA, patients with grade 2 tumours can be reclassified into two groups. Each group has a different clinical outcome similar to those with histologic grade 1 and 3 tumours respectively. This result challenges the existence and clinical value of an intermediate grade classification (Ignatiadis and Sotiriou 2008).

6.3 Tumour biology and the influence of neoadjuvant chemotherapy:

In the pathologic evaluation of tumours, various elements are essential and assist oncologists in their determination of treatment prospects and patient prognosis. In breast pathology, biologic markers, tumour histologic grade and proliferation index are among factors commonly used. Similar to studies previously performed at CHBAH, majority of patients with breast CA are hormone receptor positive (See Figure 8 and 9) and HER2 receptor negative (See Figure 10) (McCormack et al. 2013).

With the increasing use of preoperative primary chemotherapy, the question has arisen as to what consequence this has on these factors.

6.3.1) Effect of neoadjuvant chemotherapy on hormone receptor status:

In this single site analysis within a South African public sector hospital, a noteworthy proportion of tumours that received neoadjuvant chemotherapy underwent an alteration in immunohistochemical

biologic markers (See Figure 15 and 16). There was an 18% discordance in ER receptor status with the majority of cases (75%) shifting from positive-to-negative. Twenty-four percent of tumours underwent alteration of PR status with the majority (88%) of these tumours changing from PR-positive to PR-negative (Image 11).

The change in receptor status, however, only reached statistical significance for PR ($p=0.003$). Neoadjuvant chemotherapy has no statistically significant effect on expression of ER and therefore no alteration in overall hormone receptor status.

When reviewing previous studies that have considered the potential for hormone receptor status to change following neoadjuvant chemotherapy, conflicting results are observed (Table 6 and 7). Nevertheless, the majority of identified studies have concluded that neoadjuvant chemotherapy does alter hormone receptor status.

The differing conclusions from previous studies can be explained by variances in the regimens of chemotherapeutic agents used and the duration of treatment received. In addition, the values used to define hormone receptor positivity differed between studies with limits of 1% (Yang et al. 2013), 5% (Cockburn et al. 2014) and 10% (Adams et al. 2008) noted. In this study, an Allred score of greater than two (i.e. 1%) was used to reflect hormone receptor positivity. In the Allred system, the survival of patients with tumours demonstrating a receptor score of two (corresponding to less than 1% of weakly staining positive tumour cells) is similar to patients with CAs that are completely negative for ER (Harvey et al. 1999).

The absence of a control group in many studies also raises the possibility that pre-analytical factors and interobserver variability influenced interpretation of biologic markers. Finally, some studies had a very limited number of specimens and therefore study population may have been too small to identify a statistically significant alteration.

The alteration in PR status as the only marker with significant change in expression has been reported by five other authors (Kasami et al. 2008; Kinsella et al. 2012; Gahlaut et al. 2016; Xie et al. 2018; Lee et al. 2013). This alteration in biologic marker status may be explained by technical artefacts resulting in “pseudo-discordance”. Such technical artefacts may include differential sampling of the tumour on the core and surgical specimen. Breast CA is a heterogenous disease demonstrating intra-tumoural heterogeneity. PR in particular is noted to be concentrated more diversely within a tumour (See Image 12). Inconsistency of the PR immunohistochemical stain and intra- and interobserver variability with regards interpretation of the stain are also important factors to contemplate as possible causes for differences in receptor status (Kinsella et al. 2012; Zidan et al. 1997). Studies have also shown that there is a propensity for higher hormone receptor status scoring in needle core biopsies. This may be due to better fixation (Arnedos et al. 2009; Cahill et al. 2006).

In the absence of technical artefacts, neoadjuvant chemotherapy may exert a modulatory effect on hormone and HER2 expression. More specifically, the chemotherapeutic regimen used may have shown a greater degree of cytotoxicity for PR expressing tumour cells (Kinsella et al. 2012). The regimen may have targeted sensitive cells, leaving insensitive PR-negative clones behind. An alteration in receptor status may also occur as a “survival mechanism” of tumour cells, resulting in resistance to a specific agent (van de Ven et al. 2011). In addition, downregulation of intra-tumoural aromatase expression by the cytotoxic activity of certain taxanes may result in the suppression of intra-tumoural oestradiol synthesis thereby causing suppression of PR expression (Miyoshi et al. 2004). Finally chemotherapy may lower circulating levels of oestrogen thereby causing downregulation of hormone receptors, including PR (Bines, Oleske, and Cobleigh 1996).

The alteration in PR status may be unrelated to chemotherapy and simply reflect “loss of PR” over time (Broom et al. 2009). ER-positive / PR-negative tumours have higher levels of EGFR expression. This increased EGFR expression may implicate high growth factor activity with a decrease in expression or complete loss of PR (i.e. ER independent growth factor-mediated downregulation of

PR) (Arpino et al. 2005; Cui et al. 2005). Other hypothesised mechanisms include genetic loss of the PR gene locus or hypermethylation of the PR promoter (Lapidus, Nass, and Davidson 1998).

Change of PR status in the absence of alteration in ER expression following neoadjuvant chemotherapy remains of undetermined clinical significance and requires further research (Kinsella et al. 2012).

The consequence of having hormonal therapy withheld in patients who have undergone a positive-to-negative alteration in ER expression is unknown as no published literature has been identified.

Hormone therapy after surgery in these patients may be less effective. Although denying patients treatment can be considered by many as unethical, the use of any medication is not without risk and an association between tamoxifen use and endometrial CA is well documented (Fisher et al. 1994). With authors of similar studies advocating the continued use of endocrine therapy, deliberation is required regarding the need for repeat hormone receptor status in patients who have ER-positive tumours prior to neoadjuvant treatment (Gahlaut et al. 2016).

Repeat biomarker testing is recommended in ER-negative CA as a change in hormonal status may influence treatment strategy and identify those who may benefit from endocrine therapy.

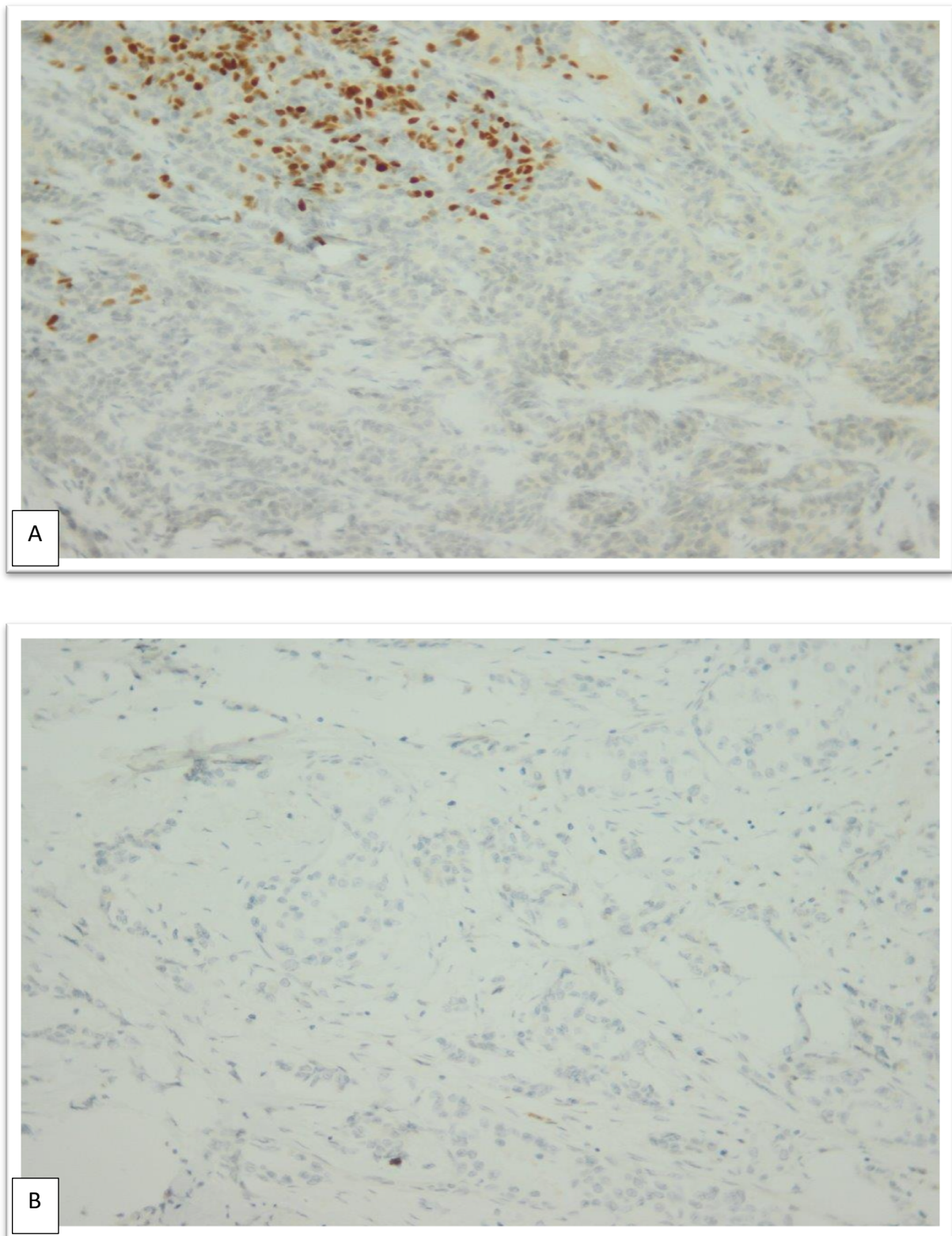


Image 12 - Micrographs showing a change in progesterone receptor status from positive (A) to negative (B) in the same tumour following neoadjuvant chemotherapy at the original magnification, x100 (A) and x200 (B). Note the intra-tumoural heterogeneity on the core biopsy (A). This receptor status alteration may reflect a “pseudo-discordance” due to differential sampling.

6.3.2) Effect of neoadjuvant chemotherapy on HER2 receptor status:

In this analysis, only a single patient (2%) showed alteration in HER2 receptor status following neoadjuvant chemotherapy. In this case, a shift from negative-to-positive was noted. HER2, therefore, appears to be more stable in response to chemotherapy. As illustrated in table 9, this is in agreement with many previous studies concluding that neoadjuvant chemotherapy does not change the HER2 receptor status (Kasami et al. 2008; Taucher et al. 2003; Bottini et al. 1996; Faneyte et al. 2003; Arens, Bleyl, and Hildenbrand 2005; Varga, Caduff, and Pestalozzi 2005; Penault-Llorca et al. 2003; Lee et al. 2013).

Unfortunately, due to its prohibitive cost and the limited resources within our setting, trastuzumab is not available in the neoadjuvant setting. Nevertheless, research shows that when neoadjuvant chemotherapy is used in combination with trastuzumab, a loss of between 32% and 43% of HER2 amplification in the residual CA has been reported (Mittendorf et al. 2009; Hurley et al. 2006).

The possibility that the isolated alteration in HER2 receptor status observed in this study is a false immunohistochemical result has strongly been considered. False-positive results can occur due to unique technical artefacts on HER2 immunohistochemistry. Factors commonly misinterpreted include edge artefact, cytoplasmic positivity and overstaining due to improper antibody titration. In addition, high grade DCIS is often HER2 positive. Therefore, misinterpretation of DCIS may also produce a false-positive result. Alternatively, a false-negative result may have been reported on the initial biopsy, justified by tumour sampling and heterogeneity (Fitzgibbons et al. 2014). Ideally, both the preoperative core and excision specimen should be re-evaluated using in situ hybridization (FISH).

The findings from this study suggest that repeating HER2 receptor status following neoadjuvant chemotherapy may be of little value especially in the absence of pre-operative trastuzumab.

Repeating HER2 status should be limited to those with a negative pre-operative result.

6.3.3) Effect of neoadjuvant chemotherapy on Ki67:

With regards Ki67, a significant reduction in proliferative activity was observed following neoadjuvant chemotherapy ($p < 0.001$) (Figures 11 and 17). This is in line with findings from similar studies (Xie et al. 2018; Dede et al. 2013; Burcombe et al. 2005; Arens, Bleyl, and Hildenbrand 2005; Koda et al. 2007; Bottini et al. 2001, 1996; Lee et al. 2013).

Tumour cell proliferation is reduced due to the microtubule stabilization effect induced by taxane containing chemotherapy regimens (Schiff, Fant, and Horwitz 1979; Horwitz 2004). A higher proliferative activity renders breast CA cells more chemo-sensitive. The newly formed CA cells are less viable due to the structural damage incurred during DNA synthesis (Makris et al. 1999; Honkoop et al. 1997; Schneider et al. 2000). A significant reduction in Ki67 is vital as it correlates with a better disease free and overall survival (Cabrera-Galeana et al. 2018).

Despite an overall reduction in Ki67 expression, 61% of tumours continued to have a high proliferative index (Ki67 greater or equal to 14%) following neoadjuvant chemotherapy. This is suggestive of a more aggressive malignant phenotype (Bottini et al. 2001). Other studies have shown that Ki67 measurement following neoadjuvant chemotherapy has more value than simply to assess for a treatment induced change. Patients with a high Ki67 proliferative index at surgery have a high risk of relapse for the first three years following diagnosis (von Minckwitz et al. 2013).

The use of Ki67 however, is controversial and continues to generate robust debate especially for its potential for significant intra- and interobserver variability. This is largely due to the lack of agreement on scoring, confusion as to the definition of low versus high expression, the absence of an appropriate cut point for positivity and the uncertainty as to what part of the tumour should be scored. Assessing Ki67 proliferation index at the “leading edge, hot spots or taking the overall average” remains unclear for many pathologists. In addition, the manual counting of cells is labour intensive, time consuming and subjective. Furthermore, there are no recognised “quality assurance schemes in place to ensure that Ki67 analysis in one laboratory leads to scores comparable to those in others”.

Regarding the effect of pre-analytic variables on Ki67, data is limited. “Consequently, routine testing of breast CA for Ki67 expression is not currently recommended by either ASCO or the National Comprehensive Cancer Network (NCCN)” (Dowsett et al. 2011; Fitzgibbons et al. 2014).

The inconsistency in Ki67 interpretation is emphasised when one analyses the results of the current report’s re-evaluation pilot study. Ki67 was the only biomarker with an interobserver discrepancy (ANNEXURE J).

Despite its controversy, Ki67 continues to be used by pathologists in Johannesburg. This is mainly due to the significant value oncologists in the area have assigned to its use. Robust prognostic information proposed by the Saint Gallen Consensus Meeting and its central role in differentiating luminal A from luminal B ER-positive breast CA assists oncologists and influences their decision on the need for cytotoxic chemotherapy (Coates et al. 2015; de Azambuja et al. 2007).

In order to reduce intra- and interobserver variability in Ki67 interpretation, standardised measurement methodologies must be adopted. A departmental protocol with clear guidelines and recommendations is mandatory.

As an alternative to manual scoring of whole sections, virtual dual staining and interpretation with digital image analysis software is essential to consider and is likely to be used with increasing frequency in the future. In combination with a cytokeratin stain, image analysis has proven to be an accurate method for the determination of Ki67 proliferation index (Koopman et al. 2018; Røge et al. 2016). Current barriers to this approach however, may include the high cost of hardware, software and system maintenance. Other important obstacles to consider may include digital slide storage, image quality, validation, regulatory hurdles, time spent in training and pathologist reluctance for its adoption (Pantanowitz, Farahani, and Parwani 2015).

An unconventional approach worth considering is employing cytotechnologists to interpret and report Ki67 assays. Cytotechnologists are highly skilled morphologists trained to distinguish neoplastic from non-neoplastic cell populations. Manual counts of Ki67 index by cytotechnologists in surgical pathology specimens have been found to be more accurate when compared with eyeball estimates by pathologists (Cottenden et al. 2018).

As Ki67 acts as a dynamic biomarker in assessment of treatment effectiveness in the neoadjuvant setting, repeating Ki67 is recommended. In patients who do not achieve a PCR, a high proliferation index is associated with a poor outcome. It is important therefore to recognise these patients as they may benefit from non-cross resistant adjuvant chemotherapy schedules (Jones et al. 2009).

6.3.4) Effect of neoadjuvant chemotherapy on histologic grade:

With regards histologic grade, assessment is dependent upon architectural and cytologic features including tubular differentiation, nuclear pleomorphism and mitotic activity.

Twelve percent of cases underwent an increase in nuclear grade following neoadjuvant chemotherapy. This increase in nuclear grade is a commonly described histologic effect of neoadjuvant chemotherapy. Nevertheless it failed to reach statistical significance in this study. A substantial proportion of tumours (47%) had grade 3 nuclear features that remained unchanged following neoadjuvant chemotherapy. There has been no correlation between the morphological changes that occur following neoadjuvant chemotherapy to increase nuclear grade and disease free survival period (Close, Tiltman, and Hacking 1987).

Neoadjuvant chemotherapy caused a reduction in mitotic activity in 24% of tumours ($p=0.160$). The decrease in mitotic activity can be explained by the effect of microtubule targeting agents such as taxanes. These drugs function by “suppressing spindle microtubule dynamics, thus inhibiting the metaphase-to-anaphase transition and preventing cell cycle progression and cellular proliferation” (Jordan and Wilson 2004).

Overall, histologic grade showed no statistically significant alteration and remained unchanged in 62% of tumours. This correlates with findings from previous studies (Moll and Chumas 1997).

6.3.5) Effect of neoadjuvant chemotherapy on pathological response:

PCR is defined in this study as the absence of residual tumour in the breast and lymph nodes following preoperative chemotherapy.

Neoadjuvant chemotherapy resulted in PCR in 9% of tumours (Figure 13). The majority (43%) of these tumours were of triple negative type. In comparison to other types of breast CA, higher rates of PCR have been described in triple negative breast CA (Denkert et al. 2017; Hahnen et al. 2017; Sikov et al. 2015; Byrski et al. 2010). Triple negative breast CA is characterised by a heterogenous phenotype with the majority of tumours being high grade invasive CA of no special type. Nevertheless, there is a significant proportion of relatively rare histology subtypes that also lack expression of hormone and HER2 receptor status. Such histotypes include medullary, metaplastic, adenoid cystic and apocrine CA (Palma et al. 2015; Nakashoji et al. 2017).

Using gene expression profiles, triple negative breast CA can be divided into six subtypes including “basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor subtype”. The basal-like 1 subtype is associated with the highest PCR rate (Lehmann et al. 2011).

Clinically, complete response in triple negative breast CA has been described in patients with less advanced tumours demonstrating less frequent mammographic calcifications. Pathologically, sensitivity of these tumours to chemotherapy has been noted in invasive CA of no special type with a negative androgen receptor status and high Ki67 (Nakashoji et al. 2017).

Comparable to the findings of similar studies, tumours of luminal A subtype are the least likely to undergo complete response (0%) (Zhao et al. 2015; Liedtke et al. 2008; Haque et al. 2018). Luminal A tumours formed a very small group within this study. Due to the poor response to neoadjuvant chemotherapy, many patients with luminal A tumours undergo primary surgery.

Five patients (7%) were reported to have no response to chemotherapy. These tumours had a high histologic grade and proliferative index. Of interest, these are features shared with tumour characteristics commonly associated with likelihood of a PCR (Faneyte et al. 2003). This may indicate that morphologically comparable, highly proliferative CA may contain two subpopulations – one that is highly sensitive to chemotherapy and one that is highly chemotherapy resistant (Caudle et al. 2010). In contrast to other studies that found these tumours to be largely triple negative however, 80% of tumours that showed no response to chemotherapy in this study had a positive HER2 receptor status. This emphasizes the critical need for neoadjuvant trastuzumab in our setting.

Research has shown that patients with a PCR have an improved outcome (Rastogi et al. 2008; Montagna et al. 2010). When considering molecular subtypes, improved disease free survival is described in luminal B, HER2 positive and triple negative breast CA that show PCR (von Minckwitz et al. 2012).

6.4 Limitations of this study:

An important limitation of this study is the absence of a non-interventional control group. Thus, part of the variation in hormone receptor expression identified in this study may either be attributed to interobserver variability or sampling error within a heterogenous tumour population. Of note, studies have reported a perceived alteration in hormone receptor status of between 5% and 6% in the absence of intervening preoperative treatment. This has been attributed to sampling (Piper et al. 2004; Lee et al. 2003). Nevertheless, if 5% is deducted from the 24% alteration identified for PR in this study, approximately 19% of PR receptor modulation could be attributed to neoadjuvant chemotherapy. As

previously discussed, there is excellent concordance with regards hormone and HER2 receptor status between core biopsy and excision specimen (Hodi et al. 2007; Dekker et al. 2013; Wood et al. 2007). In addition, findings from other studies with control groups of up to 180 patients failed to demonstrate any difference by sequential steroid hormone receptor assessment (Taucher et al. 2003).

A significant limiting factor compromising the strength of this study is incomplete data sets. Follow-up data could not be found for 209 patients diagnosed with invasive CA on core biopsy. The reason for this is likely to be multifactorial. A high mortality rate has been reported among African breast CA patients. Thus, patient demise may explain a proportion of the missing data. Various factors have been postulated for higher mortality rate including delayed presentation and predisposition to biologically more aggressive tumours (Fregene and Newman 2005).

Paper-based artefacts and poor penmanship have “historically plagued” medical professionals (Rodríguez-Vera et al. 2002). Errors made whilst completing requisition forms or during the capturing of patient and specimen details on the NHLS TrakCare system is likely to have had an impact on data retrieval. The innocuous omission or addition of a single character in a patients name or hospital number; or the use of a second name, nickname or maiden name causes considerable difficulty in attempts to review patient history. A possible solution would be to adopt a standard operating procedure in which the inclusion of the patient’s South African identification (ID) number is mandatory for specimen registration.

Looking to the future, a hospital workflow automation system and electronic health record-based referral system has many advantages. Studies have shown that the use of electronic health record systems improved clinical, organizational and societal outcomes including allowing for the improved ability to conduct research (Menachemi and Collum 2011).

Similar difficulties were experienced retrieving data on chemotherapy regimen. Information from the database was missing for 65% of patients. As a result, biomarker alteration could not be stratified

according to chemotherapy regimen. In addition, no follow-up data was available and consequently, the prognostic impact of receptor status modification could not be evaluated.

This study has been performed within a single site in Johannesburg, South Africa, thus potentially generating a sampling bias. The findings of this study may not be generalizable to other regions, particularly more rural areas in the country. Nevertheless, CHBAH is a referral centre. As such, it caters to a diverse population group including patients from nearby clinics and patients referred from hospitals in neighbouring provinces.

A particular disadvantage associated with cross-sectional studies is that results have been generated from data sets assigned for other purposes. In this particular study, data was collected from authorised pathology reports. As such, confounding factors such as fixation times have not been assessed and causality cannot be inferred.

These caveats notwithstanding, the results of this study show that neoadjuvant chemotherapy causes a reduction in PR expression and Ki67 index. The significance of neoadjuvant chemotherapy induced changes in hormone receptor expression should be further studied with specific attention given to its prognostic value.

6.5 Recommendations for pathologists:

In order to avoid false-positive and false-negative results when interpreting hormone receptor status, it is essential that pathologists take note of the staining of normal breast duct epithelium. The non-neoplastic breast epithelium serves as a valuable positive internal control. The immunohistochemical stain must be repeated if non-neoplastic breast epithelial cells are negative. In addition, the ASCO/CAP guidelines recommend that a “test be repeated on another block or subsequent specimen if normal cells are not present and the test result is negative” (Fitzgibbons et al. 2014).

External controls are valuable additions as they confirm that the reagent used has been distributed correctly onto the slide with the tissue sample. The external control must stain as expected. Finally, pathologists must show insight when interpreting a result and should correlate the findings in light of the histologic type and grade of CA. Discordant results, such as an ER-negative low-grade CA, should prompt one to repeat the study (Fitzgibbons et al. 2014).

With regards Ki67, only nuclear staining plus mitotic figures should be incorporated into the score. As with all stains, it is essential to have positive internal controls which may be identified within normal duct cells, lymphocytes, endothelial cells and stromal cells (Dowsett et al. 2011).

Ki67 staining pattern may be homogenous or heterogenous. “If a homogenous staining pattern is noted, scoring should incorporate the average of at least three randomly selected high power (x40 objective) fields. With regards heterogenous Ki67 staining, two patterns exist – a gradient of increased staining towards the tumour edge and tumour hot spots. For the former, it is recommended that three fields from the tumour edge be assessed. Assessing the whole section and recording the overall average is recommended with regards Ki67 hot spots” (Dowsett et al. 2011).

Although a 14% Ki67 cut-off was previously endorsed in St Gallen to separate luminal B CA from luminal A CA, a new cut-off of 20% is recommended by the 2013 St Gallen conference (Goldhirsch et al. 2013).

CHAPTER SEVEN: CONCLUSION

Breast CA is the most common malignancy in women worldwide with more than one million newly diagnosed patients each year. As an already noteworthy cause of death, CA incidence is projected to rise as society grows and ages. It will become an even larger crisis in developing countries as epidemiological transition continues to shift the burden of disease from infectious to non-communicable diseases (Ferlay et al. 2015; Ginsburg et al. 2017). Through revolutionary advances in CA diagnostics, pathologists are now moving towards the management forefront with sophisticated laboratory techniques to aid oncologists with targeted therapies (Stone et al. 2003; Melmed 2006).

Accuracy of immunohistochemical biomarker assessment in breast pathology is essential. In addition to being therapy predictive and prognostic, the biologic markers ER, PR, HER2 and Ki67 can also be used as surrogate markers to gene expression profiling of breast tumours with potential to predict long-term outcome (Carey et al. 2006).

With the capability to down-stage large tumours or offer patients the option of breast conserving surgery, neoadjuvant chemotherapy is a well-established treatment modality likely to be used with increasing frequency. This approach has allowed treating physicians, surgeons and pathologists the opportunity to evaluate treatment response in vivo, thus opening the door for further research (Jones and Smith 2006).

The outcome that primary chemotherapy has on hormone status, HER2 receptor status and Ki67 has generated considerable literature with studies reporting conflicting results. No such research has been published from South Africa however. This study adds to existing data and provides valuable information from the African continent.

Following neoadjuvant chemotherapy, hormone receptor status should be re-evaluated in the surgical excision specimen as an alteration in biomarker status can occur. In current practice, repeating HER2

status may be of limited value in the absence of neoadjuvant trastuzumab. The assessment of Ki67 is controversial. A departmental protocol with clear guidelines is required.

The clinical relevance and prognosis of patients who demonstrate an alteration in biomarker expression requires further investigation.

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ANNEXURE A – Project specific ethical clearance certificate

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Tools

Project Specific E... x



R14/49 Dr Micheal Jared Bromfield

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M151048

NAME: Dr Micheal Jared Bromfield
(Principal Investigator)

DEPARTMENT: Anatomical Pathology
Chris Hani Baragwanath Academic Hospital

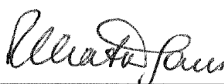
PROJECT TITLE: Alterations in Immunohistochemical Biologic Markers
Following Neoadjuvant Chemotherapy in Breast Carcinoma
(M150555)

DATE CONSIDERED: Adhoc

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Reena Mohanlal

APPROVED BY: 
Professor P Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 09/11/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature _____

Date _____

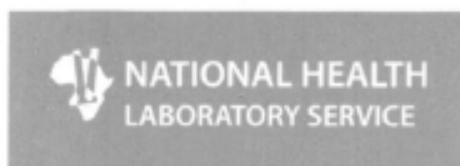
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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🔔

Sign In

ANNEXURE B – Permission regarding the use of the NHLS database



MEMO

Chris Hani Baragwanath Academic Business Unit
Chris Hani Baragwanath Road
Diepkloof
Soweto
Tel:011 48923/92

Dr. Michael J. Bromfield
Histopathology
Chris Hani Baragwanath Academic Business Unit
NHLS

Re: Request to consent to review all relevant slides from archives

Dear Dr. Bromfield

This letter serves to confirm that permission has been granted for you to review all relevant slides from the CHBAH Histopathology archives to attain related information pertaining to your MMed from our Track Care LIS.

Kind Regards

 14/8/15

Violet Gabashane
Academic Business Manager
Chris Hani Baragwanath Academic Business Unit

To whom it may concern:

This document serves to confirm that I Violet Gabashane, Business Manager of the National Health Laboratory Service (NHLS) at Chris Hani Baragwanath Academic Hospital, give Dr. Michael Bromfield, student number 0300696E, permission to review all relevant slides from our archives and attain related information pertaining to his MMed from our Trakcare database.

Kind regards

 14/8/2015

Violet Gabashane
Business Manager
National Health Laboratory Service (NHLS)
Chris Hani Baragwanath Academic Hospital
12 August 2015

ANNEXURE C – Permission to use the CHBAH breast unit database

To whom it may concern:

15.6.2015

This document serves to confirm that I Dr. H. Cubasch, Head of Breast Unit at Chris Hani Baragwanath Academic Hospital, give Dr. Michael Bromfield, student number 0300696E, permission to use all relevant clinical information pertaining to his MMED from our database.

Kind regards



Dr. H. Cubasch
Head of Breast Unit
Chris Hani Baragwanath Academic Hospital
15/06/2015

ANNEXURE D – Interpretation of hormone receptor status

The Allred score (Allred et al, 1998):

Proportion Score:	Positive nuclear staining (%):	Intensity:	Intensity Score:
0	0	None	0
1	<1	Weak	1
2	1 - 10	Intermediate	2
3	11 - 33	Strong	3
4	34 - 66		
5	67 – 100		

The 2 scores were added together for a final score with 8 possible values.

Scores of 0 and 2 : **Negative**

Scores of 3 to 8 : **Positive**

ANNEXURE E – Histologic Grade

Nottingham Grading System (Elston & Ellis, 1991):

The Nottingham grading system considers tubule differentiation, mitotic count and nuclear pleomorphism. Each component was given a score of between 1 & 3. The scores were added to give a grade.

Feature:	Score:
Tubule formation: ■ Majority of tumour (>75%) ■ Moderate (10-75%) ■ Minimal (<10%)	1 2 3
Nuclear pleomorphism: ■ Small regular nuclei ■ Moderate increase in size ■ Marked variation in size, nucleoli, chromatin clumping	1 2 3
Mitotic count (25x objective with field diameter of 0.59mm): ■ Less than 10/10 HPF ■ 10-20/10 HPF ■ >20/10 HPF	1 2 3
Mitotic count (40x objective with field diameter of 0.44mm): ■ <5/10 HPF ■ 6-10/10 HPF ■ >10/10 HPF	1 2 3

The total score was added and the grade assigned as follows:

Grade I : 3 to 5 points

Grade II : 6 to 7 points

Grade III : 8 to 9 points

ANNEXURE F – Interpretation of HER2 receptor expression

American Society of Clinical Oncology (ASCO) / College of American Pathologists (CAP) HER2 test guideline recommendations (Wolff et al, 2013):

NEGATIVE:

Immunohistochemistry:

- 0 : defined as no staining or incomplete, faint/barely perceptible membrane staining in $\leq 10\%$ of invasive tumour cells,
- 1+ : defined as weak, incomplete, faint/barely perceptible membrane staining in $>10\%$ of invasive tumour cells,

In situ hybridization negative (ISH):

- Single-probe average HER2 copy number < 4.0 signals/cell.
- Dual-probe HER2/CEP17 ratio < 2.0 with an average HER2 copy number < 4.0 signals/cell.

EQUIVOCAL:

- 2+ : defined as incomplete membrane staining that is weak/moderate or non-uniform in $>10\%$ of tumour cells or intense complete staining $\leq 10\%$ of tumour cells

POSITIVE:

Immunohistochemistry:

- 3+ : intense, complete circumferential membranous staining occurs in $>10\%$ of invasive tumour cells

In situ hybridization positive (ISH):

- Single-probe average HER2 copy number ≥ 6.0 signals/cell
- Dual-probe HER2/CEP17 ratio ≥ 2.0 with an average HER2 copy number ≥ 4.0 signals per cell

ANNEXURE G – Interpretation of Ki67 proliferative index

Manual cell counting was employed to calculate the Ki67 proliferative index:

- Positive cells (nuclear staining and mitotic figures) were counted and compared to the total number of nuclei visible in a high power field (x40 objective)
- This processes was repeated in three different fields showing the greatest expression and an average of the three areas calculated

ANNEXURE H – Response to chemotherapy

Response to chemotherapy was assessed histologically as follows:

1) Pathologic complete response (PCR):

- This was defined as the absence of any residual invasive carcinoma within the breast and lymph nodes

2) Partial response to chemotherapy:

- This was defined as the presence of residual invasive carcinoma but showing histopathological characteristics of chemotherapy exposure

3) No response to chemotherapy:

- This was defined as the absence of qualitative or quantitative pathologic evidence of tumour response to chemotherapy

ANNEXURE I – Data capture sheet

DEMOGRAPHICS AND CLINICAL INFORMATION:

Study Number:	Age:	Gender:	Race:	HIV:	Laterality:	Clinical Staging:
		F M	B W A C	+ -	RLt B/L	cTcN
1						
2						
3						

CORE BIOPSY:

Date	Number of cores:	Longest core: (mm)	Shortest core: (mm)	Grade:	Mitosis:	Pleomorphism:	Reported Grade:	LVI:
				1 2 3	1 2 3	1 2 3	1 2 3	

Receptor Status:	Oestrogen (ER):	Progestosterone (PR):	HER2:	Reported findings:
Proportion:	Intensity: + -	Proportion: Intensity: + -	0 1+ 2+ 3+	ER PR Ki67

NEOADJUVANT CHEMOTHERAPY:

Regimen:	Dose /Number of Cycles:	Hormone Therapy:	Trastuzumab:

ANNEXURE J – Pilot Study:

A re-evaluation pilot study was performed to assess consistency in histopathological reporting. Eight patients (11%) from the study population were randomly selected and slides re-evaluated in a blind manner.

The re-evaluated histologic grade of both the core and excision specimens were in 100% agreement with the reported histologic grade (Kappa: 1.0, $p=0.002$). In addition, no significant differences in re-evaluated hormone or HER2 receptor status were identified when compared with the reported receptor status.

When reclassifying Ki67 as high ($\geq 14\%$) or low ($<14\%$), findings of the re-evaluation pilot study were in 100% agreement with the reported results on core biopsies. The re-evaluation pilot study revealed 75% agreement with the reported Ki67 results of the excision specimen.

A summary of results of the re-evaluation pilot study is presented in the table below:

Summary of results of the re-evaluation pilot study:

Biomarker:	Agreement (%):		Statistics:
	Core:	Excision:	
Grade:	100	100	Kappa: 1.0
ER:	100	100	N/S
PR:	100	100	N/S
HER2:	100	100	N/S
Ki67:	100	75	McNemar $p=1.000$

ANNEXURE K – Approval of Title

Home

Tools

MMED approved... x

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Sign In



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Ms Thokozile Nhlapo
E-mail: thokozile.nhlapo@wits.ac.za

02 October 2015
Person No: 0300696E
PAG

Dr MJ Bromfield
P O Box 17608
Sunward Park
Boksburg
1470
South Africa

Dear Dr Bromfield

Master of Medicine: Approval of Title

We have pleasure in advising that your proposal entitled *Alterations in immunohistochemical biologic markers following neoadjuvant chemotherapy in breast carcinoma* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely



Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

ANNEXURE L – Turn-it-in Report

0300696e:Turnitin3(1).docx

ORIGINALITY REPORT

12%

SIMILARITY INDEX

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PUBLICATIONS

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STUDENT PAPERS

PRIMARY SOURCES

1

van de Ven, S.. "Discordances in ER, PR and HER2 receptors after neoadjuvant chemotherapy in breast cancer", Cancer Treatment Reviews, 201110

Publication

1%

2

"Breast Disease", Springer Science and Business Media LLC, 2019

Publication

<1%

3

Pinder, SE, E Rakha, CA Purdie, JMS Bartlett, A Francis, RC Stein, AM Thompson, and AM Shaaban. "Macroscopic handling and reporting of breast cancer specimens pre- and post-neoadjuvant chemotherapy treatment; review of pathological issues and suggested approaches", Histopathology, 2015.

Publication

<1%

4

Submitted to University of Witwatersrand

Student Paper

<1%

5

Robin L. Jones, Janine Salter, Roger A'Hern, Ash Nerurkar, Marina Parton, Jorge S. Reis-

<1%