THE UNIVERSITY
of EDINBURGH

Comprehensive gene assessment of estrogen receptor positive breast cancers reveals that HER2 positive status plays an important role in resistance to neoadjuvant letrozole.

Victoria L Webber

Doctor of Medicine (MD)

The University of Edinburgh

2017

I confirm that this thesis was composed by me.

I acknowledge that the work reported here was performed by me in the laboratories of the Edinburgh Breast Unit, at the Western General Hospital, Edinburgh. Exceptions to this are data from the Affymetrix microarray dataset which was generated by Dr Arran Turnbull and Dr Alexey Larionov. The Affymetrix and Illumina datasets were combined and corrected by Dr Arran Turnbull.

This thesis is presented for the degree of Doctor of Medicine (MD) and has not been submitted in canditure for any other degree or professional qualification.

Victoria L Webber

Abstract

Background

The ER positive/ HER2 positive molecular subtype accounts for up to 10% of all breast cancers, these cancers have a worse prognosis than ER positive/ HER2 negative breast cancers. There is considerable evidence that ER positive/ HER2+ positive cancers exhibit resistance to endocrine therapy, yet it is unclear what is driving this resistance to therapy. The challenge is in identifying, early in the process of treatment decision making, who will respond to neoadjuvant letrozole therapy and who might benefit from the addition of combined HER2 targeted agents.

Aims

1. To investigate which ER positive/ HER2 positive breast cancers respond to letrozole.
2. To compare the mechanisms of resistance to endocrine therapy in ER+/ HER2+ and ER+ / HER2- breast cancers.
3. To determine which cancers should be considered for combined endocrine and anti-HER2 treatment.

Methods

17 postmenopausal women with large, operable, locally advanced ER positive/ HER2 positive breast cancers treated with neoadjuvant Letrozole had their clinical response assessed using periodic 3D ultrasound. Core biopsies were taken at 0, 14 days and 3 months of treatment. RNA was extracted, amplified, labelled and hybridised to Illumina HT-12 whole genome beadarrays. A group of patients with ER positive/ HER2 negative disease were identified to compare clinical and molecular response.

Results

8 (47%) ER+/ HER2+ patients responded (R) and 9 (53%) patients did not (NR). HER2 expression was significantly higher at baseline in the NR group (p=0.005). Differences between R and NR and between the HER2+/ER+ and HER2-/ER+ groups were evident during treatment in terms of rate of change, magnitude of gene expression changes, and in change of functional molecular pathways. ER+/ HER2+ responding tumours had similar changes in gene expression over 3 months to ER+/ HER2- responding tumours. Analysis of responding tumours showed a clear association between good response and up regulation of

stromal and immune response genes and down regulation of proliferation genes. In non responding tumours, mitogen activated protein kinase (MAPK) signalling and phosphoinositide 3-kinase (PI3K) signalling play important roles in resistance to neoadjuvant letrozole.

Conclusions

- ER+/ HER2+ and ER+/ HER2- responding tumours demonstrate similar gene changes in response to neoadjuvant letrozole, suggesting ER rather than HER2 is influencing growth in these cancers.
- ER+/ HER2+ non responding tumours have fewer overall molecular changes on letrozole, maintaining high proliferation gene expression and active MAPK and PI3K signalling possibly suggesting HER2 signal transduction.
- This may allow us to select ER+/ HER2+ tumours that will not benefit from endocrine therapy but may respond to HER2 or MAPK or PI3K targeted therapies.

Lay Summary

Breast cancer is the most common cancer in the UK with 55,222 new cases of breast cancer being diagnosed in the UK in 2014. Breast cancers with receptors for the hormone estrogen are called estrogen-receptor positive or ER positive breast cancer. About 75% of breast cancers are ER positive, and these cancers respond well to treatment with hormonal therapies, also known as endocrine therapies. In post menopausal women with large or locally invading ER positive cancers endocrine therapy can be used to shrink the tumour to make it amenable to less invasive surgical resection. Whilst endocrine therapy is very effective in the treatment of patients with ER positive breast cancers, many patients will go on to develop resistance to endocrine therapy and will have progression or recurrence of their disease. HER2 (human epidermal growth factor) is a protein that can affect the growth of cancer cells and cancers which are HER2 positive tend to grow more quickly than HER2 negative breast cancers. ER positive/ HER2 positive cancers account for 10% of all breast cancers and there is evidence that these cancers exhibit resistance to endocrine therapy, although is unclear what is driving this resistance to therapy. This study investigated the response of tumours that were treated with the endocrine therapy letrozole before surgery. Biopsies of the tumour were taken before treatment started, and then after 2 weeks and 3 months of treatment. The clinical response of the tumour was measured by change in size of the tumour during the treatment period. With the tissue taken from the tumour biopsies, measurements in the expression levels of certain important genes could be recorded and in this way we attempted to determine which genes might playing an important role in resistance to endocrine therapy in this group of ER positive/ HER2 positive cancers.

Contents

Acknowledgements

I would like to thank my supervisors Professor Mike Dixon and Dr Andrew Sims for their help and support with this study.

I would also like to thank the Melville Trust who provided much of the funding for my research.

I extent huge gratitude to Dr Arran Turnbull who helped me with much of the bioinformatic interpretation of the micro-array data. Thank you Arran for your time and for your friendship.

Finally, I would like to thank my husband Nick who believes in me and supports me in every endeavor I have. Finishing this work has been so important to me and I couln't have done it without your encouragement, or the many, many hours of childcare you've provided for our wonderful boys, Christian, Louis and Felix.

List of Figures

List of Tables

Abbreviations

4HT 4-hydroxy tamoxifen

AI aromatase inhibitors

ALH atypical lobular hyperplasia

ALND axillary lymph node dissection

ASCO American society of clinical oncology

BCS breast conserving surgery

BMI body mass index

CT computerized tomography

DCIS ductal carcinoma in situ

DNA deoxyribonucleic acid

EGFR epidermal growth factor receptor

ER estrogen receptor

FISH fluorescent in-situ hybridization

FNA fine needle aspiration

FNAC fine needle aspiration cytology

GnRH gonadotrophin releasing hormone

HER human epidermal growth factor receptor

HR hormone receptor

HRT hormone replacement therapy

IGF1 insulin-like growth factor 1

IHC immunohistochemistry

ITCs isolated tumour cells

LCIS lobular carcinoma in situ

LIN lobular intra-epithelial neoplasia

LR local recurrence

MAPK mitogen activated protein kinase

MBC metastatic breast cancer

MRI magnetic resonance imaging

mRNA messenger ribonucleic acid

NHS national health service

NCCN national comprehensive cancer network

NPI Nottingham prognostic index

NSAIDs non steroidal anti-inflammatory drugs

NST no special type

OFS ovarian function suppression

PET-CT positron emission tomography

PI3K phosphoinositide 3-kinase

PR progesterone receptor

RNA ribonucleic acid

RS recurrence score

RT-PCR reverse transcription polymerase chain reaction

SERDs selective estrogen receptor down-regulators

SERMs selective estrogen receptor modulators

SLNB sentinel lymph node biopsy

TDLU terminal duct lobular unit

TNBC triple negative breast cancer

TNM tumour, node and metastases

UICC union for international cancer control

WLE wide local excision

Introduction

1.1 Epidemiology

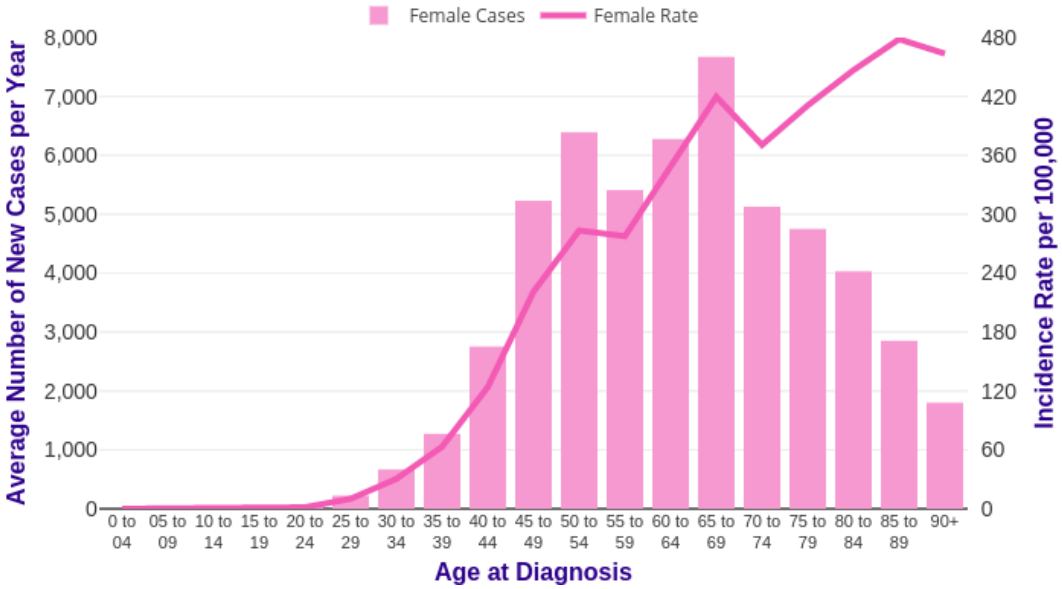
Breast cancer has been the most common cancer in the UK since 1997, and accounts for 15% of all new cancer cases. It is by far the most common type of cancer in women worldwide, in the UK breast cancer accounts for 31% of all new diagnoses of cancer in females. In 2015, there were 55,170 new cases of invasive breast cancer in the UK: 54,800 (99%) in women and 370 (less than 1%) in men, with a female:male ratio of around 148:[1,2,3,4] In the same year there were 11,400 deaths from invasive breast cancer in the UK. The crude incidence rate shows that there are 157 new breast cancer cases for every 100,000 females in the UK, and 1 for every 100,000 males. It has been estimated that breast cancer affects 1 in 8 women in the UK.

In 2015 there were 7,900 new breast carcinoma in situ cases in the UK. Incidence rates for breast carcinoma in situ in the UK are highest in people aged 65-69 years. Over the last decade, breast carcinoma in situ incidence rates have increased by 46% in the UK.

1.1.1 Incidence

The highest incidence rates of female breast cancer are in older women, supporting a link with hormonal status. In the UK in 2010, 80% of all diagnosis were in the over 50's, and 45% were diagnosed in women aged 65 and over (Figure 1.1B). Age-specific incidence rates rise significantly from around the age of 35-39 years, this incidence then levels off for women in their 50's, rises further to the age 65-69 years, drops slightly at 70-74 years, and then increases steadily to reach an overall peak over the age of 85 years. The peaks and troughs seen in incidence rates for women aged 50 and over, may partly be explained by the impact of screening for breast cancer in this age group. Although very few cases of breast cancer occur in women in their teens or early 20s, breast cancer is the most commonly diagnosed cancer in women aged under 39.

Fiure 1.1: Breast Cancer, average number of new cases per year and age-specific incidence rates per 100, 000 population, females, UK, 2013-2015.[4]



Since the 1970s, there has been an overall increase of approximately 70% in the incidence of female breast cancers in the UK, with an increase of 4% over the last decade (Figure 1.1C). This increase is in part attributable to the introduction of the national breast screening programme from the late 1980s, and to heightened patient awareness. However, there has been a subtle downward trend in the over 50 population since the mid 2000s, and this is likely to be attributable to reduced use of hormone replacement therapy, a known risk factor for breast cancer development.

The incidence of female breast cancer is strongly related to deprivation, with incidence rates being lowest for the most deprived women.[5] Many breast cancer risk factors are more prevalent in more affluent populations, such as late first pregnancy, lower parity, and use of hormone replacement therapy.

1.1.2 Risk factors

Breast cancer can result from multiple environmental and hereditary risk factors. Incidence patterns amongst migrant populations suggest that environmental factors are of greater importance. In developed countries, it has been estimated that genetic factors contribute around 25% of the differences in individual susceptibility, whilst environmental and lifestyle factors contribute the remaining three quarters.[6] However, the strongest risk factor for breast cancer, after gender, is age. The older the women, the higher her risk of breast cancer (Table

1.1B). A woman is more than 100 times more likely to develop breast cancer in her 60s than in her 20s.[1]

Table 1.1: Estimated risk of developing breast cancer, by age, in females; UK; 2008.[4]

Estimated risk at birth Up to and including:	UK (2008)
Age 29	1 in 2,000
Age 39	1 in 215
Age 49	1 in 50
Age 59	1 in 22
Age 69	1in 13
Lifetime risk	1 in 8

1.1.3 Estrogen related risk factors

A large proportion of breast cancer cases diagnosed in developed countries can be explained by factors which influence exposure to estrogen. These include reproductive factors, obesity, alcohol and physical activity. It has been estimated that around 27% of breast cancers diagnosed in the UK are linked to modifiable lifestyle and environmental factors.[1]

There are several reproductive factors that increase breast cancer risk. The overall hypothesis being the more ovulatory cycles to which the breast tissue is exposed over a lifetime, the higher the breast cancer risk.[7] Removal of both ovaries has consistently been shown to decrease the risk of breast cancer, by about 50% if performed prior to the age of 40 years.[8]

Early age at menarche is associated with an increased risk of breast cancer. This is most evident at a young age with a twofold increase risk for menarche at 11 years as compared with menarche at 13 years.[9] Early age at first birth and parity are established, independent, protective factors for breast cancer.[10,11] Early age at first birth has been shown to provide a long-term reduction in breast cancer risk, not evident for approximately 10-15 years.[12] Women with a later age at first birth, approximately 35 years or older, have an increased risk of breast cancer, and this risk has been found to be higher than that of nulliparous women.[13,14] Parous women have a reduced risk of breast cancer compared to nulliparous women and the protective effect of full term pregnancy increases with number of births.[13,14] Women in developed countries are at increased risk of breast cancer compared with women from less

developed countries. This variation can be explained by the fact that women in more developed countries have on average fewer children and a shorter duration of time spent breastfeeding. Most studies demonstrate an increased risk for breast cancer in women who have a late natural menopause at age 55 years or later, as compared with those with a natural menopause at age 45 years.[8] Women who have undergone the menopause have a reduced risk of breast cancer than premenopausal women of the same age and childbearing history. It has been estimated that for each year of increase in age at menopause, there is an average increase in breast cancer of 3.6%.[15]

High levels of other endogenous hormones such as prolactin and insulin have also been associated with increased risk of breast cancer. Higher levels of prolactin have been associated in particular with estrogen receptor positive tumours, however the role of prolactin in breast cancer aetiology is far from clear.[16] One meta-analysis showed a 27% increased risk of breast cancer in women with type 2 diabetes, although this figure decreases to 16% after adjustment for BMI.[17] Furthermore, Insulin-like growth factor 1 (IGF1) is positively associated with breast cancer risk.[18]

Use of exogenous hormone preparations including oral contraceptives and hormone replacement therapy (HRT) is associated with higher risk of breast cancer. Recent oral contraceptive use (within the prior year) is associated with an increased breast cancer risk relative to never or former oral contraceptive use. The association is stronger for estrogen receptor positive than estrogen receptor negative disease and there is a particularly elevated risk with the use of oral contraceptives containing high dose estrogen.[19] There is no significant increased risk 10 years after stopping use. It has been estimated that around 1% of breast cancer in women in the UK are linked to oral contraceptives.

The risk of post menopausal breast cancer is increased with current use of hormone replacement therapy (HRT), and this risk is larger with combined estrogen plus progestin than with estrogen-only formulations. The Women's Health Initiative (WHI) trial of combined estrogen plus progestin was stopped early when overall health risks, including invasive breast cancer, exceeded benefits.[20]

1.1.4 Non-estrogen related risk factors

A number of non-estrogen, environmental and lifestyle factors have been implicated in breast cancer risk. Breast density is strongly and independently related to the risk of breast cancer.[21,22] Breasts with a high epithelial content are described as dense in comparison with those with a higher fat content. The effect of breast density is independent of endogenous

hormones.[23] Density is affected by menopausal status, weight, number of children, and genetic inheritance.

Obesity as measured by body mass index (BMI), moderately increases the risk of post-menopausal breast cancer and is one of the few modifiable risk factors for breast cancer. As compared to lean women (BMI between 22.5-24.9), postmenopausal women who are overweight (BMI 25-29.9) have a 10-20% increased risk of breast cancer, and obese women (BMI>30) a 30% increased risk. Women with a BMI <22.5 have a 15% reduction in risk compared to women with a BMI of 22.5-24.9. In contrast, obese pre-menopausal women have a 20% reduction in breast cancer risk.[2] One study estimated that around 9% of breast cancers in women in the UK in 2010 were linked to excess body weight.[3] The link between BMI and breast cancer is likely to be due to hormones. The main endogenous source of estrogen in postmenopausal women is the conversion of hormones in fatty tissue.[24] Thus, overweight postmenopausal women are exposed to increased levels of endogenous estrogen and have higher risks of developing breast cancer. The reduced breast cancer risk seen in obese premenopausal women may be due to an-ovulatory menstrual cycles in this group.[25]

A meta-analysis of 45 studies reported that higher total fat intake increased breast cancer risk by 13%.[26] A further study has shown a small but significant risk increase for higher intakes of saturated, monounsaturated and polyunsaturated fats.[27] It has been reported that women who eat high levels of saturated have twice the risk of developing breast cancer.[28] High intake of dietary fibre and fruit have also been associated with reduced breast cancer risk.[29,30]

The association between alcohol consumption and breast cancer has been consistently shown. The relative risk associated with every unit of alcohol (10g of alcohol) consumed on a daily basis is estimated to be 7-12%.[31] This is likely to be due to the increased levels of sex hormones in people who consume alcohol. It is estimated that >6% of breast cancers diagnosed in women in 2010 were related to alcohol consumption.[32]

A 15-20% reduction in breast cancer has been shown in postmenopausal women who are very physically active.[33] The effect of physical activity on breast cancer risk may be due to effects on systemic hormone levels, with lower levels of estrogen and testosterone being reported in women with higher levels of physical activity.[34] It was estimated that >3% of breast cancers diagnosed in women in 2010 were linked to women who undertook less than 150 minutes moderate physical activity per week.[35]

It has been proposed that night shift work could increase breast cancer incidence.[36] A 2007 World Health Organisation review concluded, mainly from animal evidence, that shift work

involving circadian disruption is probably carcinogenic to humans.[37] However, this has been challenged by a recent systematic review of three prospective UK studies of post menopausal women: The Million Women Study (522 246 participant), EPIC-Oxford (22 559) and the UK Biobank (251 045). When combining the results of these studies, there was no evidence that night shift work was associated with breast cancer.[38]

The role of smoking in breast cancer risk remains equivocal. There is evidence that women who began smoking under the age of 20 years, or before the birth of their first child, have an increased risk of breast cancer.[39] It is thought that the risk increase for women who smoke compared to women who have never smoked is around 10-20%. However, the evidence remains inconsistent and more research is required.

Women with higher birth weight, length, or born to an older mother are at increased risk of breast cancer. In these women there is probably a higher exposure to estrogen in utero.[40]

There is a 7-11% increased risk of breast cancer per 5cm increment in height.[41] The underlying mechanism behind this risk is unclear but it is likely that increased height is a marker for other exposures that influence breast cancer. It may also be that hormones affecting a woman's height cause an increase in the volume of breast parenchymal tissue, and therefore increased susceptibility to breast cancer.

Exposure to ionising radiation is an established risk factor for breast cancer and is strongly related to age at exposure.[42] There is a 12-25 fold increase for secondary breast cancer in women treated with mantle radiation therapy to the chest for Hodgkin's lymphoma before the age of 30 years.[43] Women who received sequential diagnostic x-rays to the chest for tuberculosis or pneumonia between the ages of 10 and 29 years have a 3 fold increased risk of breast cancer.[44] In women treated with radiotherapy for breast cancer, the risk of developing radiation-induced contralateral breast cancer is stronger among younger women. Women >45 years of age when treated with radiotherapy for first breast cancer are not at significant risk of contralateral breast cancer.[45] It was estimated that 1% breast cancers diagnosed in women in 2010 were linked to radiation exposure. About 46% of these cases were linked to medical radiation and the remainder to background, natural radiation.[46] Breast screening mammograms are associated with a very small number of breast cancers: of 10,000 women who are screened every 3 years between ages 47 and 73 years, between 3 and 6 will develop cancer during their lifetime secondary to mammogram radiation.[47]

Regular use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) confer a 25% breast cancer risk reduction.[48] There is evidence that post-menopausal NSAID users

have lower levels of estradiol than non-users.[49] Regular use of aspirin in postmenopausal women is not recommended owing to the increased risk of gastrointestinal haemorrhage associated. The use of anti-hypertensive medications for 5 years or longer has been shown to increase breast cancer risk by 20%.[50] People treated for the autoimmune condition Graves' Disease have a 12% higher risk of breast cancer.[51] Diabetics have a 10-23% increased risk of breast cancer compared with non diabetics, the association is strongest in postmenopausal women with type II diabetes.[52] In diabetics, the risk of breast cancer may vary by treatment type, with users of metformin and pioglitazone having lower risk.[53]

Previous diagnosis of breast disease is a risk factor in itself. Women with benign, proliferative breast changes without atypia have a 2 fold increased risk of breast cancer, and those with atypical epithelial hyperplasia have 4-5 times increased risk.[54] However, women with non-proliferative, benign pathologies including cysts, complex fibroadenomata, ductal papillomata and sclerosing adenosis only carry a significantly higher risk of developing breast cancer if they have a strong family history of breast cancer.

Lobular carcinoma in situ (LCIS) and atypical lobular hyperplasia (ALH), combined now as lobular intra-epithelial neoplasia (LIN), together with ductal carcinoma in situ (DCIS), are non-invasive conditions of the breast, which can in some cases develop into invasive cancer. Overall, women with LIN are 4-5 times more likely to develop an invasive breast cancer compared to the general population. High grade lesions are more likely to develop into invasive disease than low grade lesions.[55]

A previous diagnosis of breast cancer raises the risk of developing a second primary breast cancer by up to 5 fold.[56] The risk of a contralateral breast cancer remains higher 2 years after diagnosis of a primary breast cancer, with highest risks in women diagnosed before the age of 40 years.[57]

Family history

In developed countries it is estimated that hereditary factors contribute to 25% of inter-individual differences in susceptibility to breast cancer, with environmental and lifestyle factors contributing to the remaining 75%. Epidemiological studies have shown that first-degree female relatives of women with breast cancer are at approximately twofold risk of developing the disease compared to the general population, and risk is higher if the relative is diagnosed under the age of 50 years. Although this could be attributable to shared environmental or genetic factors, or both, twin studies indicate that most of the excess familial risk is due to inherited predisposition.[4] However, more than 85% of women with

breast cancer have no family history of the disease. Furthermore, more than 85% of women who have a close relative with breast cancer will never develop the disease.

The breast cancer genes

Three well defined classes of breast cancer susceptibility alleles with different levels of risk and prevalence in the population have become apparent: rare high-penetrance alleles, rare moderate-penetrance alleles and common low-penetrance alleles. The contribution of each of these genes to phenotypic characteristics associated with them, as well as much of their biology and clinical utility is still not fully understood.

Rare high-penetrance breast cancer susceptibility genes

Disease-causing variants in *BRCA1* and *BRCA2* confer a high risk of breast cancer, approximately 10- to 20-fold relative risk. This translates into a 30–60% risk by the age 60 years, compared to 3% in the general population.[4] The prevalence of BRCA1 and BRCA2 mutation carriers is estimated to be 0.11% and 0.12% respectively, approximately 1 in 450 women. The relative risks are higher for early-onset breast cancers, and there are also elevated risks of ovarian and other cancers.[58,59] Mutations in *BRCA1* and *BRCA2* cause breast cancer by inactivation of encoded proteins, generally by causing premature truncation or nonsense-mediated RNA decay. These genes have been firmly implicated in DNA break repair.[11] Mutations are infrequent in most populations. Approximately 1 in 1,000 individuals in the UK are heterozygous mutation carries of each gene, there are many different mutations, each of which is rare.[60,61] Predisposition to breast cancer is transmitted as an autosomal dominant trait in families with mutations. *BRCA1* and *BRCA2* are recessive cancer genes, with mutations becoming homozygous in the cancers which they cause, usually through loss of the wild-type allele. Mutations in *BRCA1* and *BRCA2* account for approximately 2% of all breast cancers, and up to 20% of the familial or inherited genetic component of disease risk. Genetic testing for faulty BRCA genes is available on the NHS for women with a very strong family history.

Germline mutations in *TP53* cause Li-Fraumeni syndrome, which includes a high risk of breast and other cancers, but these mutations are thought to account for a very low proportion of familial breast cancer due to its rarity. Cancer predisposition syndromes due to mutations in *PTEN* (Cowden syndrome), *STK11* (Peutz-Jeghers syndrome) and *CDH1* are also associated with elevated risks of breast cancer, although the cancer risks and prevalence of

mutations in these genes are not well defined. It is unlikely that mutations in all six of these genes together account for more than 20% of the familial risk of the disease.[62,63]

Rare moderate-penetrance breast cancer susceptibility genes

There are 4 well documented but rare, moderate-penetrance breast cancer susceptibility genes, namely *CHEK2, ATM, BRIP1 and PALB2.* CHEK2 is a checkpoint kinase involved in DNA repair that directly modulates the activity of both p53 and BRCA1 by phosphorylation. ATM encodes a checkpoint kinase that has key functions in DNA repair, and which also phophorylates p53 and BRCA1. BRIP1 (also known as BACH1) is a binding partner of BRCA1 and is implicated in some BRCA1 activities relating to DNA repair. The PALB2 gene is called the partner and localiser of BRCA2, it provides instructions to make a protein that works with the BRCA2 protein to repair damaged DNA and stop tumour growth.

These 4 genes can result in disease-causing mutations by processes of premature protein truncation or nonsense mediated RNA decay through nonsense codons or translational frameshifts. In each of the 4 genes, there are multiple different pathogenic mutations, each of which is generally very rare. Disease causing mutations in each gene are found in less than 1% of the UK population. Overall, with respect to their effect on protein function, their prevalence in the population and their biological consequences, disease causing mutations in *CHEK2, ATM, BRIP1* and *PALB2* bear many similarities to disease causing mutations in *BRCA1* and *BRCA2*. However, mutations in *CHEK2, ATM*, *BRIP1* and *PALB2* confer less risk of breast cancer (2-3 fold risk) than mutations in *BRCA1* and *BRCA2* (10-20 fold risk). Current estimates suggest that mutations in the 4 genes together account for 2.3% of the familial risk of breast cancer, compared to 16% for *BRCA1* and *BRCA2* together.[4,60,61,62]

Common low-penetrance breast cancer susceptibility genes

A small number of statistically significant, common low-penetrance breast cancer susceptibility genes have been reported in different populations. These genes confer a very small increase in the risk of developing breast cancer. The 7 most frequently described are *CASP8* (encoding caspase 8, a member of the cysteine-aspartic acid protease family whose activation has a central role in apoptosis); *FGFR2* (encoding fibroblast growth factor receptor 2); *TNRC9* (otherwise known as *TOX3*, a transcription factor); *MAP3K1* (encoding mitogen-activated protein kinase1, involved in growth signalling); *LSP1* (encoding lymphocyte-specific protein 1); 2 of the 7 susceptibility loci are on 8q and 2q, in regions with no known protein-coding genes. The population prevalence of these genes is high, ranging from 28%-

87%, but the relative risk associated with carrying a single copy of each risk allele ranges from 1.07 to 1.26.[4,64,65,66]

1.2 Classification

Breast cancer classification divides breast cancer into categories according to histopathological type, grade of tumour, stage of tumour and the expression of proteins, receptors and genes. The purpose of classification is to select the best, tailored treatment.

Microarray-based gene expression profiling has had a major effect on our understanding of breast cancer. Breast cancer is now perceived as a heterogeneous group of different diseases characterised by distinct molecular aberrations, rather than one disease with varying histological features and clinical behaviour.

1.2.1 Histopathological Classification

Type

Histopathological classification is based upon characteristics seen at light microscopy of biopsy specimens. Carcinomas comprise the vast majority of all breast cancers and arise from the epithelial component of the breast. Within the large group of carcinomas, there are many different types of breast cancer. The first major division is between in situ and invasive carcinoma. In situ carcinoma is "pre-invasive" carcinoma that has not yet invaded the breast tissue. These in situ cancer cells grow inside of the pre-existing normal lobules or ducts. In situ carcinoma has significant potential to become invasive cancer, and that is why it must be adequately treated to prevent the patient from developing invasive cancer.

Invasive carcinoma of no special type (NST; formerly known as ductal carcinoma) accounts for 80% of all invasive breast cancers and the remaining special types of carcinoma account for the remaining 20%. Of these, invasive lobular carcinoma accounts for 10-15% of invasive breast cancers and the remaining cases of invasive carcinoma are comprised of other special types of breast cancer that are characterized by unique pathologic findings. These special types include colloid (mucinous), medullary, micropapillary, papillary, and tubular. Sarcomas are rare cancers that arise from the stromal components of the breast and these account for less than 1% of primary breast cancers.

The 3 most common histopathological types collectively represent three quarters of breast cancers. These are invasive carcinoma NST (55% of breast cancers); ductal carcinoma in situ (DCIS; 15%); and invasive lobular carcinoma (5%).

Breast carcinomas are derived from the epithelial cells that line the terminal duct lobular unit. Cancer cells that remain within the basement membrane of the elements of the terminal duct lobular unit and the draining duct are classified as in situ, or non-invasive.[67] An invasive

cancer is one in which there is dissemination of cancer cells outside the basement membrane of the ducts and lobules into the surrounding adjacent normal tissue.

The most commonly used classification of invasive breast cancer divides them into ductal and lobular types, this division was based on the belief that cancers arise from the ducts and lobules. It is now clear that both invasive ductal and lobular cancers arise from the terminal duct lobular unit. Invasive lobular cancers can be difficult to diagnose because their pattern of single-file cell infiltration does not form a well defined mass lesion that can be readily detected clinically or radiologically.[67] Inflammatory breast cancer is a form of invasive ductal carcinoma which is distinguished clinically from other carcinomas by the inflamed appearance of the breast. Inflammatory cancers are more aggressive and are associated with a poor prognosis.[68] Some tumours show distinct patterns of growth and cellular morphology which allow certain types of breast cancers to be identified. Cancers with specific features are called invasive carcinomas of special type, while the remainder are considered to be of no special type (NST). This classification has clinical relevance as certain special type tumours have a better prognosis than tumours of NST.

Ductal carcinoma in situ (DCIS) is a heterogeneous disease with increased prominence after the introduction of breast screening programmes. Before the advent of screening, DCIS represented only 2-5% of symptomatic breast cancers, compared with almost 20% of newly diagnosed symptomatic cases in the present era and up to half of screen detected breast cancer.[69,70] Not all patients with DCIS will progress to invasive disease, with proportionate estimates ranging from 25%-50% depending on grade of the lesion.[71] Not all invasive cancers arise from lesions that are recognised histologically as carcinomas in situ, although a phase involving increased epithelial proliferation is likely to precede an invasive cancer. DCIS represents a late-stage disease in terms of molecular progression, and many genetic mutations occur before invasion.[70,72]

1.2.2 Molecular Classification

There is substantial tumour heterogeneity consisting of different molecular subtypes, each with distinct biological and clinical characteristics. Cells express proteins on their surface and in their cytoplasm and nuclei that act as receptors for chemical messengers, such as hormones. The interactions between cell receptors and their ligands have profound effects in cellular behaviour. Breast cancer cells express many different types of receptor, currently three receptors are known to influence both prognosis and treatment and are included in standard breast cancer classification systems. These receptors are the estrogen receptor (ER),

the progesterone receptor (PR), and the Human Epidermal Growth Factor Receptor 2 (HER2/ neu). Breast cancer cells which do not express any of these receptors are classified as basal like, or triple negative breast cancers (TNBC). Triple negative breast cancers (TNBC), which are relatively common among BRCA1 carriers, cannot be treated with targeted therapy due to the lack of receptor expression. As such, these tumours carry a particularly poor prognosis. Approximately half of TNBC's will respond to chemotherapy.[73]

Breast cancer receptor status is determined by immunohistochemistry (IHC), using labelled antibodies to ER, PR and HER2 receptors, and is included in the pathologist's report. Fluorescent in-situ hybridisation (FISH) can be used to determine how many copies of the HER2 gene are present and is used to determine HER2 status in cases where IHC is equivocal. Results of receptor status are critical as they determine which targeted therapies will be beneficial.

Estrogen Receptor

Estrogens play a crucial role in the development and growth of both normal and neoplastic breast tissue. Approximately 75% of breast cancers express the estrogen receptor (ER). ER can be quantified by using the Allred scoring system. This scoring system is based on the percentage of cells that stain by IHC for ER (score 0-5) and the intensity of that staining (score 0-3), added together for a possible total score of 8. This score stratifies cancers into those that are likely or not to respond to endocrine therapy.

ER positive cancers tend to be ER rich, with an Allred score >6. These cancers depend on estrogen for their growth and are therefore susceptible to endocrine therapies that act either by reducing the levels of estrogen, or by modifying the activity of this hormone. These cancers are consequently associated with a better prognosis than ER negative breast cancers.

Progesterone Receptor

Approximately 65% of ER positive breast cancers also express the progesterone receptor (PR), and this combination increases the likelihood of a good response to endocrine therapy. PR is scored in the same way as ER. Very few PR positive, ER negative cancers exist.

Human Epidermal Growth Factor Receptor 2

Human Epidermal Growth Factor Receptor 2 (HER2) has been identified as an important target for breast cancer.[74] HER2 is amplified or overexpressed in approximately 20% of breast cancers, and increased HER2 expression correlates with more aggressive breast tumours and a poorer prognosis than tumours with normal HER2 expression.[75]

Fortunately, agents that target the HER2 receptor have dramatically improved survival in patients with HER2 positive disease. The monoclonal antibody trastuzumab prevents HER2 receptor activation, and in combination with conventional chemotherapy improves patient prognosis.[75]

The majority of ER positive tumours are HER2 negative. ER+/ HER2+ tumours account for approximately 10% of all breast cancers.

1.2.3 Molecular Subtypes

Recognition of the importance of the ER and HER2 in breast cancer, and the large scale use of immunohistochemistry (IHC), has enabled almost every cancer centre in the world to differentiate breast cancer patients into 3 major groups: the hormone receptor positive group (which expresses the ER and/ or progesterone receptor (PgR), the HER2 positive group (which expresses HER2 by IHC or amplification detected by fluoresce in-situ hybridisation [FISH]) and the triple negative group (which is negative for ER, PgR, and HER2).[76] Latterly, the ER positive group has been subdivided into 2 distinct prognostic groups, luminal A and luminal B, based on percentage of Ki67 or the presence of PgR.[77]

1.2.4 Gene Expression Profiling and Intrinsic Subtypes

Molecular profiling of breast cancer by gene expression studies has provided an important tool to discriminate a number of subtypes. These breast cancer subtypes have been shown to be associated with clinical outcome and treatment response. In order to elucidate the functional consequences of altered gene expressions related to each breast cancer subtype, proteomic technologies have provided further insight by identifying quantitative differences at the protein level. In recent years, proteomic technologies have matured to an extent that they can provide proteome-wide expressions in different clinical materials. This technology can be applied to the identification of proteins or protein profiles to further refine breast cancer subtypes or for discovery of novel protein biomarkers pointing towards metastatic potential or therapy resistance in a specific subtype.

In an mRNA or gene expression array, the expression levels of thousands of genes are simultaneously monitored to study the effects of certain treatments, diseases, and developmental stages on gene expression. The expression levels can be simultaneously monitored to create a molecular portrait of the investigated tumours. In 2000, Perou et al[217] were the first to show that the phenotypic diversity of breast cancers is associated with a

corresponding diversity in gene expression patterns that can be captured using cDNA arrays. Using a set of 65 surgical specimens of human breast tumours from 42 different patients, they produced complementary DNA microarrays representing 8,102 human genes. The tumours could be classified into subtypes distinguished by clear differences in their gene expression patterns. The data from Perou and colleagues, and from subsequent authors have led clinicians and scientists to reconsider the way to differentiate and treat patients with breast cancer, and has set new challenges in the search for novel, more targeted therapies. The St Gallen International Expert Consensus on the 'Primary Therapy of Early Breast Cancer 2013' proposed that identification of intrinsic subtypes is most precise using molecular technologies.[79] Where such assays are unavailable, surrogate definitions of subtype can be obtained by immunohistochemistry (IHC) measurements of ER, PgR, Ki67 and HER2 with *in situ* hybridisation, where appropriate.

Luminal A Subtype

Luminal A breast cancer is the most common subtype, representing 50-60% of the all breast cancers.[80] It is characterised by the expression of genes activated by the ER transcription factor that are typically expressed in the luminal epithelium lining the mammary ducts. It also presents a low expression of genes related to cell proliferation.[78] All cases of lobular carcinoma in situ are luminal A tumours, as are most infiltrating lobular carcinomas. The luminal A IHC profile is characterised by the expression of ER, PGR, Bcl-2, and cytokeratin CK8/18, an absence of HER2 expression, a low rate of proliferation measured by Ki67 and a low histological grade. The GATA3 marker is expressed at high levels in the luminal A subgroup. Patients with this subtype of cancer have a good prognosis, the relapse rate of 27.8% being significantly lower than that for other subtypes, and survival from the time of relapse is also longer (median 2.2 years).[80] They have a distinct pattern of recurrence with a higher incidence of bone metastases (18.7%) and less than 10% metastases to the central nervous system, liver and lung. The treatment of this subgroup of breast cancer is predominantly endocrine therapy.

Luminal B Subtype

Tumours with the luminal B molecular profile make up between 10% and 20% of all breast cancers. They have a more aggressive phenotype than the luminal A subtype, a higher histological grade and proliferative index and a worse prognosis. Although bone is still the most common site of recurrence (30%), this subtype has a higher recurrence rate in sites such as the liver (13.8%). Survival time after relapse is also lower (1.6 years).[81] Luminal A and B both express ER, although the ER level of expression is lower in luminal B, and since the

prognosis with luminal B subtype is much worse, a great effort has been made to identify biomarkers that distinguish between these 2 subtypes. There have been attempts to differentiate between luminal A and B using the protein expression of Ki67.[82] The luminal B subtype also often expresses EGFR and HER2, and has an increased expression of proliferation genes such as MKI67 and cyclin B1. The luminal A subtype has been defined as ER+/ HER2- and low Ki67, while the luminal B subtype has tumours with ER+/HER2- and high Ki67 or ER+/HER2+. Importantly however, the technique used to determine Ki67 (cut-off point to distinguish luminal A and B set at 13.25%) has not been standardised. The St Gallen International Expert Consensus on the 'Primary Therapy of Early Breast Cancer 2013' noted that the absolute values of each IHC parameter/ cut-point may vary between laboratories, and that pending improved standardization local experience might best define the locally useful cut-points between 'high' and 'low' Ki67.[79]

Whilst luminal B tumours have a worse prognosis than luminal A tumours despite treatment with tamoxifen and AI, they respond better to neoadjuvant chemotherapy achieving a rate of pathological pCR in 17% (compared with 7% in luminal A). However, this is lower than for HER2 + and basal like tumours, with values of 36% and 43% respectively.[83] The main reason for attempting distinction between luminal A and luminal B tumours is the differing implications for the use of adjuvant cytotoxic therapy between these groups. Optimal treatment of this subtype of breast cancer is challenging, clinical trial are testing inhibitory molecules of the PI3K/AKT/mTOR pathway at different levels, focussing on the treatment of luminal B tumours.

HER2 Enriched Subtype

15-20% of all breast cancers correspond to this molecular subtype. These cancers are characterized by a high expression of the HER2 gene and other genes associated with the HER2 pathway and/or HER2 amplicon located in the 17q12 chromosome. These cancers exhibit an over-expression of genes related to cellular proliferation. These tumours are highly proliferative, 75% have a high histological grade and more than 40% have p53 mutations. The IHC profile of ER- /HER2+ cancers does not correspond perfectly with the intrinsic subtype, since only 70% of HER2+ tumours classified by microarray have HER2 protein over-expressed by IHC. Conversely, not all tumours with HER2 amplification or over-expression are included in the cluster of HER2 positive subtype in the analysis of micro- arrays.[84] In addition, a significant number of tumours considered clinically as ER+/HER2+ are classified molecularly as luminal B.

Whilst the HER2 subtype is characterised by a poor prognosis, over recent years anti-HER2 treatment has substantially improved survival in both the metastatic setting and in early stage disease. This subtype, along with the basal subtype, have a high chemosensitivity, with higher response rates in neoadjuvant chemotherapy studies compared with those seen in luminal A and B tumours.

Basal-like Subtype/ Triple Negative Breast Cancer

This subtype represents 10-20% of all breast carcinomas. These cancers express genes usually present in normal breast myoepithelial cells, including high molecular weight cytokeratins CK5 and CK17, P-cadherin, caveolin 1 and 2, nestin, CD44 and EGFR. They are clinically characterised by their appearance at an early age, predominantly in women of African origin, having a large tumour size at diagnosis, a high histological grade and a high frequency of positive lymph nodes.[85]

Basal-like tumours tend to be infiltrating carcinomas of no special type with a high mitotic index, tumour necrosis, expanding margins and a clear stromal lymphocytic infiltrate.[86] They have an aggressive pattern of metastatic relapse predominantly of lung, CNS and lymph nodes.[81] An important feature of this subtype is the absence of ER, PGR and HER2 receptors. Therefore, in clinical practice this subtype is often referred to as the Triple Negative Breast Cancers (TNBC). A 'Basal Core Group' of 5 IHC markers: ER, PGR, HER2, EGFR and CK5/6, can be used to classify this subtype with a specificity of 100% and sensitivity of 76%.[87]

Basal-like tumours have a high rate of p53 mutations, which may account for their aggressive behaviour and poor prognosis.[88] Furthermore, tumours with germ line mutations in the BRCA1 are located in the basal-like subgroup in the classification by intrinsic subtypes.[89] Despite having a good response to chemotherapy, they have a worse overall prognosis than luminal tumour tumours, with a higher relapse rate in the first 3 years.[90] Identifying new therapeutic targets to treat this aggressive group of cancers is imperative.

'Normal' Breast Subtype

These tumours potentially account for 5-10% of all breast carcinomas. They are poorly characterised and have been grouped into the classification of intrinsic subtypes with fibroadenomas and normal breast samples. They do not express ER, PGR or HER2 and so can also be classified as triple negative, although they are not considered basal-like as they are negative for CK5 and EGFR. They do not respond well to neo-adjuvant chemotherapy and

due to their rarity there are few studies on this subtype. Indeed, there are doubts about their real existence and some researchers believe that they could be a technical artefact from high contamination with normal tissue during microarrays.[91]

Claudin-Low Subtype

This subtype was identified in 2007, after the initial molecular classification of subtypes. This group is characterised by a low expression of genes involved in tight junctions and intercellular adhesion, including claudin-3, -4, -7 cingulin, ocludin and E-cadherin, hence the name claudin-low. This subtype is located in the hierarchical clustering near the basal-like tumours, and both these subtypes share some characteristic gene expression such as low expression of HER2 and luminal gene clustering. Unlike the basal-like subtype, the claudin-low tumours overexpress a set of 40 genes related to immune response, indicating a high infiltration of tumour immune system cells.[92] They have a low expression of genes related to cell proliferation, and they overexpress a subset of genes closely linked to mesenchymal differentiation and epithelial-mesenchymal transition. These account for 12-14% of tumours and clinically they correspond to high grade infiltrating carcinomas of no special type, that can present metaplastic or medullary differentiation.[93] About 20% of these tumours are positive for hormone receptors. They show poor long-term prognosis[232] and have a poor response to neoadjuvant chemotherapy.

In 2011 the 12th St Gallen International Expert Consensus for Early Breast Cancer recognised the usefulness of these subtype classifications in the therapeutic decision making process.[94] In addition, the panel accepted that the different breast cancer subtypes can be defined not only by genetic array testing but by approximations to this classification using IHC. This Expert Consensus established five clinicopathological definitions, luminal A (ER and/or PGR positive, HER2 negative, Ki67<14%); luminal B-HER2 negative (ER and/or PGR positive, HER2 negative, Ki67≥14%); luminal B-HER2 positive (ER and/or PGR positive, HER2 positive, any Ki67); HER2 positive-non luminal (ER and PGR negative, HER2 positive) and Triple Negative (ductal) (ER and PGR absent, HER2 negative). It has been accepted that luminal A disease generally requires only endocrine therapy which also forms part of the therapy of the luminal B subtype. Chemotherapy is considered the recommended treatment for most luminal B, HER2 positive and Triple Negative disease.[80] The evidence given by the multiple studies that evaluate these gene expression based platforms supports that they are valuable prognostic tools. The information they provide helps clinicians predict more accurately which patients will benefit from chemotherapy, especially with regard to the ER positive tumours.

1.3 Diagnosis

It is known that earlier diagnosis of breast cancer is more likely to result in a favourable outcome. Regardless of tumour type or grade, the smaller a breast cancer is at the time of diagnosis, the more likely it is that it has not spread beyond the breast. As a result, the current strategy for reducing breast cancer mortality is to seek diagnosis as early as possible. Early detection and improvements in treatment have led to a 30% reduction in breast cancer mortality in the UK in all age groups over the past 30 years.

1.3.1 Triple assessment

Most breast cancer patients present with a painless lump. Many, particularly young women present to the breast clinic with pain but this symptom is not related to diagnosis of breast cancer. All patients with a lesion suspicious of breast cancer should undergo a triple assessment process.

Triple assessment comprises of 3 equally weighted categories:

I) Thorough clinical examination of both breasts and the regional lymph nodes in the axillae and supraclavicular fossae.

II) Radiological imaging of the affected breast with or without additional ultrasound of the breast depending on the patients age and breast density.

III) Biopsy of the breast lesion by either fine needle aspiration (FNA) for cytological assessment, trucut core biopsy for full histological assessment, or both, depending upon local clinic facilities.

1.3.2 Radiological imaging and the NHS Breast Screening Programme

Mammography

Mammography is performed on symptomatic women over the age of 35 years. Under the age of 35 years the breast tissue is radio-dense, limiting the value of mammography in these women. Two views, oblique and cranio-caudal are obtained to allow detection of mass lesions, parenchymal distortion and breast micro-calcifications, which may require further investigation. Cancers usually appear on the radiograph as solid, speculated lesions.

The NHS Breast Screening Programme

All UK women aged between 50 and 70 years, and who are registered with a GP, are invited to attend the breast screening programme. The screening is in the form of mammography. In 2010 2,020,000 women attended, out of 2,750,000 invited for screening, and 16,500 cancers were detected.

Some experts have questioned the overall benefit of screening in terms of reduced breast cancer mortality and how substantial the harm is in terms of over-diagnosis, which is defined as cancers detected at screening that would not have otherwise become clinically apparent in the woman's lifetime. An independent UK panel conducted a review on breast cancer screening in 2012 concluded that screening reduces breast cancer mortality but that some over-diagnosis occurs.[69] From this review, it was estimated that for every 10000 UK women aged 50 years invited to screening for the next 20 years, 43 deaths from breast cancer would be prevented and 129 cases of breast cancer, invasive and non-invasive, would be over-diagnosed; that is 1 breast cancer death prevented for about every 3 over-diagnosed cases identified and treated. Crucially, this information should be made available in a transparent and objective way to women invited to screening so that they can make informed decisions.

Ultrasound

Under the age of 40 years, the breast tissue is more dense and in these women breast ultrasonography (US) is the most useful diagnostic imaging modality. In older women US is used to better define localised palpable, and mammographically detected lesions and to guide excision biopsy for diagnosis. Patients with invasive cancer also have axillary US to detect nodal disease, and guided biopsy of suspicious lymph nodes.

US can also be used to monitor clinical response to therapy by measuring tumour size and sequential stages during a patients treatment.

Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) sensitivity is high and can be a valuable tool for assessing the extent of invasive and non-invasive disease. Whilst contrast enhanced breast MRI has demonstrated a sensitivity of 94%-100% in the detection of breast cancer, its specificity has generally been lower and more variable with ranges from 37%-97%. This tool is not in routine clinical use for breast cancer diagnosis and assessment. MRI is indicated in patients for whom a potential benefit of local staging is expected including women with mammographically heterogeneous or extremely dense breasts, at high risk for breast cancer

diagnosed with invasive lobular carcinoma and/ or with multifocal, multi-centric or contralateral disease. It is used in the screening of high-risk women between the ages of 35 and 50 years, and is the optimum method for imaging the breast in patients who have had previous implant surgery.[95]

1.3.3 Breast Biopsy

Breast biopsy is required of any suspicious palpable lesion. Impalpable or very small lesions can be biopsied with the guidance of ultrasound.

Fine Needle Aspiration Cytology (FNAC)

This technique can differentiate between solid and cystic lesions. If the breast lesion is suspected to be fluid filled and is easily accessible then FNAC is the preferred biopsy technique. Tissue is extracted for cytological analysis that can quickly differentiate between a benign and a malignant lesion. The advantages of FNAC over core biopsy are: the results are readily available, often during the clinic; quicker to perform; local anaesthetic is often not required; less traumatic and so more appropriate for some patients on anticoagulative therapy. The main limitations of FNAC are the inability to discriminate in situ from invasive carcinoma and difficulty in rendering a definitive diagnosis in several categories (eg papillary breast lesions, atypical hyperplasias). Furthermore, training in smear preparation and cytological experience is required for interpretation. Whilst core biopsy is considered best practice for non-operative breast biopsy, FNAC is used for sampling axillary lymph nodes.

Core Biopsy

The main advantage of core biopsy over FNAC is that this technique provides an architecturally intact specimen for full histological diagnosis and can differentiate between invasive and non-invasive cancers. It takes longer than FNAC, requires local anaesthetic, and results are not readily available. However, in experienced hands this is a highly sensitive and specific technique and for this reason it has largely superseded FNAC in the role of diagnostic breast biopsy in the clinic. Ultrasound is the imaging method of chose for sampling non-palpable soft tissue lesions where it provides real time demonstration of the needle traversing the lesion. X-ray stereotaxis is used for image-guided biopsy of most indeterminate and suspicious microcalcifications, areas of parenchymal distortion/ stellate lesions and small soft tissue masses which cannot be adequately visualised by ultrasound.

The ER, PR and HER2 status should be requested for all invasive breast cancers simultaneously at the time of initial histopathological diagnosis.

1.3.4 Grade

Histological grade is an independent prognostic factor for invasive breast cancer, used internationally to aid treatment decisions. Together with lymph node status, tumour size, hormone and HER2 receptor status, it gives important information relating to risk of both local and distant recurrence, and influences patient management. Grade has significant impact on selection of patients for adjuvant systemic therapy after breast conserving surgery or mastectomy, and for post-mastectomy radiotherapy.[96,97]

The internationally accepted grading system is that proposed by Elston and Ellis (EE)[98,99] based on a cohort of 1951 patients treated in Nottingham, UK between 1973 and 1989 with up to 15 years follow-up. Grading focuses on the appearance of breast cancer cells with respect to that of normal breast tissues. Normal breast cells are differentiated, meaning that they have specific morphological characteristics that reflect their function as part of the breast as a whole. Breast cancers have lost their differentiation and the cells have become disorganised. Control of cellular division is lost and the cell nuclei become larger and less uniform (nuclear pleomorphism).

The differentiation of breast tumours is graded by consideration of glandular formation, nuclear pleomorphism and frequency of mitoses. Each of these are scored using the Nottingham Modification of the Scarff-Bloom-Richardson grading system from 1-3, and their values are added to produce three overall grades. This derived histological grade is an important predictor of both disease free and overall survival.

> Grade I cancers (score 3-5): low grade, well differentiated tumours. These have the most favourable prognosis, can be treated less aggressively and are associated with the best survival rate.
>
> Grade II cancers (score 6-7): intermediate grade, moderately differentiated tumours.
>
> Grade III (score 8-9): high grade, poorly differentiated tumours which have lost many of the normal features seen in normal breast cells. They appear large and immature, they divide rapidly and have a greater propensity to spread. These cancers are associated with the poorest prognosis.

1.3.5 Quantification of ER

ER status is usually measured by IHC, using monoclonal antibodies. It is generally considered that there is a bimodal distribution of ER values in which almost all tumours are found to be either strongly ER positive or negative, with only a small proportion showing intermediate values.

The Allred scoring system is the ER scoring system used in the UK. The score is a combination of the percentage of cells that stain by IHC for ER (on a scale of 0-5), and the intensity of that staining (on a scale of 0-3, for a possible total score of 8).
Percentage score:

0= No staining

1= Staining in <1% of cells

2= 1 to 10%

3=10 to 33%

4= 33 to 67%

5=67 to 100%

Intensity score:

0= Negative

1= Weak

2= Intermediate

3= Strong

Tumours with a score of 6-8 are considered to show strong ER positivity, and those with a score of 2-5 are weakly positive.

1.3.6 HER2 Testing

Given the importance of HER2 as a prognostic and treatment related factor, almost all invasive cancers are now tested for the presence of HER2 over expression.[100] Various methods are used, and guidelines for testing have been produced.[101] The most widely used method is an IHC test, which is simple, relatively inexpensive, and easily accommodated within existing surgical or pathology laboratories. The test classifies HER2 on a subjective scale of 0, 1+, 2+, and 3+. Patients with 0 and 1+ are considered to have low expression and to be HER2 negative. Patients with 2+ are considered indeterminate and those with 3+ are considered to be HER2 positive. This IHC assay is about 90% accurate.[102] Various in situ hybridisation techniques, which use one or two tags for the centromere on chromosome 17 and the HER2 gene, are also available.[103,104] If the ratio of HER2 to the centromere on chromosome 17 is greater than 2.2 then HER2 is considered to be amplified.[102] Some centres

use this method for all cancers, but most use it only for samples that are indeterminate (2+) on IHC. Newer methods that measure mRNA for HER2 are in development.

1.3.7 Staging

Following diagnosis and histological classification of a breast cancer, the presence and extent of local and distant disease must be assessed. This process is known as staging and is a key factor used to define treatment and to assess the chance of successful treatment outcome.

The most widely used staging system among clinicians is the TNM system maintained by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC).[56] This system codes the extent of the primary tumour (T), regional lymph nodes (N), and distant metastases (M) and provides a 'stage grouping' based on T, N and M. The UICC system groups combinations of the TNM factors into stages:

Stage 0: Non-invasive or in-situ disease.

Stage 1-3: Disease confined to the breast and lymph node basins.

Stage 4: Metastatic disease.

Patients with small tumours (stage I and II) have a low risk of metastatic disease and require no further investigations, unless directed by clinical signs and symptoms. For patients with larger tumours, and in whom there is clinical suspicion, CT and PET-CT scans should be considered.

TNM is periodically updated based on advances in understanding of cancer prognosis, to remain current and relevant to clinical practice. Historically, cancer staging and treatment planning was based solely on the anatomic extent of the cancer. Whilst anatomy continues to be a key prognostic factor for cancer, the relevance of the TNM staging of breast cancer in the era of biomarkers, genomic analysis, and personalised medicine is becoming increasingly limited. The emergence of tumour biology as an essential component to breast cancer care has allowed clinicians to understand why patients who are staged similarly using the TNM staging system have significantly different outcomes based on tumour biology. The 8th edition of the AJCC staging manual (effective Jan 2018; UICC Jan 2017), outlines a new prognostic staging system that relies not only on the anatomic extent of disease, but also on prognostic biomarkers.[105] It now encorporates T, N, M and tumour grade; and HER2, ER and PR status.

The results of a multigene assay should also be incorporated into the prognostic staging for patients with hormone receptor positive, HER2 negative, node negative tumours that are <5cm. Specifically, a recurrence score (RS) <11 on Oncotype Dx[106] denotes a prognosis similar to those with T1a to b N0M0 tumours, and they are assigned a prognostic stage of I1.

The Oncotype Dx is a genomic assay of 21 genes assessed by reverse transcription polymerase chain reaction (RT-PCR). A score <11 denotes a favourable prognosis with a 5 year distant recurrence-free survival of 99.3% for patients treated with adjuvant endocrine therapy alone.

1.3.8 Staging the Axilla

The status of the axillary lymph nodes in non-metastatic, lymph node positive breast cancer patients is the single most important determinant of overall survival.[107,108] In patients with invasive breast cancer, full pathological assessment is only complete following surgical sampling of the lymph nodes. For patients with pathologically proven axillary lymph node involvement, the number of positive lymph nodes correlates with the incidence of distant metastases and overall survival, and more than 3 positive nodes is associated with a 13-24% locoregional recurrence rate.[67] Both the number of involved nodes and their anatomical level have predictive value for survival.[109] There is axillary lymph node involvement in up to 40% of women with early, invasive breast cancer, and as such accurate staging of the axilla is critical.

Pre-operative identification of nodal malignancy allows for prompt and definitive axillary surgery. Clinical and radiological assessment of lymph node status is not reliable. Routine US of the axilla followed by FNAC or core biopsy of clinically suspicious nodes can detect up to 40% of patients with axillary node involvement.

Complete axillary node dissection remains the standard approach for patients who are clinically node positive. This involves removal of all tissue between the anatomical landmarks of the axillary vein (superiorly), the thoracodorsal bundle (laterally), and the long thoracic nerve (medially); 10 to 40 nodes are removed, and this is referred to as a level 1 and 2 node dissection.[110] Level 1 and 2 lymph node dissection surgery is associated with an increased risk of adverse outcomes, including lymphoedema (14%), limited shoulder/ arm motion (28%), and neuropathic pain (31%).[111] A desire to minimize morbidity led to the development of the sentinel lymph node biopsy technique.

Sentinel Lymph Node Biopsy (SLNB)

The sentinel lymph node is defined as the first node draining the primary tumour in the regional lymphatic basin. Various studies have now shown that histopathological examination of the sentinel node is reliable in predicting axillary lymph node status in breast cancer.[112,113,114] With improvements in breast cancer screening, more patients now present without palpable or sonographically evident nodal metastases.[115] Sentinel lymph node biopsy,

first described in 1994, exploits the orderly pattern of lymphatic drainage. Radioactive technetium Tc 99m and/ or blue dye (isosulfan or methylene blue), are injected directly onto the breast or into the skin of the breast. The first 1 to 4 nodes that take up Tc 99m and/ or blue dye are subsequently removed and evaluated for metastases, because these nodes are presumed to be those to which metastatic disease would first spread. False negative rates are less than 5% in experienced centres. The five year axillary recurrence rate is reported to be between 0.5%-1.5% in patients with a negative SLNB,[116,117,118] and continues to be low at 10 years.[119] In a meta-analysis of 48 studies including 14959 patients, axillary recurrence rate was reported as 0.3% after a median follow-up time of 34 months.[120]

For patients with DCIS undergoing breast conserving surgery, surgical assessment of the axilla is not recommended. However, SLNB is recommended for patients with DCIS undergoing mastectomy, as it is not possible after a mastectomy in the event that invasive disease is subsequently found.

1.4 Prognosis in Breast Cancer

Using prognostic factors, patients may be stratified into low or high risk groups, giving a relative prediction of the future behaviour of their disease. Prognostic factors are measured objectively at the time of diagnosis and can predict clinical outcome independent of therapy. Prognostic factors can be divided into clinical, histological and molecular factors.

1.4.1 Clinical Factors

Clinical prognostic factors include tumour size, nodal status and presence of metastatic disease. Tumour size is measured by the pathologist following surgical excision and correlates with survival, with smaller cancers having better survival rates than larger cancers.[121]

Histological axillary nodal status is the most powerful prognostic factor in breast cancer. Survival directly correlates with the number and level of axillary node involvement.[122] The new TNM staging system includes a definition of micrometastases as one measuring between 0.2mm and 2mm in diameter. The clinical significance of the micrometastasis is unclear and it is treated as node negative disease.[123]

5% of patients have metastatic disease at presentation, and have a poorer prognosis than patients with localised disease. Predicted survival depends upon the site of metastases, thus patients with supraclavicular fossa disease will have a better overall survival than those with disease at other sites. Furthermore, patients with bony metastases alone have better survival outcomes than those with visceral disease.[97,124]

1.4.2 Histological Factors

The histological type and grade of the breast cancer provide important prognostic information, as well as the expected biological behaviour and pattern of spread of that type of cancer. Special types of invasive breast cancers, including tubular, mucinous and cribiform cancers are associated with a better prognosis than invasive ductal cancer of no special type.[98]

25% of breast cancer cases will have lymphovascular invasion and this is associated with increased local disease recurrence and short term systemic relapse.[125] Currently, there is no clear evidence of prognostic significance of peri-tumoural angiogenesis or lymph node micrometastases.

Patients who have an extensive in-situ component of disease (>25% of the main tumour with non-invasive disease) and who have positive margins at the time of resection also have an increased risk of local recurrence after breast conserving surgery. These tumours are not associated with increased recurrence risk if the resection margins are clear.[67]

1.4.3 Molecular and Biological Factors

The identification of molecular and biological markers that provide prognostic and predictive information to add to standard clinical and pathological factors is an exciting area of current research. A molecular risk profile will be clinically useful if higher-risk patients can be selected for specific treatments and those at lower risk can avoid adjuvant therapies such as chemotherapy or radiotherapy, which impair quality of life and increase health-care costs. Interest in novel prognostic markers is based on the fact that a significant number of patients with early-stage breast cancer harbour microscopic metastasis at the time of diagnosis. Many molecular markers that have been studied have both prognostic and predictive value. Prognostic markers are indicators of aggressiveness, invasiveness, extent of spread, and thus correlate with survival, independent of systemic therapy. Predictive markers allow clinicians to expect therapeutic outcomes and decide future treatment plans.

Clinical decisions regarding breast cancer management are influenced largely by tumour expression of ER, PR and HER2. These biomarkers have prognostic and predictive significance in breast cancer and have important implications for tumour growth and metastatic patterns.

Estrogen Receptor

In general, ER positive breast cancers recur at a low but steady rate over time.[126] In a recent meta-analysis of 62,923 patients with ER positive, early-stage breast cancer, treated with 5 years adjuvant endocrine therapy, there was a persistent risk of recurrence and death from breast cancer for at least 20 years after the original diagnosis.[127] ER positive tumours have a constant long term recurrence rate of 2% per year.[128,129] This contrasts with other breast cancer subtypes where recurrences peak early (often within the first three years of initial diagnosis) and then decline in frequency thereafter. Overall, ER positive tumours are less likely to recur or cause death from breast cancer than other subtypes.[130] Tumours that co-express ER and PR have the best outcomes, which may be driven by better responses to endocrine therapy.[129] Furthermore, the median survival from diagnosis of recurrence is higher for women with ER positive breast cancer than those with ER negative disease.[130] Epidermal

growth factor receptor (HER1) correlates inversely with ER status and is associated with poor survival.[131]

HER2

In the absence of treatment, HER2 positivity is associated with inferior overall survival compared with other breast cancer subtypes, regardless of other known prognostic features such as age, nodal status, tumour size, tumour grade, hormone receptor status and adjuvant treatment.[132] However, trastuzumab, the humanised monoclonal antibody which targets HER2, has been shown to prolong disease free survival and overall survival, both in the metastatic[133] and the adjuvant settings.[134] In recent years a series of other anti-HER2 agents have been developed. The introduction of lapatinib and more recently pertuzumab and ado-trastuzumab-emtansine are further improving outcomes in the arena of HER2 positive breast cancer. Therefore, the median survival for HER2 positive metastatic breast cancer (MBC) is now over 2 years.[135]

Triple Negative Breast Cancers (TNBC)

In general, TNBC is associated with a worse prognosis than other stage-matched tumour phenotypes, partly reflecting tumour biology and partly due to the lack of validated targeted therapies for these patients. The poorer outcome associated with a TNBC phenotype appears to be independent of grade, tumour size, nodal status and therapy.[136] The overall survival from diagnosis of metastases is short in patients with TNBC (7-12 months).[137] In contrast to ER positive breast cancer, women who do not experience tumour relapse within the first 5 years of early stage TNBC are likely to be cured.

Ki67

An increased expression of cellular proliferation markers, indicative of uncontrolled growth, is associated with poor clinical outcomes in breast cancer.[138] Immunohistochemical (IHC) staining of Ki67, a nuclear antigen that is only present in proliferating cells, has been shown to be a reliable marker to quantify the growth fraction of normal and neoplastic cell populations.[139,140] The Ki67 labelling index is based on the percentage of cells with positive Ki67 staining, and correlates with mitotic index. Ki67 is expressed in cells throughout the cell cycle, but not during the resting G0 phase, and is thus most frequently expressed in poorly differentiated tumours with high rates of mitotic activity.[141] Studies have shown that baseline tumour Ki67 is a prognostic factor for breast cancer.[142,143,144] However, lack of consistency across laboratories has limited Ki67's value. A working group has been formed to create a Ki67 reproducibilty study. This reported substantial variability in Ki67 scoring among some

of the word's most experience laboratories. The group concluded that Ki67 values and cutoffs for clinical decision making cannot be transferred between laboratories without standardazing scoring methodology.[145]

Novel biological markers

P53, p14ARF, cyclin D1, cyclin E, TBX2/3, and VEGF are novel molecular markers which have been identified through their involvement in the regulation of the p53 and RB tumour suppressor pathways, DNA damage response, and angiogenesis/ metastasis, which play critical roles in human breast cancer.

1.4.4 Prognostic indices

There are tools in current clinical use which attempt to combine clinical, histological and biological factors in order to estimate prognosis and to plan appropriate treatment for breast cancers. Examples of such methods include the Nottingham Prognostic Index (NPI),[146,147] St Galllen consensus criteria,[148] the National Comprehensive Cancer Network (NCCN) guidelines[149] and Adjuvant Online![150]

The NPI is used to determine prognosis following surgery for breast cancer. It is calculated using three pathological criteria: invasive tumour size; number of involved lymph nodes; and histological grade. The index is calculated using the following equation:

> NPI=: 0.2 x tumour size (cm) + lymph node stage (score 1 for negative lymph nodes; 2 for 1-3 positive nodes; 3 for >4 positive nodes) + histological grade (score 1 for grade 1; 2 for grade 2; 3 for grade 3).

The NPI can be used as a risk stratifier in unselected cohorts of operable, early-stage primary breast cancer patients. Prognosis worsens as the NPI numerical value increases and by using cut off points patients may be stratified into 10 year survival groups. (Table 1.5A)

Table 1.2: 10 year breast cancer survival according to Nottingham Prognostic Index[151]

Nottingham Prognostic Index (NPI)		
Group	**Index Value**	**10 Year Survival (%)**
Excellent	2.0-2.4	96
Good	2.41-3.4	93
Moderate 1	3.41-4.4	82
Moderate 2	4.41-5.4	75
Poor	5.41-6.4	53
Very Poor	>6.41	39

The NPI does not consider any biological or molecular markers and as such could be improved to support more accurate personalised management of breast cancer patients.

Improved prognostication is an extremely promising area of translational clinical genomics in which the patterns of altered gene expression in tumors are used to construct classifiers for prognostication (gene-expression signatures). Several 'high throughput' methods have been introduced into research and routine laboratories. These have provided a new approach to the analysis of genomic alterations and RNA or protein expression patterns. Gene expression microarrays provide a comprehensive view of gene activity in a biological sample. Unlike most traditional molecular biology tools, which generally allow the study of a single gene or a small set of genes, microarrays facilitate the discovery of novel and unexpected functional roles of genes. The power of these tools has been applied to discovering novel disease subtypes, developing new diagnostic tools, and identifying underlying mechanisms of disease or drug response. However, this technology produces a vast amount of data and introduces new challenges in data interpretation, requiring further exploitation of modern computational and statistical tools.[152]

Some microarray studies have led to the development of commercially available molecular testing kits which can be used in the breast cancer clinic. These tools use gene signatures which are predictive of response to treatment. To date, only 2 commercial multigene tests have received FDA clearance.

MammaPrint

The microarray based MammaPrint is used to assess the prognosis in patients less than 62 years of age with cancers that are lymph node negative or positive (1-3 nodes) and ER positive or negative and are smaller than 5cm.[153] MammaPrint includes 70 genes including 55 established genes and 15 genes with unknown function. Patients can be categorized into low or high risk cohorts, which has been shown to be more accurate when predicting 5 year metastasis free survival than conventional clinicopathological parameters.[154] Results are now available for the prospective MINDACT trial for MammaPrint. Microarray In Node-negative and 1 to 3 positive lymph node Disease may Avoid ChemoTherapy (MINDACT) clinical trial demonstrates that 46% of breast cancer patients considered for chemotherapy, whose tumours are classified MammaPrint Low Risk, have excellent survival without chemotherapy, and can thus be candidates to avoid this toxic therapy.[155,156]

PAM50 (Prosigna, Nanostring Techologies, USA)

This is the 2nd FDA approved assay which uses a panel of 50 genes plus 5 housekeeping genes to compute a risk of recurrence score which enables to identify the intrinsic BC subtypes. PAM50 also provides estimation for distant relapse-free survival and likelihood of recurrence at 10 years for ER-positive, tamoxifen treated patients. It was validated in 2 retrospective trials including more than 2,400 patients and can also support identification of node-positive and ER positive patients who do not necessarily need adjuvant chemotherapy.[157,158]

Oncotype DX (Genomic Health, USA)

This test measures the expression of 21 genes including 16 cancer related genes and 5 reference genes to compute a continuous recurrence score (RS) ranging between 0 and 100.[159] The most important genes in the assay include HER2, ER and Ki67, with highest score given to the 2 genes related to the HER2 pathway (HER2 and GRB7). RS is prognostic for ER breast cancer treated with tamoxifen regardless of nodal status (up to 3 positive lymph nodes). Patients with a low recurrence score (0-10) have a very low risk of recurrence with endocrine therapy (ET) alone. Patients with a high RS (26-100) demonstrated poorer outcomes with higher event rates despite the addition of chemotherapy to ET. For patients in the mid-range RS (11-25), the benefit of adding chemotherapy is uncertain. TAILORx is a trial which attempted to bring clarity to the interpretation of this midrange RS group. TAILORx enrolled 10,273 women, making this the largest breast cancer treatment trial ever conducted. The investigators concluded that adjuvant chemotherapy may be spared in all women older than 50 years with an RS of 11-25 and in 36% of those 50 years or younger. Of patients 50 years or younger (14% of the overall population), 64% had an RS of 16-25 and this subset can derive some befefit from chemotherapy.[160]

1.4.5 Predictive indices

While prognostic markers correlate with survival independent of systemic therapy, predictive markers provide information on the likelihood of a favourable response to a particular treatment. A most important question is regarding adjuvant chemotherapy, since uniform treatment for all tumours would result in substantial over or under treatment for the individual patient.

Some prognostic factors are predictive and vice versa. For example in addition to providing prognostic information, ER status predicts response to hormone treatment. Similarly HER2 positivity predicts response to immunological treatment with Trastuzumab (Herceptin),

Lapatanib, Pertuzumab and T-DM1 and HER1 (EGFR) positivity to treatment with Lapatanib, Pertuzumab and Gefitinib. With this in mind, a number of attempts have been made to incorporate prognostic and predictive information into clinically useful tools, which aim to provide prognostic and predictive information.

Adjuvant! Online

Adjuvant! online (www.adjuvantonline.com) is a frequently updated site that assists healthcare professionals and patients with early stage breast cancer to discuss the risks and benefits of adjuvant therapy after surgery. It does this by presenting estimates of the risk of cancer-related mortality or relapse, which can be used in patient consultations. Patient details including age, health and comorbidities, and details of the tumour including size, nodal status, estrogen receptor and HER2 status. The information is processed to provide details of recurrence rates and survival with and without adjuvant therapies.

There has been some uncertainty about how applicable the Adjuvant! Model is to patients diagnosed and treated in the UK. One study of 1,065 women with early breast cancer treated in the UK showed that Adjuvant! overestimated the overall survival by 6%.[161] In the UK, PREDICT.nhs (www.predict.nhs.uk) is now widely used. This is a mathematical model accessed by the internet and designed for patients and doctors to help them decide on the ideal course of treatment following breast cancer surgery. The model was developed on information collated for 5,694 women who had surgery for invasive breast cancer in East Anglia from 1999-2003. Breast cancer mortality models for ER positive and ER negative tumours were derived and a prognostication model for early breast cancer that predicts breast cancer survival following surgery was developed. Data entry for an individual patient includes patient age, tumour size, grade, number of positive nodes, ER status, HER2 status, Ki67 status and mode of detection. Survival estimates, with and without adjvant therapy, are presented. Treatment benefits for endocrine therapy and chemotherapy are calculated and predicted mortality reductions are available for both second generation (anthracycline-containing) and third generation (taxane containing) chemotherapy regimens.[162] It is the first model of this type to include tumour HER2 and KI67 status.[163,164]

1.5 Management Pathways- Local Treatments

The aim of breast cancer treatment is to achieve long-term disease control with minimal morbidity to the patient. Most patients benefit from a combination of local and systemic therapies. Local therapy includes surgery and radiotherapy and is intended to treat the tumour at its site within the breast, and in the axilla, without affecting the rest of the body.

1.5.1 Surgical Management of the Breast

Surgery is considered the primary treatment for breast cancer. The main aim of surgery is the complete resection of the primary tumour with negative margins to reduce the risk of local recurrences. Surgery also allows for pathological staging of the tumour and axillary lymph nodes to provide necessary prognostic information. Procedures include breast conserving surgery (BCS), when tumour is excised with a margin of surrounding tissue whilst preserving the healthy breast tissue, and mastectomy.

Large, randomized, clinical trials have reported no significant difference in disease-free and overall survival between BCS and traditional mastectomy.[165,166,167] BCS is considered to be associated with a diminished psychological burden compared with mastectomy, offers better cosmetic results, and reduces wound infection risk.[168] The most important disadvantage of BCS is the lifelong risk for local recurrence, in which case additional surgery is necessary. Large clinical trials have reported local recurrence rates between 6% and 16%.[169,170]

Breast Conserving Surgery

Breast conserving surgery is the complete surgical resection of a primary tumour with a 1cm margin of macroscopically normal tissue, a wide local excision (WLE). It may be performed with palpation guidance or by pre-operative localisation with image guidance and placement of a wire. It is applicable in most patients with stage I or II invasive carcinomas. This procedure is combined with excision of the sentinel node or all of the axillary nodes. Patients who have BCS will subsequently have radiotherapy.

Relative contraindications to BCS include small breast size, large tumour size (>5cm), and collagen or vascular disease. Absolute contraindications to BCS are considered to be:

(i) Multifocal disease
(ii) History of previous radiation therapy to the area
(iii) Inability to have radiation therapy for invasive disease
(iv) First or second trimester of pregnancy
(v) Persistent positive margins after attempts at conservation.

Accurate localization is essential for adequate surgical removal of breast tumors, in which an optimal balance between good cosmetic results and preservation of resection margins is the primary goal. Obtaining tumor-free surgical margins decreases the incidence of LR of the primary tumor. To ensure the complete excision of all invasive and in-situ disease, specimens can be sent for imaging intra-operatively. In the literature, the best reports result in at least 20% of patients returning to theatre for re-excision of positive margins.[171] A negative margin of 1-2mm is considered to be accurate.[172] Whilst the influence of a 'close' margin, usually defined as tumour cells being present within >0 and ≤2mm from the cut edge, has been controversial, margin 'closeness' is currently not seen as an indication for re-excision.[173] Wider margins do not reduce local recurrence further but may adversely effect cosmesis.

Patients with tumours which are too large for routine BCS may be offered neoadjuvant medical therapy in order to shrink the tumour preoperatively, rendering if suitable for BCS. Neoadjuvant endocrine therapy has been shown to reduce tumour volume to a better extent than neoadjuvant chemotherapy, resulting in better rates of subsequent complete excision following neoadjuvant endocrine therapy.[174,175,176,177] This will be discussed in further detail later. Where tumours are too large for BCS, consideration may also be given to oncoplastic surgery. Options for breast reconstruction include fasciocutaneous local tissue advancement flaps; breast parenchymal local flaps; and latissimus dorsi myocutaneous flaps. These procedures are associated with reduced psychological morbidity than that following mastectomy.[126]

Mastectomy

Patients not suitable for BCS will be offered mastectomy. Some patients opt to have mastectomy through personal preference, or where the cosmetic result from BCS is deemed unacceptable.

A total mastectomy involves complete removal of all breast tissue to the clavicle superiorly, the sternum medially, the infra-mammary crease inferiorly, and the anterior axillary line

laterally, with en bloc resection of the pectoralis major fascia. The following variants are performed:

- Modified radical mastectomy – A total mastectomy with axillary lymph node dissection (ALND)
- Skin-sparing total mastectomy (SSM)
- Nipple-sparing total mastectomy (NSM)

Increasingly, skin and nipple sparing mastectomies are performed in conjunction with immediate breast reconstruction to achieve optimal aesthetic results. Radical and extended radical mastectomy (total mastectomy plus en bloc resection of the pectoralis major and ALND, with resection of the internal mammary lymph nodes in the extended procedure) are now deemed historical.

1.5.2 Surgical Management of the Axilla

In patients with invasive breast cancer, the histopathology result from SLNB determines the subsequent management of the axilla. Where the sentinel node has no pathological evidence of disease, no further treatment to the axilla is required. In patients with a positive lymph node, axillary node clearance has long been the treatment of choice to achieve regional disease control. The presence and number of cancer-containing lymph nodes detected has been used to inform decisions regarding adjuvant chemotherapy and radiotherapy.[178] Around 15-20% of patients will have tumour-positive sentinel lymph nodes after preoperative axillary ultrasound, despite no clinically suspicious findings on initial axillary examination.[179] There are three options in the case of a positive SLNB: proceed to axillary lymph node dissection (ALND); irradiate the axilla; observe. Although ALND provides excellent regional control, it is associated with harmful side effects. The AMAROS trial was a randomised, multicentre, non-inferiority trial in which patients with a positive sentinel node were assigned to receive axillary radiotherapy or axillary lymph node dissection. This trial concluded that for the treatment of macrometastases (>2mm), axillary radiotherapy and axillary node clearance are equivalent in their prevention of regional relapse; but there is less morbidity and/ or lymphoedema associated with radiotherapy.[180]

In patients with clinically node-negative disease, the sentinel node is the only involved node in 40% to 60% of patients undergoing SLNB.[112] Completion axillary lymph node dissection (ALND) for women with micrometastases or isolated tumour cells (ITCs) is controversial because of the uncertain clinical significance of micrometastases and the low yield of additional positive axillary lymph nodes. The American College of Surgeons Oncology Group (ACOSOG) Z0011 trial was designed to compare outcomes of patients with sentinel

node metastases who were randomised to have completion ALND or managed without completion ALND and without third field axillary radiation.[181] At a median follow-up time of 6.3 years, there were no statistically significant differences in local recurrence or regional recurrence between the 2 groups.

The Positive Sentinel Node: adjuvant therapy alone versus adjuvant therapy plus Clearance or axillary radiotherapy (POSNOC) trial has recently completed recruitment of 1900 participants. This is a UK wide randomised, non-inferiority trial for women with early stage breast cancer and 1 or 2 sentinel node macrometastases, to assess whether adjuvant therapy alone (chemotherapy and/ or endocrine therapy) is no worse than adjuvant therapy plus axillary treatment, in terms of axillary recurrence within 5 years. Results of the trial are expected to be published after March 2023.

1.5.3 Radiotherapy

Radiation therapy is an integral part of management in breast carcinoma treatment. Ionising radiation can kill malignant cells, as well as normal cells. It works by damaging the cells DNA and cancerous cells are more sensitive to radiotherapy than normal cells as they have a higher mitotic rate. Careful pre-therapy planning is necessary to optimise targeting the tumour tissue within the radiation field and to minimise damage of the surrounding healthy tissues. Most radiotherapy is delivered as an external beam, however, brachytherapy in which a radiation source is placed within the breast tissue or tumour bed, can be used to further minimise exposure to healthy tissue.

Other considerations must also be made in the context of radiotherapy. Radiotherapy poses a cardiac risk and cardiac exposure should always be minimised as much as possible. The risks of treatment are however outweighed by the reduction in breast cancer recurrence. Furthermore, there is a slight increased risk of lymphoedema and shoulder restriction after radiotherapy.

Current guidelines state that at least 95% of patients should receive radiotherapy within 4 weeks of BCS or final dose of chemotherapy.[182,183]

Radiotherapy after breast conserving surgery

For most patients treated with breast conserving surgery, whole breast radiation therapy is recommended. The exceptions to this approach are patients ≥65 years old with node-negative, hormone receptor positive primary tumours up to 3cm for whom endocrine therapy is planned, where radiotherapy may reasonably be excluded; and for patients ≥50 years old with ≤3cm, hormone receptor positive, node negative tumours where accelerated partial breast irradiation may ba a reasonable alternative to whole breast radiotherapy.

Two meta-analyses of individual patient data have shown significant reduction in breast cancer recurrence with radiotherapy given after breast conservation surgery.[184,185] The rate of recurrence is approximately halved at 10 years from 35% to 19.3%. Adjuvant radiotherapy should also be offered to patients with DCIS following adequate breast conserving surgery. Shorter fractionation schedules can now be used safely in patients with early breast cancer (eg 4,005cGy in fractions over 3 weeks rather than 5,000 cGy in 25 fractions over five weeks).[186] A randomised control trial comparing intraoperative radiotherapy (IORT) with external beam radiotherapy (EBRT) has shown no significant increase in local recurrence after 4 years. [187] The risk of local recurrence after standard radiotherapy can be reduced by the addition of a boost to the tumour bed. Radiotherapy boost is recommended in all patients aged 50 years or under at diagnosis and it should be considered in patients over 50 years at diagnosis, especially those with high-grade cancers.[188]

Post mastectomy radiotherapy

Post mastectomy radiotherapy (PMRT) should be considered in patients with lymph node positive breast cancer if they have a high risk of recurrence (≥4 positive lymph nodes or T3/4 tumours). PMRT results in a three fold reduction in local recurrence at 15 years, with the most significant difference in the first five years.[189] The results of the SUPREMO trial which was set up to determine the benefits of PMRT in women considered 'intermediate' risk of recurrence are awaited.

Radiotherapy following axillary surgery

Following complete axillary dissection (level I/II), post operative radiotherapy is deemed unnecessary and may add to morbidity. In patients with ≥4 positive lymph nodes, irradiating the supraclavicular field confers a survival benefit.

1.6 Management Pathways- Systemic Treatment

Systemic therapies include chemotherapy, endocrine and biological therapies and may be delivered before or after locoregional treatment.

1.6.1 Chemotherapy

Locoregional Disease

In general patients with an estimated relapse risk of more than 10% over the course of 10 years are viewed as potential candidates for neoadjuvant or adjuvant chemotherapy. In early breast cancer, preoperative chemotherapy is equally as effective as postoperative chemotherapy regarding disease-free survival and overall survival.[190] Traditional chemotherapeutic agents specifically target cells undergoing mitosis, thereby preferentially affecting the rapidly dividing cancer cells. In trials of neoadjuvant chemotherapy, pathological response has consistently been shown to be a powerful determinant of long-term outcome. Patients with a complete pathological resonse (pCR) have a significantly better diease free and overall survival.[192]

The current standard chemotherapies in early breast cancer are anthracyclines and taxanes, given as a combination or in sequence over a period of 18-24 weeks. The EBCTCG meta-analysis suggested that anthracycline containing and taxane containing chemotherapy reduced 10 year breast cancer mortality by about a third.[192] An anthracycline and taxane sequence is as effective as their combination.[193] Results of several trials in node-positive high risk disease have shown that dose-dense chemotherapy improves outcome in early breast cancer compared with standard interval chemotherapy.[194] Data from chemotherapy trials exist for patients with early breast cancer up to about 70 years of age, however biological age is more important than chronological age when indicating chemotherapy in elderly patients.[193] Dose and schedule can be tailored according to the special requirements of an elderly patient, as stated by the International Society of Geriatric Oncology (SIOG).[195]

For patients with triple negative breast cancer, standard regimens containing anthracycline and taxane should be used, preferably as neoadjuvant therapy. A 6-9 times higher risk for relapse has been reported for patients with TNBC or with HER2 positive breast cancer who do not achieve a pCR with neoadjuvant treatment. Since 2014, trials have indicated that adding platinum to a neoadjuvant anthracycline-taxane combination or sequence improves the rate of pathological complete response.[196,197,198] The results of the GeparSixto trial showed

that adding neoadjuvant carboplatin to a regimen consisting of taxane-anthracycline chemotherapy and targeted therapy (bevacizumab) substantially increased pCR in patients with stage II-III TNBC,[199]

Metastatic Disease

In contrast with early breast cancer, metastatic breast cancer is considered incurable with currently available therapies, however, the concept of metastatic breast cancer as a chronic disease controlled by sequential therapies over a long period is realistic for certain subgroups. Next to prolongation of life, therapeutic goals in metastatic breast cancer are maintenance of quality of life and palliation of symptoms. Chemotherapy is always indicated in triple-negative breast cancer, after endocrine options have been exhausted in luminal disease or if rapid response is needed in life-threatening situations or in patients who are highly symptomatic.[194] If not already given in the adjuvant setting, patients with metastatic breast cancer should receive anthracyclines and taxanes. Unless patient symptoms require combination chemotherapy, sequential mono-chemotherapies are recommended, as combination chemotherapy does not prolong survival.[200]

1.6.2 Biological Therapies

HER2 positivity accounts for about 20% of breast cancers and is defined as evidence of HER2 protein over-expression measured by immunohistochemistry status (IHC3+) or by fluorescence in-situ hybridisation (FISH) measurement of a HER2 gene copy number of six or more or a HER2/CEP17 ratio of 2·0 or greater. Anti-HER2 treatment for HER2-positive breast cancer has changed the natural history of this disease. Trastuzumab (Herceptin; Genentech, Inc, USA) is a humanised mouse monoclonal antibody that binds to the extracellular domain IV of HER2, thereby inhibiting ligand-independent HER2 and HER3 signalling. The approval of trastuzumab in 1998 has been a milestone in the treatment of HER2 positive breast cancer. It has consistently been shown to improve disease free survival (DFS) and overall survival (OS) in early and breast cancer and improved time to disease progression and OS in patients with metastatic breast cancer (MBC).[201,202,203] However, despite appropriate treatment with trastuzumab, up to 40% of patients with HER2 overexpression may be resistant to therapy (de novo and acquired resistance).[204] To combat resistance, dual therapy combinations have been trialled, including combination of anti-HER2 agents with chemotherapy, combination of 2 anti-HER2 agents with complementary mechanisms of action targeting HER2/ HER3 dimerization, and testing of irreversible dual HER1/ HER2 inhibitors, such as afatinib and neratinib.[204] Trastuzumab, combined with chemotherapy in the neoadjuvant setting almost doubles the rate of complete pathological

response and in the adjuvant setting reduces recurrence and improves survival.[279, 280] Newer anti-HER2 treatments include lapatinib, a small molecule that blocks HER1 and HER2 activation, and pertuzumab, a monoclonal antibody that targets HER2 and prevents HER2/ HER3 dimerization. Combining either pertuzumab or lapatinib with trastuzumab and chemotherapy before surgery appears even more effective than dual treatment with chemotherapy and trastuzumab.[207,208,209]

Several randomised trials have shown that adjuvant treatment with trastuzumab leads to a significant improvement in disease-free survival in women with HER2-positive, operable breast cancer. Furthermore, follow up data from the NSABP B-31 and NCCTG 9831 trials[210] and the BCIRG 006[211] trial show overall survival rates of 83-86% at 8-10 years in women with HER2 positive, operable breast cancer. This is a vast improvement for a disease subtype previously associated with poor prognosis. Further studies have investigated ways of targeting the HER2 receptor with new drugs and combinations. The ALLTO study[212] compared trastuzumab with three experimental regimens: lapatinib, lapatinib with trastuzumab, or sequential therapy with trastuzumab followed by lapatinib. The net result was that addition of lapatinib to trastuzumab did not improve the therapeutic benefit of adjuvant trastuzumab and was associated with additional toxic effects that were directly due to lapatinib.

In a phase III, randomized study, the combination of trastuzumab and lapatinib improved progression-free survival (PFS) when compared to lapatinib alone in patients with progression of MBC on prior trastuzumab-containing therapy.[213] Clinical Evaluation of Pertuzumab and Trastuzumab (CLEOPATRA) trial demonstrated a significant improvement in OS to 56.5 months in the pertuzumab, trastuzumab, and docetaxel group versus 40.8 months in the trastuzumab and docetaxel group. Additionally, the PFS and duration of response were prolonged in the pertuzumab group, supporting the use of dual HER2 blockade in MBC.[214]

The latest new agent targeted at HER2 over expressing cancers is ado-trastuzumab (T-DM1; Kadcyla®), which is a three part immunoconjugate consisting of trastuzumab, a stable linker, and the potent cytotoxic emtansine derivative, DM1. Compared with lapatinib and chemotherapy, T-DM1 significantly increased progression-free survival and was better tolerated in patients who had progressed while receiving trastuzumab and taxane chemotherapy.[216] Unlike pertuzumab which has low single-agent activity and must be partnered with trastuzumab as part of a regimen that frequently includes anthracyclines and taxanes in earlier stage disease, T-DM1 has very good single-agent first line metastatic breast cancer activity. The MARIANNE study was designed to assess the efficacy and safety of T-

DM1 and T-DM1 plus pertuzumab compared with trastuzumab plus taxane in patients with HER2 positive, advanced breast cancer and no prior therapy for advanced disease.[217] In the study, although not demonstrating superiority in PFS to a taxane plus trastuzumab, T-DM1 was noninferior. Furthermore, the duration of response to T-DM1 with or without pertuzumab was 20.7 months and 21.2 months, respectively, versus 12.5 months for a taxane and trastuzumab. The single-agent toxicity profile of T-DM1 is also highly favourable given that it seldom causes alopecia and is well tolerated subjectively. On the basis of these findings, T-DM1 may provide an alternate first line treatment option to trastuzumab plus taxane in patients with HER2 positive metastatic breast cancer.
In April 2017 the Scottish Medicines Consortium approved the use of T-DM1 for the treatment of patients with HER2 positive, unresectable lovally advanced or metastatic breast cancer who previously received trastuzumab and a taxane. Of note is that Pertuzumab, following a second submission for approval in June 2017, is not approved by the Scottish Medicines Consortium and as such is not available for prescription on the NHS in this country.

1.6.3 Neoadjuvant Trials of anti-HER2 therapies

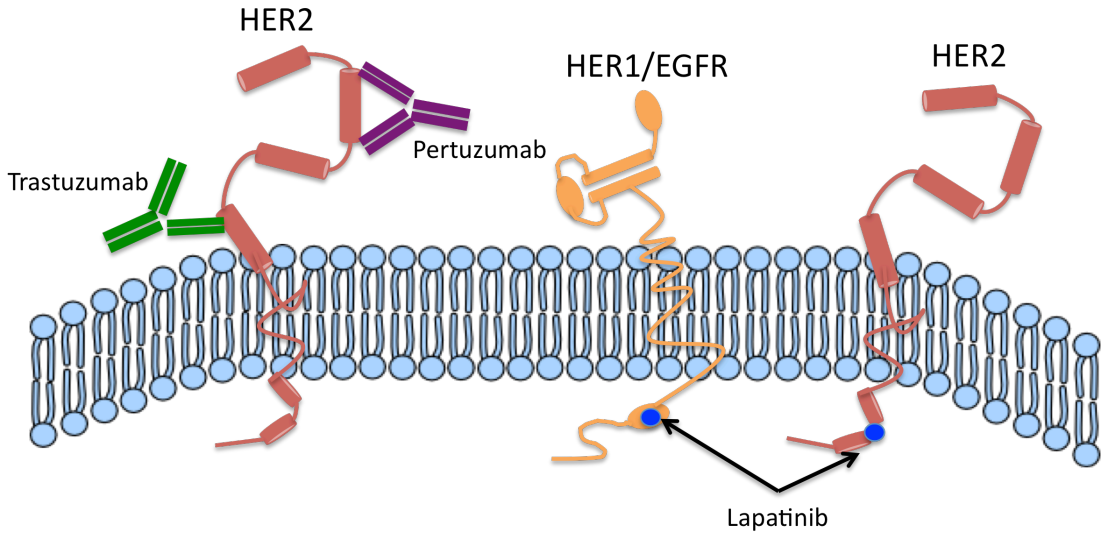
In the Neoadjuvant Lapatinib and/or Trastuzumab Treatment Optimisation (NeoALTTO) trial, a phase III, multicenter study, pCR was significantly higher in patients treated with lapatinib and trastuzumab than with trastuzumab alone (51.3% vs 29.5%, p=0.001).[218] Furthermore, in women who achieved pCR in the NeoALTTO study, there was a statistically significant improvement in both event free survival (EFS) and overall survival (OS).[219] The CHER-LOB trial (Chemotherapy, Herceptin and Lapatinib in Operable Breast Cancer) also demonstrated significant improvement in in pCR with dual therapy compared to trastuzumab or lapatinib alone, demonstrating an 80% increase in pCR rate.

Trastuzumab in combination with pertuzumab in the neoadjuvant setting has also been investigated. In the NeoSphere trial patients were assigned to 1 of 4 treatment arms: 1) trastuzumab alone with docetaxel; 2) pertuzumab alone with docetaxel; 3) trastuzumab and pertuzumab with docetaxel; or 4) trastuzumab and pertuzumab without chemotherapy prior to breast surgery.[220] Results demonstrated that patients who were given pertuzumab and trastuzumab plus adjuvant docetaxel had a significantly improved pCR (45.8%) compared with those given trastuzumab with docetaxel (29%) or pertuzumab with docetaxel (24%; P=0.01). These results were considered practice changing and drove the FDA approval of pertuzumab for neoadjuvant treatment of HER2+ BC. Notably, the dual anti-HER2 therapy without chemotherapy arm resulted in a pCR rate of 16.8%, which could suggest a potential

role for a treatment regimen without chemotherapy in select groups of patients who may not be candidates for cytotoxic chemotherapy.

Most recent results from the ADAPT trial of ER positive/ HER2 positive breast cancers, are particularly relevant to the current study of ER positive/ HER2 positive breast cancer.[221] In this prospective, neoadjuvant, phase II trial, 375 patients with early breast cancer with HER2 positive and HR positive status were randomly assigned to 12 weeks of T-DM1 with or without endocrine therapy or to trastuzumab with endocrine therapy. The primary end point was pCR. pCR was observed in 41% of patients treated with T-DM1, 41.5% of patients treated with T-DM1 and ET, and 15.1% with trastuzumab and endocrine therapy ($p<0.001$). The ADAPT trial demonstrates that neoajuvant T-DM1 (with or without endocrine therapy) given for only 12 weeks results in a clinically meaningful pCR rate, thus a substantial number of patients might be spared the adverse effects of systemic chemotherapy.

Figure 1.2 : Trastuzumab, Pertuzumab, Lapatinib: Mechanisms of action.
Trastuzumab binds to the extracellular domain IV of HER2, thereby inhibiting ligand-independent HER2 signalling. Pertuzumab binds to the extracellular domain II of HER2, inhibiting ligand-dependent HER2-HER3 dimerization and signalling. Lapatinib binds to the cytoplasmic binding sites of the kinases and blocks downstream signmalling through homodimers and heterodimers of HER1/EGFR and HER2.



1.7 Endocrine Therapy

Endocrine therapy (ET) for breast cancer consists of 1) ovarian function suppression; 2) selective estrogen receptor modulators (SERMs); 3) selective estrogen receptor down-regulators (SERDs); and 4) aromatase inhibitors (AIs), or a combination of 2 or more drugs. The 2 major strategies are directed at either blocking the estrogen receptors of cancer cells (SERMs), or by reducing estrogen production by acting against the key enzyme involved in its biosynthesis (AIs).

1.7.1 Blocking Estrogen Function

The SERMs include tamoxifen, raloxifene and toremifen. Tamoxifen is the most commonly used endocrine therapy and acts as an antiestrogen by inhibiting the binding of estrogen to estrogen receptors. It was initially studied as an anti-fertility drug, and soon demonstrated favourable effects in patients with ER+ breast cancer.[222] Tamoxifen acts as a partial non-steroidal agonist in some tissues, such as liver, uterus and bone, but is a competitive receptor inhibitor in the breast and brain.[223] In these tissues, tamoxifen selectively blocks signalling at the level of ERs, also inhibiting the proliferation of ductal cells. It is hydroxylated by the cytochrome P450 enzyme system into 4-hydroxy tamoxifen (4HT), and further metabolised in the liver to endoxifen. Endoxifen and 4HT are the main active metabolites of tamoxifen and exhibit potent ER binding capacity and suppression of estrogen dependent cancer cells. The effects of tamoxifen binding are similar to that of estrogen, with dimerization causing a conformational change in the shape of the ER. However, rather than activating AF2, the tamoxifen bound receptor reduces or eliminates the activity of AF2. Consequently, the association of the ER with co-activator proteins does not take place, co-repressor proteins are not displaced and the transcription of estrogen-dependent genes is greatly reduced. Tamoxifen only benefits women with ER+ breast cancer, and ASCO (American Society of Clinical Oncology) guideline recommendations consider a cut off of 1% for IHC testing of ERs in breast cancer cells.[224] This drug can be used in both pre and post menopausal breast cancer patients.

SERMs have effects on other tissues containing ER, including bone, uterine and genitourinary tissues, the brain, and have an impact on cardiovascular risk. Whilst tamoxifen is an effective antagonist in the presence of high levels of estrogen, it can also exhibit some agonist activity on the breast and on other estrogen dependent tissues, particularly in the presence of low levels of estrogen. The estrogen agonist effects of tamoxifen in stimulating the endometrium have been well documented. Postmenopausal breast cancer patients treated with tamoxifen have increases in endometrial thickness and a high incidence of abnormal histopathological

findings in the endometrium.[225, 226] A meta-analysis of breast cancer prevention trials found that tamoxifen therapy increases the risk of endometrial cancer by about 2.4 fold when compared with placebo.[227] Furthermore, tamoxifen therapy was shown to increase the risk of thromboembolic events by 1.9 fold. Another estrogen agonist effect of tamoxifen is that it helps to prevent bone demineralization in postmenopausal women, and can improve the lipid profile in these patients.

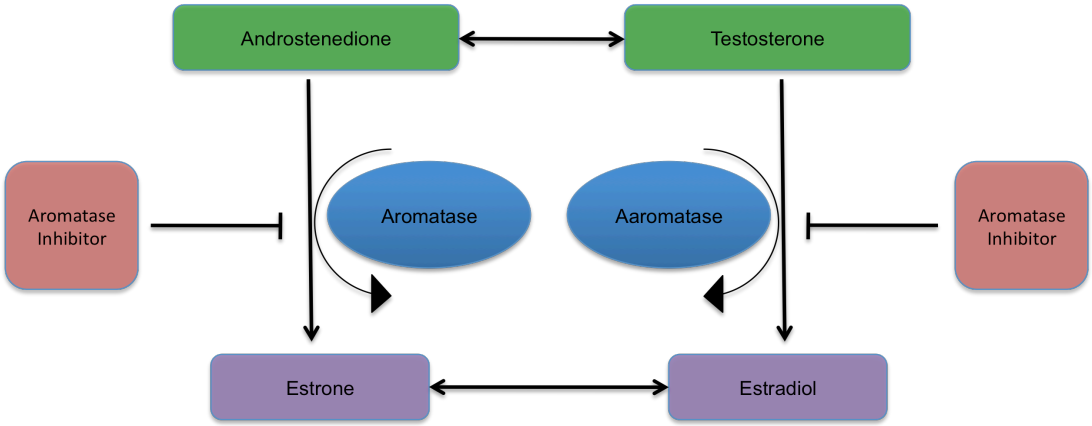
In postmenopausal women, the ER signalling pathway may be targeted by fulvestrant ('Faslodex'), a newer type of ER antagonist with no agonist effects. Fulvestrant, known as the 'pure anti-estrogen', binds, blocks and causes degradation of the ER, culminating in complete suspension of estrogen-sensitive gene transcription. Clinical studies have demonstrated that fulvestrant is an effective treatment option in postmenopausal women with advanced breast cancer who have progressed on prior endocrine therapy.[228]

1.7.2 Limiting Estrogen Production

Aromatase Inhibition

Aromatase, an enzyme of the cytochrome P-450 super family and the product of the *CYP19* gene, is expressed in several tissues, including subcutaneous fat, liver, muscle, brain, normal breast tissue, and mammary adenocarcinoma.[229] It is responsible for the conversion of the adrenal androgen substrate androstenedione to estrogen in peripheral tissues (Figure 1.3),[230] and this is the predominant source of estrogen in postmenopausal women. Aromatase inhibitors (AIs) can reduce estrogen production by more than 90%.[231,232] Unlike tamoxifen, however, AIs lack estrogen-agonist activity. Because AIs do not affect the ovarian production of estrogen, only women without functioning ovaries benefit from the use of AIs.

Figure 1.3: Mechanism of action of aromatase inhibitors. The adrenal androgen substrate. androstenedione is converted by aromatse to estrogen in the peripheral tissues. Aromatase inhibitors act by blocking the action of aromatase.



AIs are classified as first, second, or third generation according to the specificity and potency with which they inhibit the aromatase enzyme. They are further sub-classified as type 1 or type 2 inhibitors, according to the reversibility of their inhibitory activity. Type 1 inhibitors, steroidal analogues of androstenedione, irreversibly inhibit the aromatase enzyme by covalently binding to it. Permanent inactivation persists after discontinuation of the drug until the peripheral tissues synthesize new enzymes. In contrast, non-steroidal type 2 inhibitors bind reversibly to the aromatase enzyme, resulting in competitive inhibition[233]. Third generation AIs (i.e., anastrozole, letrozole, and exemestane) are the most potent, most selective, and least toxic AIs known today and can reduce serum estrogen by more than 95%. The AIs switch off peripheral estrogen biosynthesis almost completely and are highly effective in the treatment of postmenopausal breast cancer. Type 1 steroidal AIs (Exemestane) bind non-covalently and irreversibly to aromatase, whereas the type 2 steroidal AIs (Letrozole and Anastrozole) bind covalently and reversibly.[234] Several clinical trials have evaluated the efficacy and safety of these agents. They are well tolerated and have become the endocrine therapy of choice in post-menopausal women with ER positive breast cancer.

In pre-menopausal women AIs cannot be used in isolation for a number of reasons. Firstly, higher circulating levels of androgens present in premenopausal women compete with aromatase inhibitos for the aromatase enzyme complex, resulting in less efficient suppression of estrogen production. Secondly, hypothalamic/ pituitary feedback mechanisms in premenopausal women mean that lower serum estrogen levels lead to compensatory changes in the ovary, including upregulation of aromatase enzymes in the ovary, that render aromatase inhibitors ineffective.[235] AI's may be used as treatment in premenopausal women in special circumstances, such as prior tamoxifen failure or medical contraindications to tamoxifen.

When AIs are used in premenopausal women, they must be combined with surgical or medical ovarian ablation. There are several clinical studies evaluating the use of AIs in premenopausal women combined with ovarian suppression with a LH releasing hormone (LHRH) analogue. In 2003, the International Breast Cancer Study Group initiated two randomised trial, the Suppression of Ovarian Function Trial (SOFT) and the Tamoxifen and Exemestane Trial (TEXT), involving premenopausal women with hormone-receptor-positive early breast cancer. The 5-year rates of recurrence of breast cancer were significantly lower among premenopausal women who received the aromatase inhibitor exemestane plus ovarian suppression than among those who received tamoxifen plus ovarian suppression.[236,237]

1.7.3 Adjuvant Endocrine Therapy

Historically, tamoxifen has been the standard treatment for hormone receptor-positive breast cancer, resulting in a significant improvement in disease-free survival (DFS) regardless of nodal status.[238] For women with ER positive early breast cancer, treatment with tamoxifen for 5 years substantially reduces the breast cancer mortality rate throughout the first 15 years after diagnosis. However, resistance to tamoxifen therapy in early breast cancer may occur as early as 12–18 months after the initiation of therapy.[239] Therefore, the role of more effective, less toxic agents, such as the third-generation AIs, has been evaluated in adjuvant therapy for early breast cancer.

Several adjuvant randomized studies of tamoxifen versus an AI as single agents or given in combination or sequentially have been conducted.

Pre-menopausal Adjuvant Endocrine Therapy

Ovarian function suppression (OFS) by GnRH agonists, ablation or radiotherapy is used in premenopausal patients to diminish the ovarian function in combination with tamoxifen or AIs. Five years of adjuvant treatment with tamoxifen versus no treatment has shown a relative risk reduction in 15 year recurrence risk of 40% with an absolute gain of 13.2%.[240] The results of the TEXT and SOFT trials revealed that for premenopausal patients, addition of ovarian function suppression should be considered for patients younger than 35 years (5 year breast cancer free interval of 67.7% for tamoxifen vs 78.9% for tamoxifen plus OFS and 83.4% for exemestane plus OFS) or who received chemotherapy (5 year breast cancer free interval 78% for tamoxifen vs 82.5% for tamoxifen plus OFS vs 85.7% for exemestane plus OFS).[241]

Extended Adjuvant Endocrine Therapy

Despite the benefits of 5 years of tamoxifen, more than 50% of breast cancer relapses and more than two-thirds of deaths occur after the initial 5 years after surgery. More recent studies

have shown that a longer course of adjuvant endocrine therapy reduces the risk of breast cancer recurrence, breast cancer mortality and reduced overall mortality. The ATLAS (Adjuvant Tamoxifen: Longer Against Shorter) trial showed that continuation of tamoxifen to 10 years rather than stopping at 5 years produces a further reduction in recurrence and mortality, particularly after year 10. The results of this trial taken with results from previous trials of 5 years of tamoxifen treatment versus none, suggest that 10 years of tamoxifen treatment can approximately half breast cancer mortality during the second decade after diagnosis.[242]

Furthermore, evidence in favour of extended AI therapy comes from the MA.17 Canadian trial. This trial showed that letrozole when given following five years of tamoxifen as extended adjuvant therapy, reduced the risk of local recurrence in ER positive, node negative patients. Patients with node positive disease had significantly improved overall survival with letrozole.[243] For postmenopausal women with ER positive breast cancer, extended adjuvant therapy with letrozole has become the best standard of care.

Current guidelines recommend 10 years of adjuvant endocrine therapy for the majority of pre- and postmenopausal patients who have ER positive disease. More recently the MA.17R trial was reported which studied the outcome of patients receiving up to 15 years of endocrine therapy, including 10 years of AI.[244] Extension of AI treatment to 10 years resulted in significantly better 5-year DFS that included disease recurrence local/ distant or the occurrence of contralateral breast cancer events. No overall survival benefit was seen. Based on the results of the MA.17R trial, adjuvant treatment with 5 years tamoxifen followed by 10 years of AI is considered appropriate for a specific subset of patients who have a high residual risk of late relapse.

A recent meta-analysis conducted by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) involved the results of 88 trials involving 62,923 women with ER positive breast cancer who received 5 years of endocrine therapy.[127] The aim was to identify subgroups of women who stop endocrine therapy after 5 years in whom the long term risks of disease recurrence were so small that any additional benefits from extended therapy would be unlikely to outweigh the additional side effects. However, even among women with T1N0 disease, the cumulative risk of distant recurrence was 13% during years 5 to 20. It was comcluded that breast cancer recurrences continued to occur steadily throughout the study period from 5 to 20 years. The risk of distant recurrence was strongly correclated with the original TN status.

1.7.4 Neoadjuvant Endocrine Therapy

The aim of neoadjuvant endocrine therapy is to downstage disease either to allow surgical resection in otherwise inoperable tumours, or to allow breast conserving surgery for tumours that might otherwise have required a mastectomy. Such treatment is typically used for postmenopausal women with breast cancer. Few neoadjuvant endocrine studies have been conducted in premenopausal women.[245]

In postmenopausal women with large, locally advanced, estrogen receptor rich breast cancers, neoadjuvant endocrine therapy is an appealing option as many older women may not be able to tolerate the toxicities of chemotherapy. In these women, neoadjuvant endocrine therapy can be used effectively to shrink tumours, rendering them amenable to breast conserving surgery, as opposed to mastectomy. In tumours suitable for breast conserving surgery, neoadjuvant endocrine therapy can shrink the tumour so that a smaller surgical resection is required, allowing for improved cosmetic outcome. Furthermore, patients with locally advanced breast cancer have a poorer prognosis than patients with early breast cancer, and whilst neoadjuvant treatment was initially dominated by chemotherapy, studies have shown response rates to neoadjuvant treatment with aromatase inhibitors in selected hormone receptor positive patients is comparable to that seen with neoadjuvant chemotherapy. [246] Women with locally advanced breast cancers may also benefit from endocrine therapy in terms of survival, and it allows women to avoid the more unpleasant toxicities associated with chemotherapy. This is all the more important as hormone receptor positive tumours have repeatedly been shown to have lower response rates to neoadjuvant chemotherapy than hormone receptor negative tumours, in terms of pathological complete response.[247,248,249] pCR rates of less than 15% have been reported with neoadjuvant anthracycline-taxane therapy in luminal-type breast cancer.[238] Because endocrine therapy has a different mechanism of action (focussed mainly on reducing the estrogen-induced effects on proliferation), pCR is rarely observed with endocrine therapy.[250] However, it is possible that some clinical trials of only 3 months of neoadjuvant endocrine therapy, have underestimated the true pCR potential, given what may be a premature cessation of treatment. [251] It is often considered that assessment of pCR is a suitable surrogate endpoint for paitnets with HER2 positive (nonluminal) and triple negative breast cancer, less valid in those with luminal B (HER negative or positive) and probably not a good predictive test for patients with luminal A tumours.[252]

Changes in tumour morphology and histology have been reported with neoadjuvant endocrine therapy and include decreases in cellularity, increases in fibrosis, so called ‘central scar formation’.[253,254] Endocrine therapies do not appear to enhance tumour cell apoptosis and therefore apoptosis is not a useful predictor of benefit from therapy.

Whilst systemic treatment of HER2 positive disease has been dominated by chemotherapeutic agents, there is a need to explore whether the combination of HER2 targeted and endocrine therapy in this group of ER+/HER+ patients might firstly tackle the problem of endocrine resistance in these patients, and secondly, whether some of these patients might be treated safely and effectively with this combined therapy, and might avoid the need to use chemotherapy in these women.

Several trials have assessed the efficacy and safety of neoadjuvant endocrine therapy using aromatase inhibitors in patients with postmenopausal breast cancer.[255,256,257] In a randomised phase II study in which neoadjuvant endocrine therapy and neoadjuvant chemotherapy was compared in patients with hormone receptor positive breast cancer, no significant difference in the clinical response rate was observed between the two groups.[258] Notably the rate of breast conserving surgery was numerically but not significantly higher and the incidence of most adverse events was lower in the endocrine therapy group compared with the chemotherapy group. These results support the use of neoadjuvant endocrine therapy in postmenopausal patients with hormone sensitive breast cancer as an alternative to neoadjuvant chemotherapy.

Currently, there is little data which allows one to identify which patients benefit from treatment with neoadjuvant endocrine therapy. The Edinburgh Breast Unit has pioneered investigation of gene expression changes during neo-adjuvant treatment in so called 'window of opportunity' studies,[258,259,260,261] and have published the first attempts to characterise the effects of the aromatase inhibitor, letrozole and the mTOR inhibitor, Everolimus on breast cancers[261]. The largest 'window of opportunity' neoadjuvant endocrine study, the PeriOperative Endocrine Therapy for Individualizing Care (POETIC) trial of 4,486 postmeopausal patients with ER positive breast cancer who are randomised to nonsteroidal aromatase inhibitor or to no treatment for 2 weeks before and 2 weeks after surgery, has now completed recruitment in the UK. The primary endpoint is to assess whether endocrine therapy prior to surgery improves outcome and it is anticipated that the on treatment Ki67 assessment may be of such significance that it could warrant its routine assessment.[262]

The neoadjuvant setting allows prompt testing and evaluation of therapies and provides reliable results to inform and direct the design of adjuvant trials. This approach is becoming increasingly important in the investigation of endocrine resistance, and can be used to look at specific subgroups such as the ER positive and HER2 positive population.

Neoadjuvant Aromatase Inhibitor or Tamoxifen

In the neoadjuvant setting, 4 phase III randomised clinical trials assessed whether an AI or tamoxifen should be given, three in postmenopausal and one in premenopausal women. Three trials of postmenopausal women were the P024 trial of letrozole vs tamoxifen before surgery,[263] the IMPACT trial of anastrazole, tamoxifen, or a combination of tamoxifen and anastrozole for 12 weeks before surgery,[264] and the PROACT trial of anastrozole vs tamoxifen.[265] A meta-analysis of 3 studies, including 1160 patients, indicated superior outcomes in terms of clinical objective response rate, ultrasound response rate and breast conserving surgery rate with AI as compared to tamoxifen.[191]The STAGE trial of 197 premenopausal women who were treated with neoadjuvant anastrozole or tamoxifen, both given in combination with goserelin, confirmed that anastrozole was superior to tamoxifen in terms of calliper response, ultrasonography response, and MRI or CT response. [266] Thereby, even though the role of neoadjuvant endocrine therapy in premenopausal women remains largely investigational, the results of the STAGE trial are consistent with the findings of other major adjuvant studies and it could be expected that the superior activity of neoadjuvant AI would translate into improved cancer outcomes with continued treatment in the adjuvant setting.

Optimum Duration of Neoadjuvant Endocrine Therapy

A three to four month duration has been proposed in the majority of the clinical trials such as P024,[263] IMPACT[264] and PROACT.[265] However, there is evidence to suggest that three to four months duration could be insufficient to achieve maximum reduction in tumour volume. Dixon et al of the Edinburgh Breast Unit investigated the potential benefits of prolonged treatment with neoadjuvant letrozole in 182 patients.[267] Of these, 63 patients were continued on letrozole beyond 3 months because of different reasons: 26 responded but not enough to allow breast conserving surgery, 15 responded but still had inoperable disease, 13 were unfit and considered unsuitable for surgery and 9 refused surgery. The response rate in the 182 patients increased from 69.8% at 3 months to 83.5% after prolonged treatment, and importantly, breast conserving surgery increased from 60% to 72%. In another multicentre, prospective study of 146 patients with early BC, initially unsuitable for breast conserving surgery, treatment with letrozole was for a maximum of 12 months or until the patient became eligible for BCS, progressed or withdrew for scheduled mastectomy.[268] The median time to achieve a tumour response sufficient to allow BCS was 7.5 months. Only 9 out of the 146 patients had disease progression during neoadjuvant endocrine therapy. Until further data is available, the optimal duration of neoadjuvant endocrine therapy should be individualised based on careful and serial evaluation of clinical response.

Neoadjuvant Endocrine Therapy vs Neoadjuvant Chemotherapy

There is limited evidence comparing neoadjuvant chemotherapy with neoadjuvant endocrine therapy. 2 randomised phase II trials have tried to address this question. In one trial of 239 postmenopausal women were randomised to receive preoperative AI or chemotherapy, there was no significant difference between AI and chemotherapy in terms of clinical response rate, time to response, or pCR.[269] Similarly, a second trial of exemestane or chemotherapy also found no significant difference between the 2 arms in terms of clinical response rate.[270] The NEOCENT trial (neoadjuvant chemotherapy versus endocrine therapy), in postmenopausal women was prematurely closed because of slow accrual.[271] Thus, clarity remains to be established in this important issue.

1.8 Breast Cancer Endocrinology

This thesis specifically relates to endocrine therapy in post-menopausal women, and as such the focus of the remainder of the introduction will be largely directed towards endocrine therapy. The relationship between estrogen and the breast is fundamental to the understanding of breast development, tumorigenesis and endocrine therapies for breast cancer. Estrogens have been implicated in the development of breast cancer since Beatson discovered that oopherectomy prevented tumour recurrence and induced regression of breast cancers in the 19th century.[272]

1.8.1 Breast Development and Tumorigenesis

The mammary gland is not completely formed at birth, but begins to develop in early puberty when the primitive ductal structures enlarge and branch. Once ovulatory menstrual cycles have begun, branching of the ductal system becomes more complex and lobular structures form at the ends of the terminal ducts to produce terminal ductal lobular units (TDLUs), which become more complex with successive menstrual cycles. During early pregnancy, there is another burst of activity in which the ductal trees expand further and the number of ductules within the TDLUs increases greatly. These ductules differentiate to synthesise and secrete milk in late pregnancy and subsequent lactation. Histological studies have shown that most human breast tumours appear to be derived from TDLUs and have morphological characteristics of luminal epithelial cells. Human tumours also contain receptors for estradiol and progesterone that, in the normal breast, are expressed only in the luminal epithelial cell compartment. [273]

The clinical and epidemiological evidence for the role of estrogen in human mammary gland development and tumorigenesis is considerable. There is complete failure of breast development in the absence of intact ovarian function, and estradiol-replacement therapy is necessary to induce breast development.[274] Increased exposure to the fluctuating levels of estradiol of the menstrual cycle through early menarche, late menopause or a late, first, full-term pregnancy increases breast cancer risk, as does use of exogenous estrogens in the form of the oral contraceptive pill or hormone replacement therapy.[275]More compellingly, treatment with anti-estrogens reduces the incidence of breast cancer in high-risk women.

There is less evidence for a role of progesterone in human breast development. Studies on mouse models in which the PR has been knocked out suggest that, whereas estradiol stimulates ductal elongation and PR expression, progesterone induces lobulo-alveolar development.[276] As far the role of progesterone in breast tumorigenesis is concerned, there is

data to suggest that exogenous progestins taken in the form of combined hormone replacement therapy increase the risk of postmenopausal breast cancer to a greater extent than use of estrogen replacement therapy alone.[277]

1.8.2 Estrogen Production

The estrogens are a family of steroid hormones that stimulate the development and maintenance of female secondary sexual characteristics and sexual reproduction. The most prevalent forms of circulating estrogens are estradiol and oestrone, but their primary site of production differs according to menopausal status. In pre-menopausal women the ovaries are the primary source of estrogen production, with small amounts produced by the adrenal cortex and other organs. In postmenopausal women, with declining ovarian function, estrogen production occurs predominantly by the peripheral conversion of ovarian and adrenal androgens to estrogen in peripheral muscle and bone as well as within breast tumours. This conversion is a function of the aromatase enzyme, a product of the CYP-19 gene of chromosome 15.[278]

1.8.3 The Estrogen and Progesterone Receptors

The steroid hormones estradiol and progesterone are lipophilic, they enter the cell nucleus primarily by diffusing through the plasma and nuclear membranes. Once in the nucleus, the steroids encounter proteins known as receptors and they bind their ligands with high affinity and specificity. There are two receptors for estradiol, the ERα and the ERβ. Both these ERs are members of the steroid/thyroid hormone nuclear receptor superfamily and both may be described as ligand-dependent nuclear transcription factors.[273] It is likely that ERα is the key mediator of estrogen in the normal mammary gland and that ERβ mediates some of the non-classical effects of the estrogens and may even negatively modulate the activity of ERα.

Progesterone also has two receptors, PRA and PRB. They are also members of the steroid/thyroid hormone nuclear receptor superfamily, and they function as ligand dependent nuclear transcription factors. It has been suggested that PRB is the major activator of gene transcription and that PRA is a repressor of PRB activity.[279]

Most data on ER and PR expression in the normal human breast have been obtained in the course of studies on tissue from adult women who are not pregnant or lactating. These studies show that ERα is expressed in approximately 15–30% of luminal epithelial cells and not at all in any of the other cell types within the human breast.[280] Initial studies indicate that the ERβ is expressed in most luminal epithelial and myoepithelial cells, as well as being detectable in

fibroblasts and other stromal cells within the normal human breast.[281] The PR is thought to be present in 15-30% of luminal epithelial cells and not elsewhere in the breast.

1.8.4 Estrogen Receptor Signalling

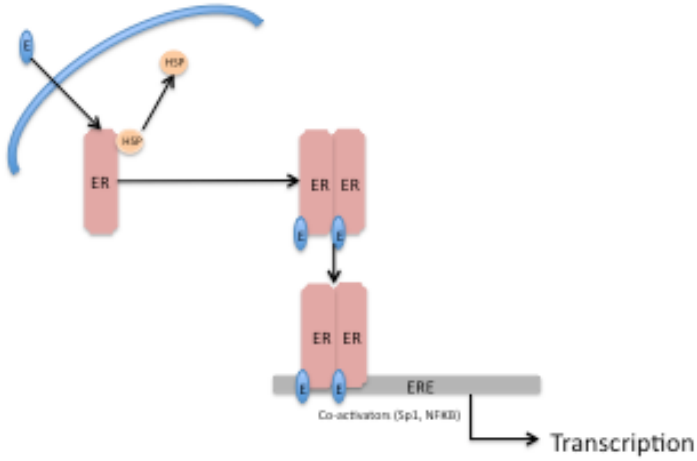
Once inside the cell nucleus, steroid hormones bind to their respective receptors with high affinity and specificity. This is known as the 'classical estrogen signalling pathway' (Figure 1.4). In the absence of this highly specific binding, the receptors are inactive and exist in a monomeric form bound to heat shock proteins such that they exert no influence on a cells DNA expression. However, upon binding, the ER dissociates from its heat shock protein and undergoes a process of homo-dimerization and protein phosphorylation on a number of specific residues. This leads to conformational change in the shape of the AF2 region. Consequently, the ER dimers bind to specific DNA sites, known as estrogen response elements (EREs). These consist of palindromic nucleotide sequences and they lie upstream of estrogen responsive genes. The ER dimers simultaneously associate themselves with receptor co-activator proteins and together these associations are responsible for the activation of a large number of estrogen dependent genes, the transcription of their messenger RNA (mRNA) and the subsequent translation of this mRNA into proteins which invariably impact on the properties of the cell, the cell cycle and that of their adjacent cells.[282]

Additional estrogen signalling pathways have been described. It has been suggested that the estrogen receptor may bind to and activate the activity of 2 further transcription factors, jun and fos, which may recognise alternative receptor binding sites and perpetuate cell proliferation signalling. Another suggestion is that estrogen receptors within the plasma membrane may participate in the activation of growth factor receptor signalling, by activating the ER1/2 gene, and thus influencing the nearby activity of dividing cells via protein kinase signalling pathways.

In many neoplastic breast cells, the ER signalling network contributes to controlling the relative rates of cell proliferation and programmed cell death, with pro-survival and proliferation signals overwhelming pro-death and quiescence signals. To make substantial new advances in the treatment of advanced breast cancer, we need a better understanding of the ER signalling network. Studies have shown the functional relevance of several factors involved in ER signalling, including nuclear factor κB (NF-κB), a pro-survival transcription factor that is highly expressed in hormone resistant cells compared to hormone sensitive cells,[283] interferon regulatory factor 1 (IRF1),[284] a pro-death transcription factor that is down regulated in endocrine-resistant cells, and X-box binding protein 1 (XBP1), a transcription

factor that is involved in the unfolded protein response (UPR) and the induction of autophagy and is highly expressed in its active variant in endocrine resistant cells.[285]

Figure 1.4: Classical estrogen signalling pathway. In the absence of the estradiol ligand the estrogen receptors exist in a monomeric form bound to heat shock proteins. Upon binding the ER dissociates from its heat shock protein and homodimerizes, leading to a conformational change in the shape of its AF2 region. The activated ER dimers bind to estrogen response elements allowing for transcriptional activation.



1.9 ER Positive/ HER2 Positive Subtype

Whilst the ER negative/ HER2 positive subtype of breast cancers is a relatively well characterised group in terms of tumour biology and behaviour, the ER positive/ HER2 positive (ER+/HER2+) subtype has been less well investigated. HER2 positive cancers account for 15-20% of all breast cancers and 50% of these are ER positive. The ER+/HER2+ molecular subtype accounts for up to 10% of all breast cancers, and is an important subset because even small HER2 positive cancers have a much worse prognosis than similar sized ER+/HER2 negative cancers. There is now considerable pre-clinical and clinical evidence demonstrating that ER+/HER2+ tumours exhibit both intrinsic and acquired resistance to endocrine therapy. This is pertinent as endocrine therapy to block ER activity and signalling, remains at the forefront of systemic treatment for all women with ER positive disease. It is unclear as to whether ER signalling or HER2 signalling drives resistance to endocrine therapy in the ER+/HER2+ subset.

Resistance to endocrine therapy, both primary and acquired, is a major clinical problem in the management of hormone receptor positive breast cancers. Extra tumoural mechanisms of resistance include patient factors, such as poor compliance with treatment and adverse drug metabolism reducing the levels of active drug metabolites. More specific mechanisms of resistance include 1) Loss or reduction of ER expression; 2) ER mutations leading to desensitisation to ER targeted therapies; 3) HER2 overexpression; 4) Activation of redundant pathways bypassing the need for ER. Mechanisms of endocrine resistance will be discussed specifically with regard to the ER+/ HER2+ subtype.

1.9.1 ER/ HER2 Crosstalk

Both the ER and HER receptor pathways play important roles in driving growth and proliferation of breast cancers. It is evident that there exists crosstalk between ER and the EGFR/HER2 pathway and that this plays an important role not only in the physiological action of ER as part of growth factor receptor signalling, but also plays an important role in endocrine resistance. There are different biological mechanisms which may explain how HER2 signalling could result in endocrine resistance. HER2 signalling may impair endocrine sensitivity by increasing ERα function, either directly by phosphorylating and activating the AF-1 domain of the receptor (at Ser167 or Ser118), or indirectly by recruiting coactivators to ERα, which leads to increased ERα transcriptional activity.[286,287,288] Another possible mechanism is via signalling through membrane receptors and downstream effectors such as extracellular signal-regulated kinase (ERK) which can suppress ERα expression and function, thereby promoting ERα independence. Alternatively, conversion to an ER negative phenotype

will result in endocrine resistance.[289,290,291,292] While membrane ER can activate HER2 signalling, the kinase cascade downstream of HER2 can phosphorylate and activate ER and its co-regulatory proteins. This pathway interaction was first implicated when it was noted that transfection of ER+ breast cancer cells with HER2 resulted in down-regulation of ER and resistance to tamoxifen.[293] These were the first studies to implicate ER/HER2 crosstalk as important in primary endocrine resistance. Later studies have subsequently demonstrated and confirmed its role in acquired endocrine resistance. Tamoxifen treated breast cancer cells in long-term culture have been reported to show increased levels of expression of EGFR and HER2, increased activation of EGFR/HER2 heterodimers, and increased phosphorylation of p42/44 MAPK, AKT and nuclear ER.[294,295] These findings indicate that enhanced growth factor signalling, can up-regulate both the genomic and non-genomic activities of ER, which could be a key contributor to at least one mechanism of acquired endocrine resistance. Crosstalk between ER and HER2 signalling pathways may not only play a role in resistance to endocrine therapy, but also in resistance to HER2 targeted agents.[245]

1.9.2 HER2 'escape/ survival' route

The human epidermal growth factor receptor family (HER) family comprises four homologous transmembrane receptors (HER1, also known as EGFR, HER2, HER3 and HER4), which form a complex system of growth factors involved in growth regulation.[203] As a result of ligand binding, HER receptors form a series of dimers that result in autophosphorylation, with activation of tyrosine kinases and downstream signalling pathways. Overexpression of HER2 leads to transformation in the absence of a ligand. None of the HER family of ligands binds to HER2 directly. Therefore, HER2 is regarded as an orphan receptor. It appears that HER2 is the preferred dimerization partner for all other HER receptors, and as such HER2 functions as a shared co-receptor. Indeed, both HER1 and HER3 have been shown to be upregulated in breast cancers with HER2 overexpression.[296]

HER2 has been demonstrated to play a role in both primary and acquired resistance to endocrine therapy. A meta-analysis on the interaction between response to endocrine treatment and over expression of HER2 in metastatic breast cancer concluded that HER2 positive metastatic breast cancer is less responsive to any type of endocrine treatment.

Three trials have shown in the metastatic setting that the combination of an aromatase inhibitor with an anti-HER2 agent is superior to aromatase inhibitor alone as first line therapy in patients with HER2 positive and ER positive disease, in terms of progression free survival. The randomised phase III TANDEM trial included 207 patients with HER2 positive, ER positive metastatic breast cancer demonstrated a doubling of progression free survival time

with the addition of trastuzumab to anastrozole versus anastrozole alone (4.8 vs 2.4 months).[297] Johnston et al also showed a significantly increased progression free survival (8.2 vs 3 months) for a combination of letrozole plus lapatinib compared with letrozole alone in a randomised phase III first line trial in HER2 positive disease. The PFS rate in the AI alone arm indicates that endocrine therapy alone may not be sufficiently effective in triple positive disease.[298] In the eLEcTRA study, women with ER positive/ HER2 positive disease were randomised to receive either letrozole monotherapy or letrozole plus trastuzumab as first line treatment. Median time to progression was 3.3 months in patients who received letrozole alone, and 14.1 months in the combined therapy arm. In patients with HER2 negative disease who received letrozole monotherapy, median time to progression was 15.2 months.[299] Importantly, these 3 studies provide support the view that carefully selected ER+/HER2+ cancers can be effectively treated with combined endocrine and HER2 targeted therapies, without the need for chemotherapy.

Response rates to aromatase inhibitors range between 35% and 70% in neoadjuvant studies, and benefits may be lower in advanced disease.[255,264] Acquired resistance after initial successful treatment also occurs and it seems that HER2 signalling may play an important role in this acquired resistance to endocrine therapy.[300] In the group of patients with ER positive cancers who do develop resistance, it may not be adequate to treat their disease with second-line anti-estrogen therapy alone. In one in vitro study of human breast cancer cells, modest adaptive increases in HER2 expression occurred in 20% of patients who relapsed on tamoxifen. In cell models of ER+/HER2- breast cancers, with acquired endocrine resistance and modest adaptive increases in HER2, the combination of lapatinib and endocrine therapy resulted in synergistic growth inhibition.[301] These cells did not achieve HER2 levels comparable with HER2 amplified breast cancers, and indeed in clinical practice conversion from HER2 negative to HER2 2+/3+ FISH positivity remains a rare event. What these results suggest is that even with a modest increase in HER2 expression, crosstalk between the HER2 and ER pathways can develop and may be important in resistance to endocrine therapies. In other studies, trastuzumab has been shown to inhibit growth of endocrine resistant ER positive breast cancers which have increased HER2 protein expression, but are not HER2 amplified.[302,303] The combination of lapatinib and letrozole has also been shown to be effective in some endocrine resistant, HER2 negative metastatic breast cancers.[298] HER2 expression in some patients is higher in metastatic cancers compared with primary disease. Given the plasticity of endocrine resistance in breast cancers, treatment strategies should be based on the phenotype of the tumour at relapse rather than that at diagnosis.

Unfortunately, there is limited available information on the use of combined endocrine therapy and anti-HER2 therapy in the non-metastatic setting. The current observations suggest that HER2 does play an important role in primary endocrine resistance. Furthermore,

studies lead us to question the effectiveness of endocrine therapy alone in some patients with ER+/HER+ breast cancers and it is almost certain that a proportion would benefit from the addition of a HER2 targeted agent.

1.9.3 ER 'escape/survival route'

In the same way that HER2 signalling can provide an 'escape route' to endocrine therapy in ER positive patients, ER signalling can provide an 'escape route' in patients treated with anti-HER2 therapy who do not get total ER blockade. In an in vitro study of HER2 resistant cell lines ER+/HER2+ cells were shown to exploit ER activity as a mechanism of primary and acquired resistance. In ER+/HER2+ tumours, whilst either ER or HER2 may initially function as the driver of proliferation and survival, with sustained, effective HER2 inhibition with lapatinib, or lapatinib plus trastuzumab, ER will eventually become the primary driver of cell survival, resulting in resistance to anti-HER2 therapy.[304] These findings are consistent with results from several neoadjuvant clinical trials investigating HER2 targeted agents with or without chemotherapy in patients with early HER2+ breast cancers. Whilst the results differed between trials depending on the type of HER2 targeted agents and chemotherapy used, there is a consistently lower pCR in ER+/ HER2+ cancers, compared with ER-/ HER2+ cancers. In the clinical NeoSphere and NeoALLTO trials, patients with HER2 positive disease received neoadjuvant chemotherapy in addition to HER2 targeted therapy, but no ER targeted therapy.[219,220] There was a significantly lower pCR in ER+/HER2+ in both trials compared with ER-/HER2+ tumours. The NeoSphere trial combined docetaxel with trastuzumab alone, pertuzumab alone, or trastuzumab plus pertuzumab, and also included an arm without chemotherapy (trastuzumab and pertuzumab only). In this latter arm there was a 5.9% pCR rate in ER/PR positive tumours treated only with trastuzumab and pertuzumab compared with 29.1% in the ER/PR negative group. This demonstrates the importance of blocking the ER pathway in ER+/HER2+ cancers and supports the efficacy of the trastuzumab/ pertuzumab combination in ER/PR negative cancers. In a further phase II multicenter study, neoadjuvant lapatinib and trastuzumab was given to patients with HER2 and ER positive disease, combined with letrozole. The pCR rate in patients with ER positive disease was 21%.[305] Neoadjuvant treatment of patients with ER-/HER2+ breast cancers with combined trastuzumab and chemotherapy has been shown to change ER status from negative to positive in up to 20% of cases. This suggests that blocking HER2 signalling might result in an up regulation of ER and ER transcriptional activity, allowing for an ER driven escape route.[306] Taken together, these studies highlight the importance of providing blockade of both ER and HER2 signalling in the group of ER+/HER2+ patients.

Summary

The ER+/HER2+ subtype accounts for up to 10% of all breast cancers and is an important subtype as these cancers have a worse prognosis than ER+/HER2- breast cancers. While there is now considerable preclinical and clinical evidence that ER+/HER2+ cancers exhibit intrinsic and acquired resistance to endocrine therapy, it remains unclear what is driving this resistance to therapy. This is a pressing clinical issue as endocrine therapy remains at the forefront of systemic treatment for all women with ER+ disease. However, many patients with ER+/HER2+ disease do respond well to endocrine therapy. The challenge, therefore, is in identifying, early in the process of treatment decision-making, who will respond to endocrine therapy and who might benefit from combined endocrine and HER2-targeted agents, or HER2-targeted agents alone.

Neoadjuvant therapy with third-generation aromatase inhibitors such as letrozole and anastrozole have an established role in the treatment of estrogen-receptor alpha positive postmenopausal breast cancer. This neoadjuvant approach can result in downsizing of large, locally advanced tumours allowing for increased rates of breast conserving surgery and curative resections. Furthermore, neoadjuvant therapy affords the use of 'window of opportunity studies' where tumour biopsies taken before treatment onset, during the treatment and ultimately from the surgical excision. Not only can response to treatment be monitored biologically and clinically, this approach improves statistical power by reducing patient-patient variation. However, these studies are typically difficult to perform owing to the demands of effective tissue collection and storage and are largely dependent on patient and sample numbers.

2. Aims and Objectives

There is considerable preclinical and clinical evidence that ER positive/ HER2 positive tumours exhibit resistance to endocrine therapy. This is a pressing clinical issue as endocrine therapy remains at the forefront of systemic treatment for all women with ER positive disease. However, many patients with ER+/ HER2+ disease will respond well to endocrine therapy. The challenge therefore, is in identifying, early in the treatment process, who will respond to endocrine therapy, and who might benefit from combined endocrine and HER2 targeted agents.

The current study uses the largest data set of matched ER positive/ HER2 positive breast cancer samples before and during endocrine treatment. The aims of this study are:

1. To investigate which ER positive/ HER2 positive breast cancers respond to letrozole.
2. To compare the mechanisms of resistance to endocrine therapy in ER+/ HER2+ and ER+ / HER2- breast cancers.
3. To determine which cancers should be considered for combined endocrine and anti-HER2 treatment.

3. Materials and Methods

3.1 Study Design

This study involved the prospective enrolment of postmenopausal patients presenting with locally advanced or large operable, ER positive breast cancer for whom the current clinical guidelines suggest neoadjuvant treatment with the aromatase inhibitor Letrozole.

All patients included in the study were diagnosed and treated at the Edinburgh Breast Unit, Western General Hospital. Patients who met the eligibility criteria were discussed at a weekly multi disciplinary meeting (MDM) and referred to a member of the medical or research staff involved. Patients were provided with detailed written information about the study design and a meeting was arranged for patients to discuss this further. All patients gave informed consent and the study was approved by the local regional ethics committee (2001/8/80 and 2001/8/81; *Appendix 1*). The patients general practitioner was sent information about the study and the patients enrolment.

Of note is that several studies were undertaken simultaneously to maximise the research potential of the information and tissue samples collected from the respective patients. The extended conduct and findings of further studies are out with the scope of this thesis.

Letrozole (Femara; Novartis Pharmaceuticals AG, Basel, Switzerland) is a reversible, non-steroidal aromatase inhibitor. It is licensed in the UK for the adjuvant treatment of postmenopausal women with hormone-receptor-positive invasive early breast cancer, and for the treatment of postmenopausal women with hormone-receptor-positive invasive early breast cancer who have already received standard adjuvant tamoxifen therapy for 5 years. Letrozole is also licensed in the UK for various indications in advanced breast cancer and as neo-adjuvant treatment.[339] Patients were treated with a neoadjuvant protocol in which letrozole 2.5mg was given daily for at least 3 months.

3.2 Patients

A consecutive series of 255 postmenopausal women presenting to the Edinburgh Breast Unit (Western General Hospital) with large primary histologically confirmed invasive breast cancer, immunohistochemically (IHC) determined to be estrogen receptor (ER) positive (Allred score≥6) were recruited between 2003 and 2011. Patients were excluded based on strict predefined criteria: (i) if the tumour was multifocal, (ii) if histological assessment by a pathologist determined low cellularity or less than 40% malignancy, (iii) if extraction failed to yield sufficiently good quality RNA to be suitable for further study, (iv) if follow-up records for clinical assessment of response were unavailable or incomplete, (v) if less than two

tumour biopsies were available or carried out, including a before-treatment biopsy, or (vi) if the drug was changed from letrozole to another agent during the 3 month treatment window due to an adverse drug reaction.

Of the patients who met the predefined criteria, 17 were classified as ER positive/ HER2 positive at diagnosis and had 3 or more tumour biopsies available (comprising pre-treatment, 14 day and approximately 3 month biopsies) and were selected for microarray analysis. A patient was determined to be HER2+ with an IHC score of 3; or IHC 2+ and FISH positive.

3.3 Tumour Samples

Tumour biopsies were taken with a 14-guage needle: before, and approximately 14 days and 3 months following commencement of continuous letrozole treatment (Figure 4.1A). Samples were snap-frozen in liquid nitrogen and frozen sections taken, stained with haematoxylin and eosin (H&E) and the cellularity and percentage presence of cancerous tissue within each specimen was assessed by a pathologist.

3.4 Assessment of Response

Clinical response was determined using dynamic changes in tumour volumes assessed by repeated measurements taken over the 3 month treatment period. Primary assessment was based on ultrasound measurements performed by a single observer (JMD) and these were verified by mammographic measurements. Clinical response was reassessed for samples using strict predefined criteria. In order to be consistent with the validation dataset, we also classified response independently using the modified International Union Against Cancer IUAC/WHO criteria. As a secondary endpoint, pathological response was determined from the clinical records, originally assessed by a pathologist.

3.5 RNA Processing and Microarray Hybridisation

Biopsies were homogenised and RNA was extracted using the RNeasy Mini Kit with RNAse-free DNAse treatment (Qiagen). RNA quantity and quality was verified on a Bioanalyser 2100 with RNA 6000 Nano Kit (Agilent) and Nanodrop 2000c (Thermo Scientific). RNA was reverse transcribed and amplified using the WT-Ovation FFPE System Version 2 (NuGEN), purified using the Qiaquick PCR Purification Kit (Qiagen), biotinylated using the IL Encore Biotin Module (NuGEN), purified using minElute Reaction Cleanup Kit (Qiagen) and quantified once again using the Nanodrop 2000c (Thermo Scientific). Labelled cDNA was hybridised to Human HT-12v4 whole-genome expression beadarrays (Illumina) according to the standard protocol for NuGEN amplified samples. Samples in the same response group were assigned random positions across all the Beadarrays with samples from the same patient

on the same array. Beadarrays were hybridised and processed in 5 batches and each batch included a replicate Universal Human Reference RNA(UHRR) control sample in order to assess the need for batch effect correction. The Human HT-12v4 whole-genome expression Beadarray covers more than 48000 transcript probes and its annotation is publically available. Raw gene expression files are publicly available from the caBIG supported Edinburgh Clinical Research Facility Data Repository (https://catissuesuite.ecmc.ed.ac.uk/caarray/). Data were extracted using the GenomeStudio software (Illumina).

3.6. Affymetrix Dataset

Affymetrix gene expression data was generated from primary breast tumour tissue biopsies taken before, approximately 14 days after and again approximately 3 months after commencement of continuous neoadjuvant letrozole treatment in 58 patients (data was available online from 55 patients) as part of a previously described clinical study.[340,341] Patients were selected from a consecutive series recruited between 2001 and 2003 as part of the aforementioned letrozole clinical audit. RNA was extracted, amplified and labelled as previously described before hybridisation to HGU-133A GeneChips (Affymetrix) according to the standard protocol.

3.7 Data Processing and Analysis

All data was processed using the R/Bioconductor software and packages, and the TM4 Microarray software suite (MeV). Data generated on the Illumina microarray platform (this study, samples from 34 patients) and data generated on the Affymetrix platform (published dataset[340,341] samples from 45 patients) were each independently pre-processed and re-annotated to Ensembl gene identifiers, then combined and batch corrected.[296] UHRR control samples were removed from the Illumina dataset prior to pre-processing. Illumina probe profiles were quantile normalised using the lumi package (R/Bioconductor) and mapped to Ensembl gene sequences using a composite list comprising mappings from reMOAT, Ensembl BioMart and a custom BLAST sequence search of the online Ensembl gene database where there was agreement between at least two of the resources. Where multiple Illumina probes represented an Ensembl gene, the mean expression level was calculated. A custom Chip Definition File (CDF)[297] was used to map the Affymetrix data to Ensembl gene annotations and RMA implemented by the affy package (R/Bioconductor) was used for normalisation. The datasets were then filtered using Illumina or Affymetrix probe detection P-values, removing probes that were undetected ($P>0.05$ in the total minus 3 samples). Both datasets were then combined and batch-corrected with cross-platform normalisation (XPN; ArrayMining) to reduce platform associated bias. The combined, corrected Affymetrix and Illumina dataset (79 patients; 237 samples) was used for all further analysis. These datasets

were considered suitable for integration as both studies were designed with a similar experimental focus and both studies have similar composition in terms of patient/sample numbers and clinical parameters.

Differential gene expression analysis was performed using Rank Product and Significance Analysis of Microarrays (MeV; TM4 Microarray Software Suite). Pathway enrichment analysis was performed in DAVID Bioinformatics Resources 6.7 using the Kyoto Encyclopaedia of Genes and Genomes (KEGG).[298] The most informative features differentiating between responders and non-responders were identified using Random Forest (RF) (Salford Predictive Miner, Salford Systems, San Diego, USA) and predictive signatures were assessed using Support Vector Machines (SVM;*e1071* package), centroid classification and logistic regression (*glm*package). Gene expression heat-maps were generated in MeV using Euclidean distance with complete linkage following gene mean-centering performed in Cluster 3.0.[343] Multidimensional scaling was performed in R with 3D scaling plots generated in JMP10 (JMP Software, USA). Statistical analyses were carried out in Prism 6 (Graphpad Software, California, USA).

3.8 Immunohistochemistry

Formalin-fixed, paraffin embedded sections were stained with rabbit antibodies to ERK-p44/42 MAPK (#9102) Cell Signalling, 1:200; and pERK-P-p44/42 MAPK (#9101) Cell Signalling, 1:25. Antigen retrieval was carried out with 0.1M sodium citrate/0.1M citric acid pH6 and detection using the EnVision TM kit (Dako, Agilent Technologies) as per the manufacturer's standard protocol (*Table 3.1*).

Protein expression was assessed by 2 independent scores, blinded to clinical response. Images were interpreted visually using the 'NDP.view2' software. Semi-quantative protein expression was determined on staining intensity and scored 'High', 'Intermediate' or 'Low'.

The available patient tissue is highly valued and as such breast cancer mouse xenograft tissue was employed for controls.

Table 3.1: Immunohistochemistry, antibodies and protocol.

Antibody	Catalogue Number	Dilution	Antigen Retrieval	Incubation Time	Company	Animal Type	Clonality	Secondary Used
p44/42 MAPK (Erk1/2)	9102	1/200	Sodium Citrate	1 hour	Cell Signalling Technology	Rabbit	Monoclonal	DAKO anti-rabbit
Phospho-p44/42 MAPK (Erk1/2)	9101	1/25	Sodium Citrate	1 hour	Cell Signalling Technology	Rabbit	Monoclonal	DAKO anti-rabbit

3.9 Validation with treatment naïve ER+/ HER2+ cohort

Affymetrix gene expression data was generated from primary breast tumour tissue biopsies taken from surgical resection specimens of 13 tumours identified as being ER+/ HER2+ by standard classification. RNA was extracted, amplified and labelled as previously described before hybridisation to HGU-133A GeneChips (Affymetrix) according to the standard protocol.

3.10 HER2 Survey

Appropriate treatment with the available anti-HER2 therapies depends on prompt assessment of HER2 status on all patients with invasive breast carcinoma at diagnosis. All invasive breast cancers are tested for the presence of HER2 overexpression. The method most widely used is an immunohistochemical assay which classifies HER2 on a subjective scale of 0, 1+, 2+, and 3+. Patients with 0 and 1+ are considered to have low expression and to be HER2 negative. Patients with 2+ are considered indeterminate and those with 3+ are considered to be HER2 positive. This IHC assay is about 90% accurate.[278] Various in situ hybridisation techniques, which use one or two tags for the centromere on chromosome 17 and the HER2 gene, are also available.[274, 275] If the ratio of HER2 to the centromere on chromosome 17 is greater than 2.2 then HER2 is considered to be amplified.[278] These tests are performed on tumour samples that are indeterminate (2+) on IHC.

A survey of UK surgeons was performed to determine how many breast units had readily available access to HER2 assessment, and in particular how many units had HER2 results available in time for the post operative multidisciplinary meeting, when decisions are made on adjuvant treatment. A questionnaire was sent to 220 breast units throughout the UK, 187 completed questionnaires were returned for analysis (*Appendix 3*).

4 Results

4.1 Patient Numbers and Clinical Response

Of the 76 patients studied, a total of 17 were ER+/ HER2+ at diagnosis and tissue biopsies and tumour volume measurements at baseline, 14 days and 3 months was available for each of these. Response to neoadjuvant letrozole therapy was defined as a volume reduction of at least 50% by 45 days and 70% by 3 months. Non response was defined as a volume increase or partial reduction that never exceeded 50% (*Figure4.1A)*.

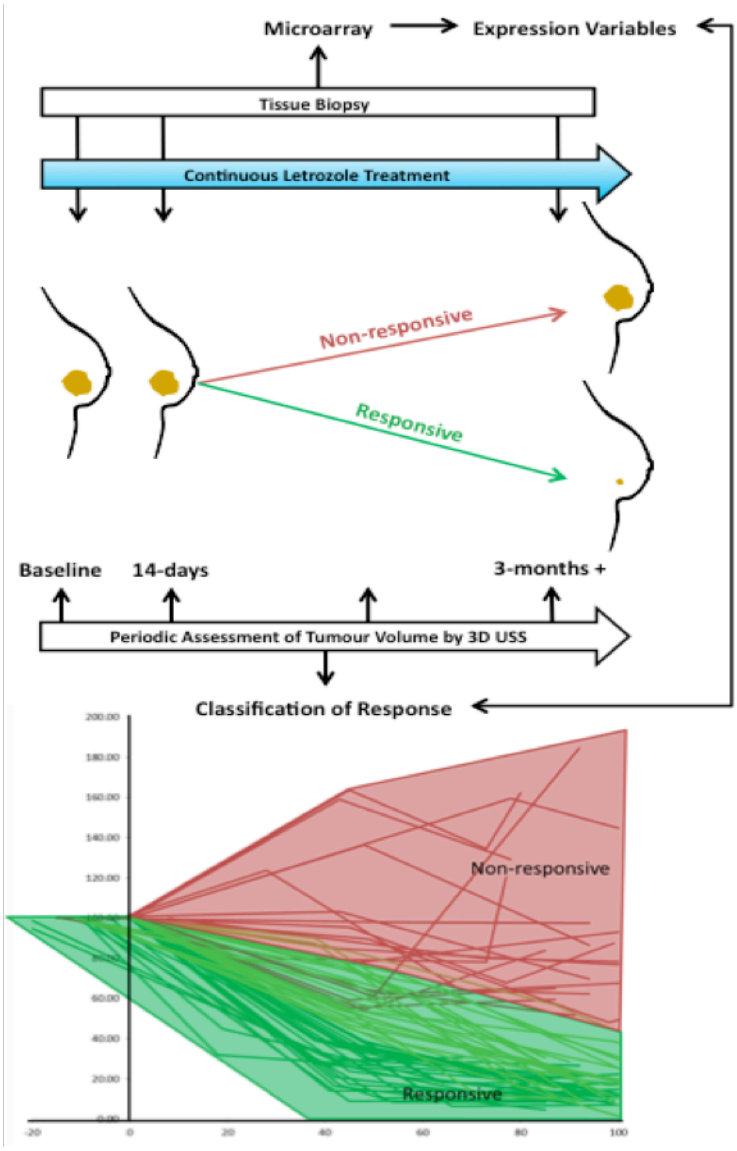


Figure 4.1A: Fresh frozen biopsy and initial tumour volume by 3D USS at baseline. Neoadjuvant letrozole therapy commenced, interval biopsy and USS at 14 days and 3 months. Microarray data generated from baseline, 14 day and 3 month samples. Patients divided into Responders or Non Responders depending on serial USS measurements.

Of the 17 ER+/HER2+ patients, almost half (8/17; 47%) were responders (R) to neoadjuvant letrozole, and the others (9/17; 53%) were non responders (NR) to neoadjuvant letrozole therapy (*Figures 4.1B)*.

This group of ER+/ HER2+ patients was compared with 59 patients who were ER+/ HER2 negative (ER+/ HER2-). Of these, 48 (81.%) were responders (R) to neoadjuvant letrozole therapy and 11 (19%) were non responders (NR) to neoadjuvant letrozole therapy, based on the same clinical response criteria.

Overall, just over half of the patients in the ER+/HER2+ subgroup (9/17; 53%) did not respond to neoadjuvant letrozole therapy, a much larger proportion compared with 19% (11/59) of patients in the ER+/ HER2- group who did not respond to neoadjuvant letrozole therapy.

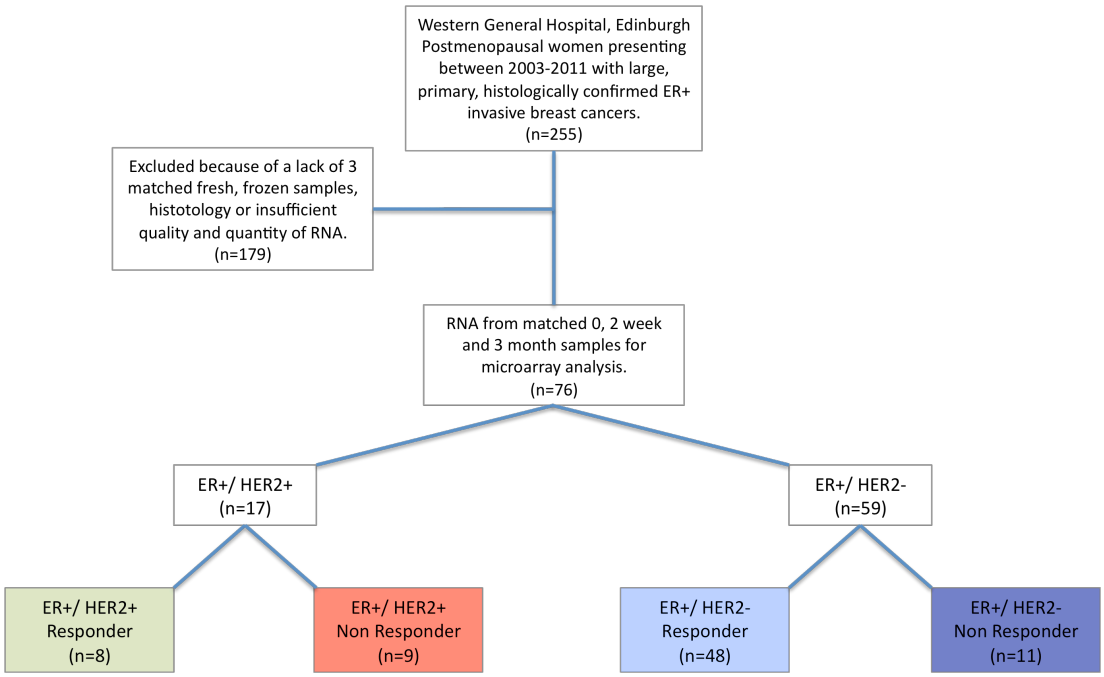


Figure 4.1B: Study consort flow diagram. Number of patients recruited between 2003-2011, along with inclusion criteria and reasons for sample exclusions. Number of patients who were either ER+/ HER2+ or ER+/ HER2- and their clinical response group.

4.2 *ERBB2* Expression at Baseline

The level of expression of the HER2 gene (*ERBB2*) was significantly higher at baseline in the ER+/ HER2+ NR group of patients than in any of the other subgroups.

Expression of *ERBB2* at baseline in ER+/ HER2+ Non Responders was significantly higher than in ER+/ HER2+ Responders (p=0.005).

Less surprisingly, the baseline expression of *ERBB2* was also significantly higher than in both the ER+/ HER2- subgroups Responders (p<0.0001); and Non Responders (p<0.0001) (*Figure 4.2)*

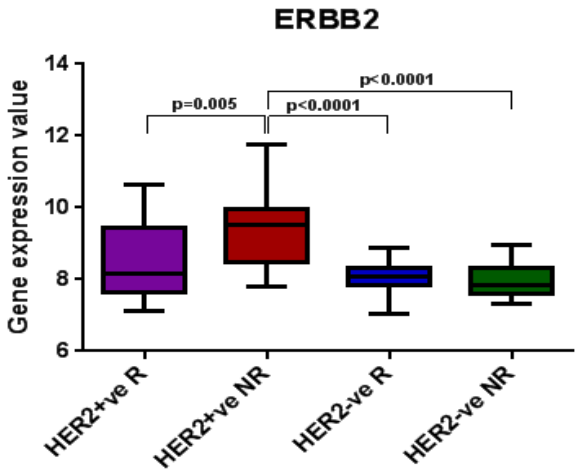


Figure 4.2: Baseline expression of *ERBB2*. ER+/ HER2+ Non Responding subgroup has a significantly higher baseline expression of *ERBB2* than ER+/ HER2+ Responders (p=0.005); ER+/ HER2- Responders (p<0.0001) and ER+/ HER2- Non Responders (p<0.0001).

4.3 *ERBB2* Expression in ER Positive/ HER2 Negative Groups

The expression level of *ERBB2* was almost the same at baseline in both the ER+/ HER2- Responding and Non Responding subgroups. However, in the ER+/HER2 – Non Responding group, expression of *ERBB2* increased over the 3 month neoadjuvant letrozole treatment period and at 3 months was significantly higher in the ER+/ HER2- Non Responding group than in the ER+/ HER2- Responding group (p=0.015). This might suggest that even in HER2 negative patients, HER2 signalling could play a role in resistance to neoadjuvant letrozole (*Figure 4.3)*

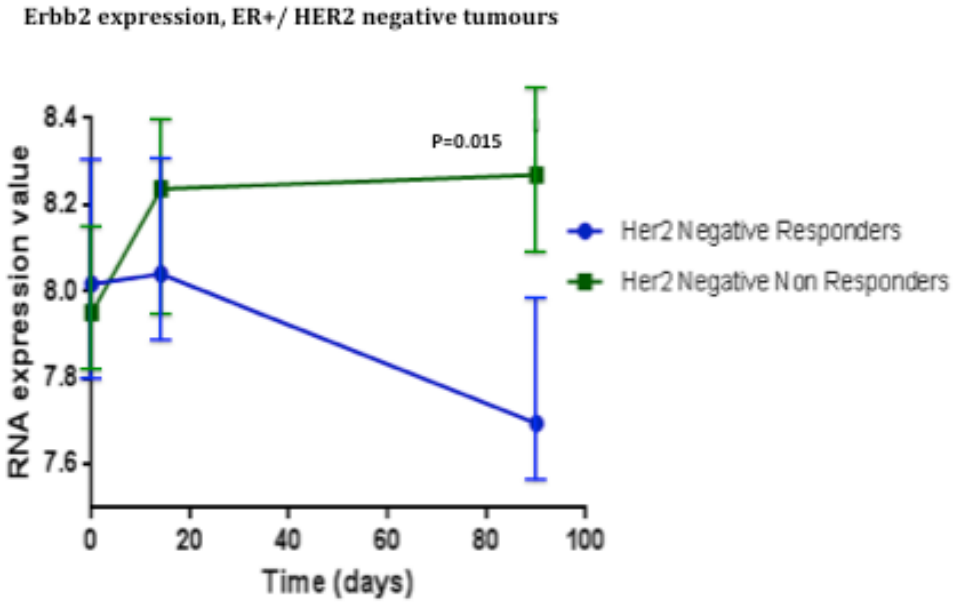


Figure 4.3: Change in level of expression of ERBB2 over 3 month neoadjuvant letrozole. At 3 months ERBB2 expression in ER+/ HER2- Non Responders is significantly higher than in ER+/HER2- Responders (p=0.015).

4.4 Estrogen Signalling

Whilst all of the patients included in the study had ER rich tumours (Allred score between 6-8), there were significant variations seen in the expression of *ESR1* before neoadjuvant letrozole treatment started.

The expression level of the *ESR1* gene was significantly lower at baseline in the ER+/ HER2+ Non Responding group than in all the other subgroups including ER+/ HER2+ Responders (p=0.0003); ER+/ HER2- Responders (p=0.0007); and ER+/ HER2- Non Responders (p=0.009) (*Figure 4.4A)*.

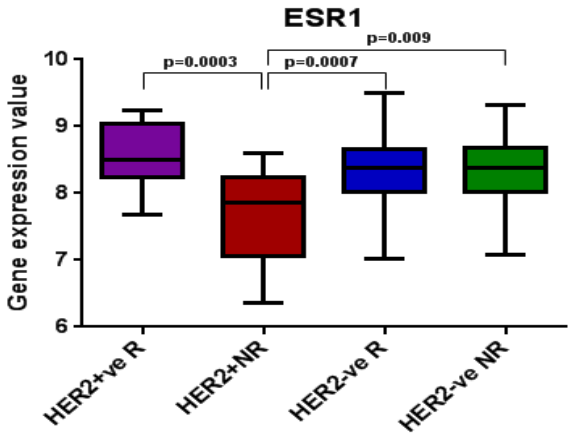


Figure 4.4A: Baseline expression of ESR1. ER+/ HER2+ Non Responding subgroup has a significantly lower baseline expression of *ESR1* than ER+/ HER2+ Responders (p=0.0003); ER+/ HER2- Responders (p=0.0007) and ER+/ HER2- Non Responders (p=0.009).

This evidence suggests that even before treatment with neoadjuvant letrozole commences, the ER+/ HER2+ Non Responding subgroup is different in terms of estrogenic signalling pathways, and would support the hypothesis that this group of patients who do not respond to neoadjuvant letrozole can be identified early on in the treatment process.

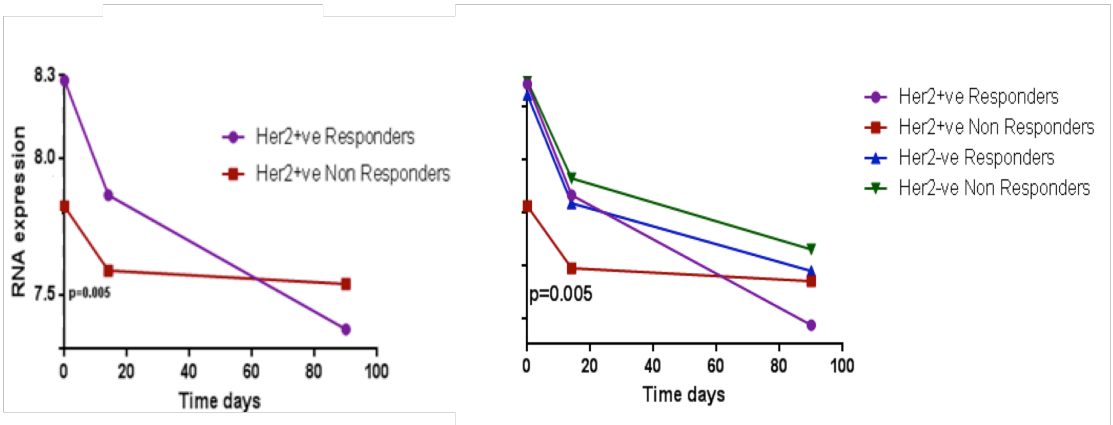


Figure 4.4B: Mean expression of estrogen signalling genes (ESR1; ESR2; PGR; GATA3; FOXA1; AGR2; NAT1; and BCL2) throughout 3 month treatment period with neoadjuvant letrozole in all 4 subgroups.

Using a set of genes known to be involved in estrogen signalling, differences were noted in the expression levels between the subgroups.[330] The estrogen signalling genes analysed were: *ESR1; ESR2; PGR; GATA3; FOXA1; AGR2; NAT1;* and *BCL2*.

At baseline, the mean expression level of these genes was significantly higher in the ER+/ HER2+ Responding group than in the ER+/HER2+ Non Responding group (p=0.005) *(Figure 4.4B)*.

During the 3 month neoadjuvant letrozole treatment period, the value of expression of these estrogen signalling genes fell dramatically in the ER+/ HER2+ Responding subgroup, in keeping with the anti-estrogenic effects of letrozole in this clinically responding group *(Figure 4.4B)*.

In the ER+/ HER2+ Non Responding subgroup, the level of expression of these estrogen signalling genes fell during the first 14 days of treatment with neoadjuvant letrozole but thereafter, between the 14 days and the 3 months periods, there was virtually no further change in the expression of these genes. This would suggest that after the initial 14 days the anti-estrogenic effects of letrozole had no effect on this clinically Non Responding ER+/ HER2+ group of patients *(Figure 4.4B)*.

When compared with the ER+/ HER2- subgroups, the mean expression level of these estrogen signalling genes is the same at baseline in the ER+/HER2+ Responding subgroup as in both the ER+/HER2- Responding and Non Responding subgroup. At this early stage, the ER+/ HER2+ Responders would appear to be more similar to the ER+/ HER2- tumours in terms of estrogenic signalling properties *(Figure 4.4B)*.

This evidence suggests that even before treatment with neoadjuvant letrozole commences, the ER+/ HER2+ Non Responding subgroup is different in terms of estrogenic signalling pathways, and would support the hypothesis that this group of patients who do not respond to neoadjuvant letrozole can be identified early on in the treatment process.

4.5 Differences in the Molecular Profiles Between ER+/ HER2+ Responders and Non Responders, at Baseline

Differential gene expression analysis between the ER+/ HER2+ Responding and ER+/ HER2+ Non Responding subgroups at baseline, revealed distinct molecular profiles between the 2 subgroups (*Figure 4.5)*.

Analysis of the 230 most highly expressed genes in ER+/ HER2+ Non Responders, revealed pathways which were functionally enriched (DAVID Bioinformatics tool) in *immune response* pathways (67/230 genes; 29.3%) and in pathways involved in *extracellular matrix remodelling (ECM) and focal adhesion (FA)* (36/230 genes; 15.5%) (*Figure 4.5)*.

The 230 most highly expressed genes at baseline in the ER+/ HER2+ Responders subgroup include genes involved in molecularly distinct pathways (using DAVID Bioinformatics tool), from the ER+/HER2+ Non Responding group. These functional pathways include *transcription and translation* (40/230 genes; 17.4%); *cell signalling* (16 genes; 7%)*; metal ion binding* (16 genes; 7%); *and oxidative phosphorylation* (15 genes; 6.5%) (*Figure 4.5).*

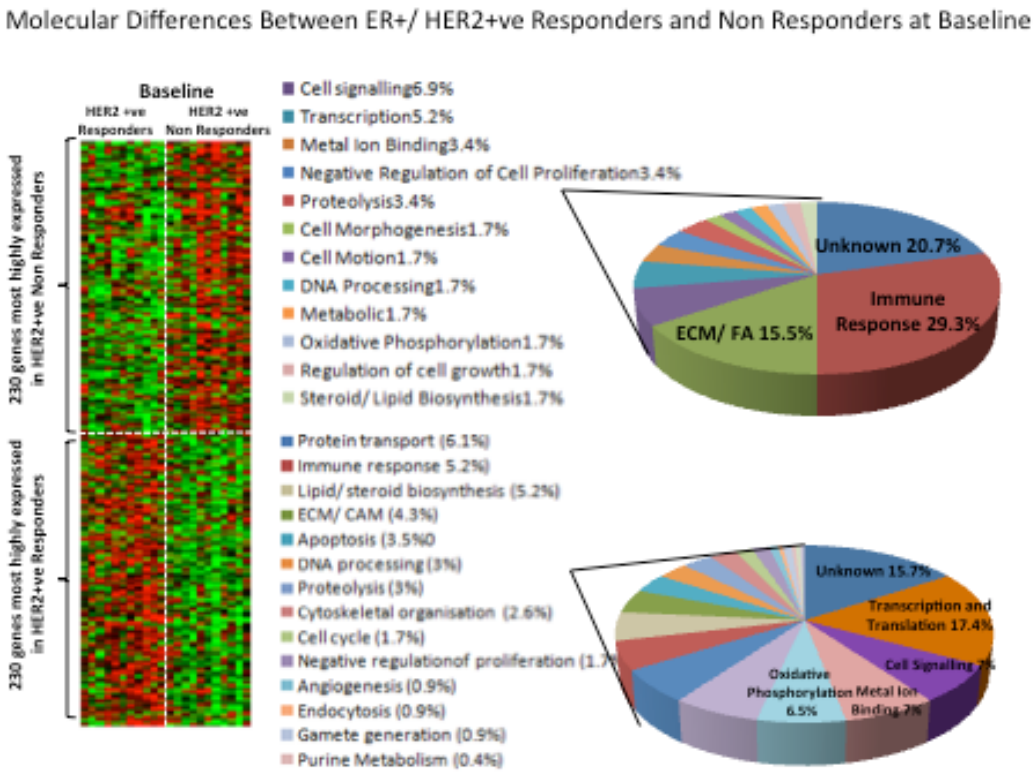


Figure 4.5: 460 most differentially expressed genes between ER+/ HER2+ Responders and Non Responders at baseline reveal molecularly distinct pathways between the 2 subgoups.

4.6 Most Consistently Changed Genes in Responders

Analysis of the 551 most consistently changed genes in ER+/ HER2– responders to letrozole therapy (pairwise RP, FDR=0.01) revealed pathways that were functionally enriched (DAVID Bioinformatics tool) in terms of both down-regulated and up-regulated genes at both the 14 day and 3 month time-points.

The most down regulated genes between pre-treatment and 14 days of letrozole were genes involved in the following pathways: proliferation/ cell cycle/ DNA replication; transcription; protein processing.

The most up-regulated genes in this group over the same treatment period were involved in the immune/ inflammatory response and ECM/ stromal remodelling /adhesion and angiogenesis. (*Figure:4.6A)*

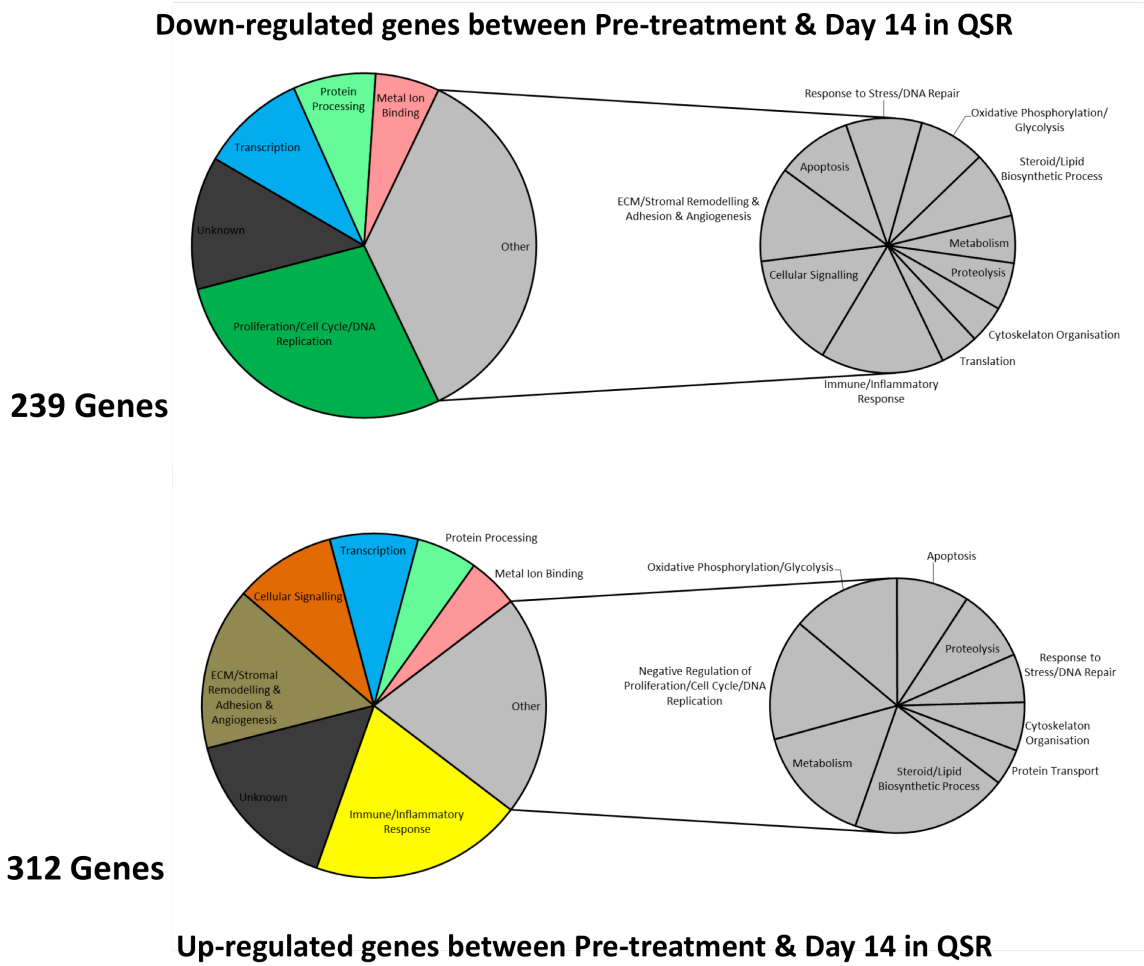


Figure:4.6A: Pie charts showing the functional groups which were most down regulated over 14 days of treatment (above) and most up regulated over 14 days of treatment (below).

Proliferation associated genes including cyclins (CCNA2, CCNB1 and CCND1), the mini chromosome maintenance genes (MCM2, MCM4 and MCM6) and the mitotic spindle associated genes (ASPM and AURKA) were found to be down-regulated in both ER+/ HER2+ and ER+/ HER2- responding tumours by 14 days. Glycolysis and oxidative phosphorylation genes were found to be consistently down-regulated by 3 months in responding tumours (both ER+/ HER2- and ER+/ HER2+).

Genes involved with the *Immune/ inflammatory response* and *ECM/ stromal remodelling* were found to be significantly up-regulated by 3 months in the responding tumours (both ER+/ HER2+ and ER+/ HER2- subgroups) *(Figure 4.6B)*. The same genes remained at a relatively low expression level at both on-treatment time points (14 days and 3 months) in non responding tumours (both ER+/ HER2+ and ER+/ HER2- subgroups).

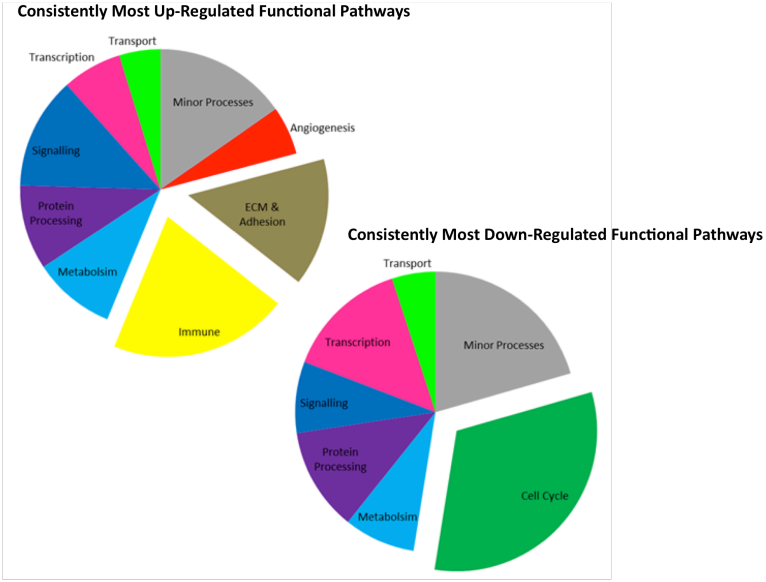


Figure 4.6B: Pie charts of up-regulated and down-regulated functional pathways in Responders: The 551 consistently most changed genes between baseline and 3 months of neoadjuvant letrozole therapy in Responding letrozole treated patients (pairwise Rank Product analysis; FDR=0.01) were functionally enriched for up-regulation of immune and extracellular matrix (ECM) remodelling genes and down-regulation of proliferation associated genes.

4.7 Proliferation, Immune, Stromal, and ECM Remodelling Pathways

A heatmap of the proliferation and immune/ stromal/ ECM genes effectively demonstrates the different patterns of gene expression change between the different ER+/HER2- and ER+/HER2+ Responders and Non Responders between pre-treatment and 3 months of neoadjuvant letrozole.*(Figure 4.7A).* The ER+/ HER2- Responding tumours and the ER+/ HER2+ Responding tumours had identical changes in these key genes, indicating that some ER+/ HER2+ tumours behave biologically like ER+/ HER2- tumours. It may be that in these responding ER+/ HER2+ tumours, HER2 signalling is inactive.

The ER+/ HER2- Non Responders showed a similar down-regulation of proliferation genes but only minimal up-regulation of the immune/stromal/ECM genes.

The heatmap demonstrates two different groups of ER+/HER2+ Non Responders. In the first group (pink) there was some down-regulation of proliferation genes over the 3 months of treatment with minimal up-regulation of the immune/stromal/ECM genes. This group would appear to behave in a similar way to the ER+/HER2- Non Responders.

The remaining ER+/HER2+ Non Responders (purple) did not show any change in proliferation or immune/stromal/ ECM genes. In terms of change in gene expression, letrozole therapy would appear to have no effect on these tumours at all.

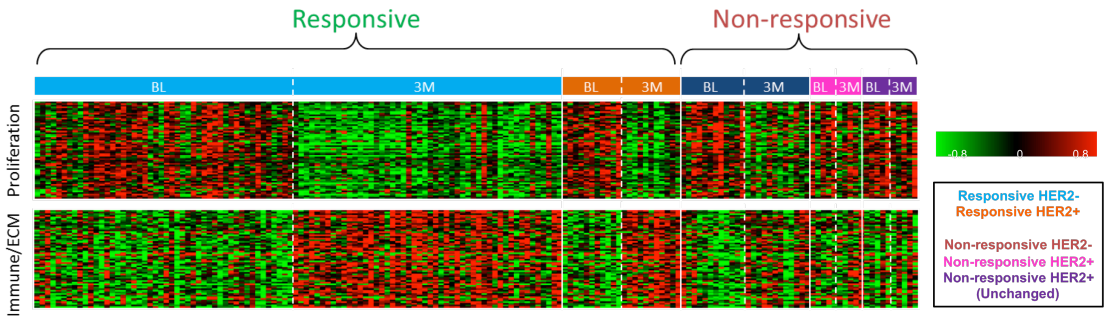


Figure 4.7A: Heatmaps comparing expression over time (baseline to 3 months) of the 2 main functional groups (immune/ ECM and proliferation) in all Responders and Non Responders: ER+/ HER2- (light blue) and ER+/HER2+ (orange) Responders had identical changes in these key genes. ER+/ HER2- Non Responders (navy) had a decrease in proliferation genes but no increase in immune/ ECM genes. ER+/ HER2+ Non Responding tumours (pink/ purple) did not change in respect of either gene set. Higher proliferation was observed in the subset with no changes on treatment (purple).

Multidimensional scaling *(figure 4.7B)* was used to plot the relationship of gene expression changes between pre-treatment and 3 months of letrozole therapy for each tumour. To

demonstrate the direction and extent of change in gene expression in each tumour, a line connecting baseline to 3 months was used to plot the trajectory of movement. An arrow starting in the blue ellipse on the left and moving to the green ellipse on the right represents the down-regulation of proliferation genes and up-regulation of immune/ stromal/ ECM remodelling genes as described above.

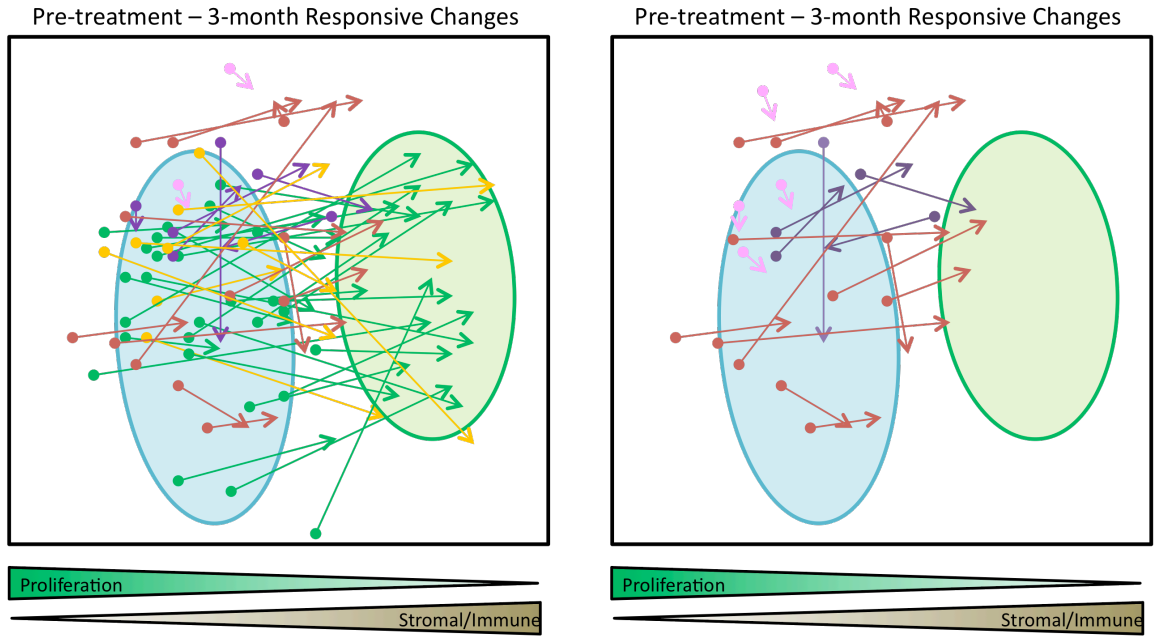


Figure 4.7B : Multidimensional scaling (MDS) plot: Trajectory of changed genes from baseline samples (circles) and 3 month on-treatment samples (arrow heads). Left plot demonstrates trajectory of movement of all tumours (green: ER+/HER2- R; yellow: ER+/HER+ R; red: ER+HER2- NR; pink/purple: ER+/HER2+ NR). Right plot show all Non Responding tumours.

Responding tumours moved from left to right (from the large light blue ellipse to the smaller green ellipse). This represents increased expression of immune/ ECM remodelling genes, and a decreased expression of proliferation genes. Both ER+/ HER2- Responders (green arrows) and the ER+/ HER2+ Responders (yellow arrows) responding tumours had similar path trajectories, reflecting a similar change in gene profiles over the 3 months of treatment.

Non responding tumours (MDS plot on the right) demonstrated less overall change in gene expression than responding tumours, with most not reaching the green ellipse. The majority of ER+/ HER2- Non Responding tumours (red arrows) appeared to change in the same direction as the responding tumours but not to the same extent. The ER+/ HER2+ Non Responding tumours either didn't move at all (pink arrows) or showed an erratic pattern of movement (purple).

4.8 Immune cell profiling for ER+/ HER2+ tumours at baseline

We have demonstrated that response to letrozole involves an increase in the expression of immune related genes (*Figure 4.6A; Figure 4.6B; Figure 4.7A; Figure 4.7B)*. Gene expression signatures for individual immune cell types[308] including All T cells; CD8+ T cells; T regulatory cells; B cells; and dendritic cells were used to create gene expression heatmaps for all ER+/ HER2+ tumour samples at baseline

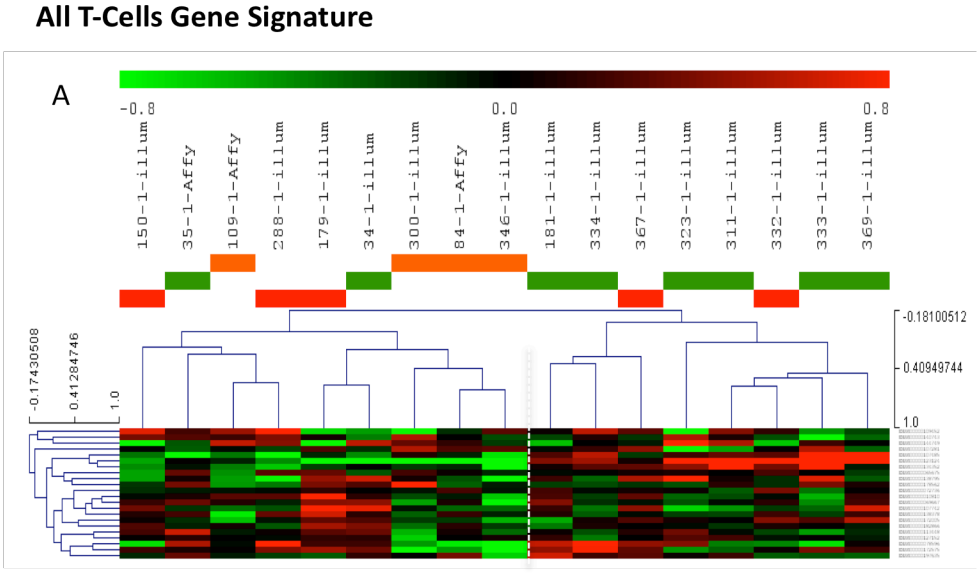


Figure 4.8A: Heatmap of All T cells gene signature (gene list Appendix 4). Red: ER+/ HER2+ Non Responders; Orange: ER+/ HER2+ Progressors; Green: ER+/ HER2+ Responders.

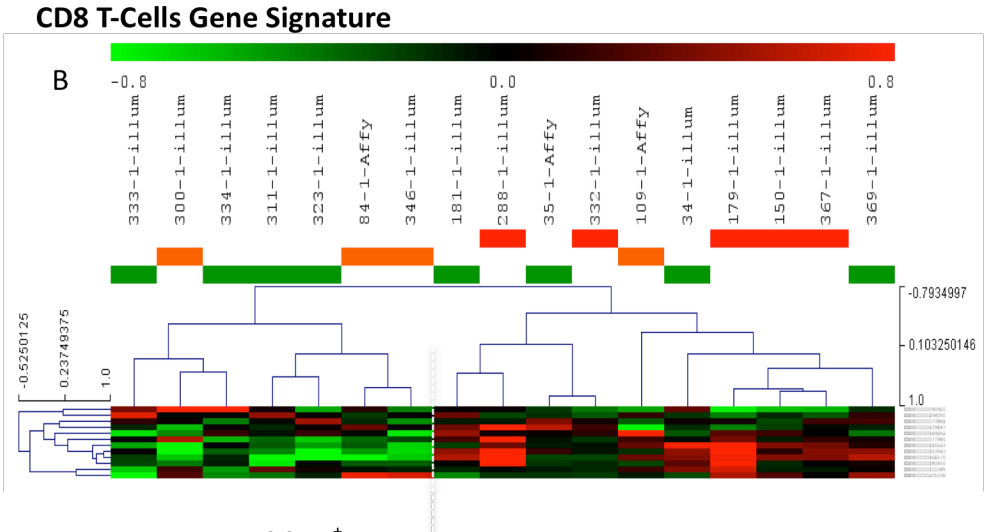


Figure 4.8B: Heatmap of $CD8^{+}$ T cells gene signature (gene list Appendix 4). Red: ER+/ HER2+ Non Responders; Orange: ER+/ HER2+ Progressors; Green: ER+/ HER2+ Responders.

T Regulatory Cells Gene Signature

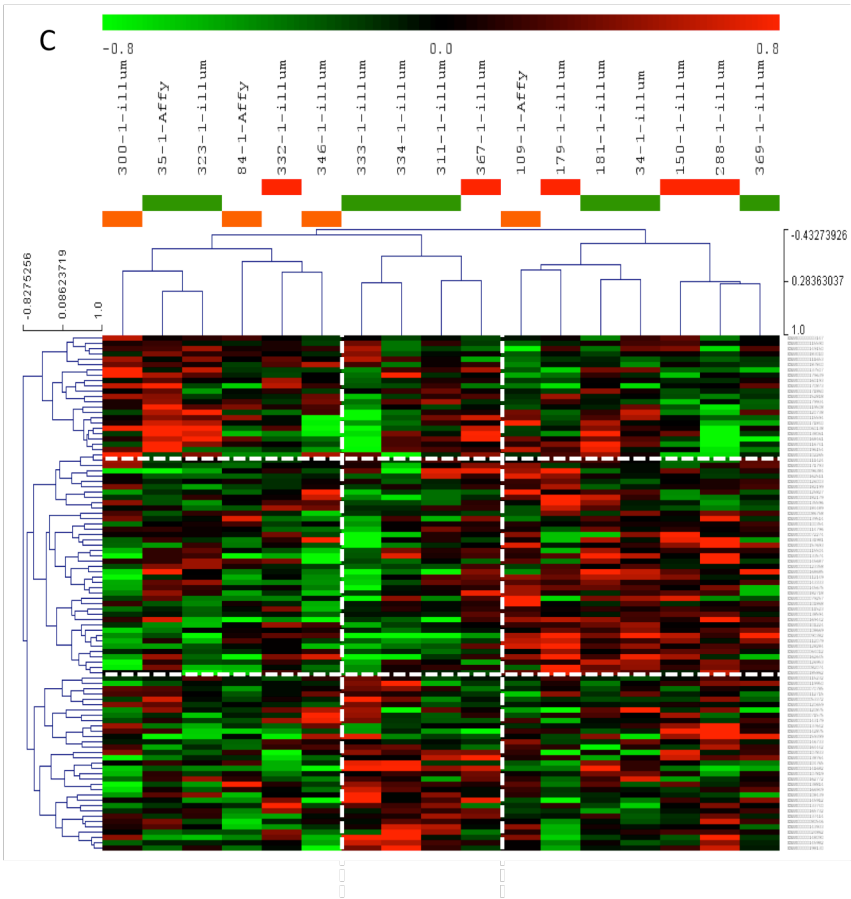


Figure 4.8C: Heatmap of T regulatory cells gene signature (gene list Appendix 4). Red: ER+/ HER2+ Non Responders; Orange: ER+/ HER2+ Progressors; Green: ER+/ HER2+ Responders.

B-Cell Gene Signature

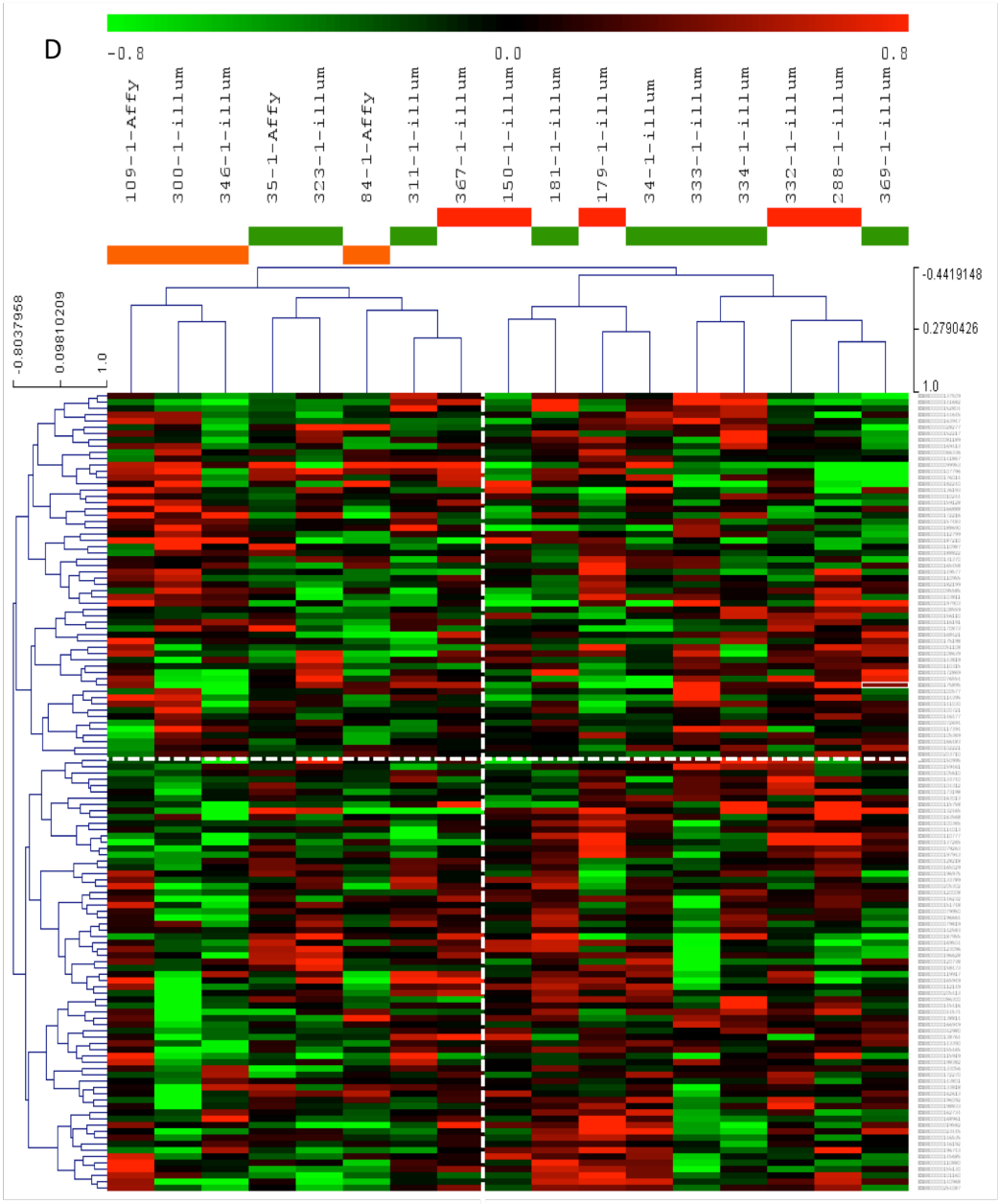


Figure 4.8D: Heatmap of B cells gene signature (gene list Appendix 4). Red: ER+/ HER2+ Non Responders; Orange: ER+/ HER2+ Progressors; Green: ER+/ HER2+ Responders.

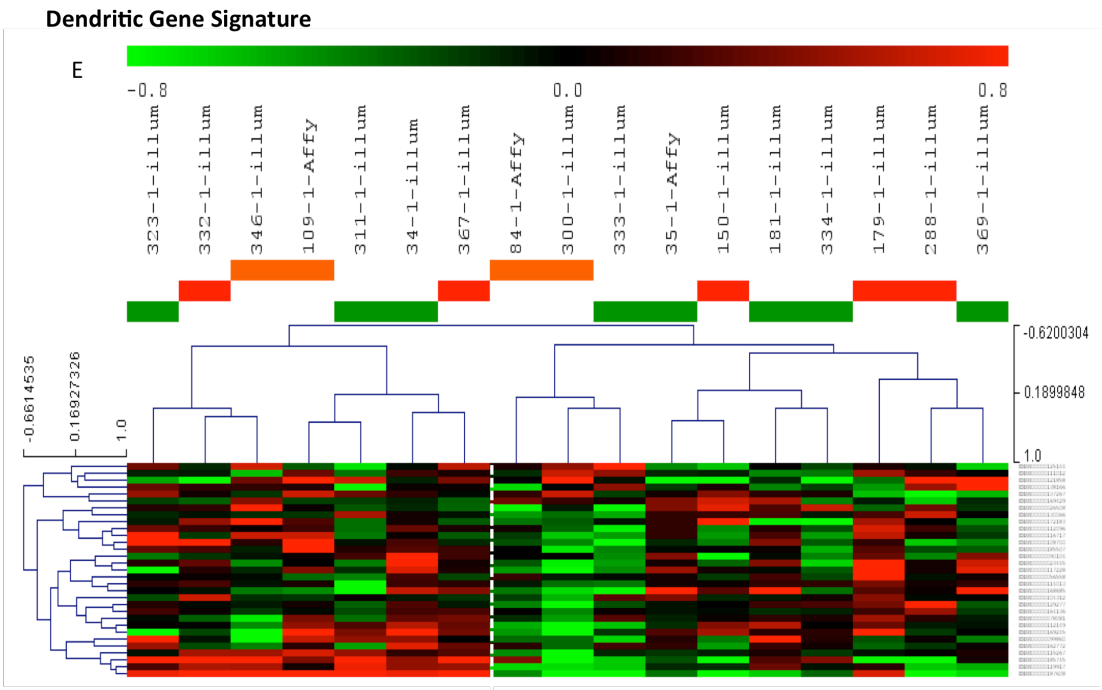


Figure 4.8E: Heatmap of Dendritic cells gene signature (gene list Appendix 4). Red: ER+/ HER2+ Non Responders; Orange: ER+/ HER2+ Progressors; Green: ER+/ HER2+ Responders.

Using the 'All T cells' gene signature there did appear to be clustering of the ER+/ HER2+ responders from the non responders (*Figure 4.8A)*. This separation was not quite so evident when the other immune gene signatures were applied to heatmaps.

4.9 ER Positive/ HER2 Positive Responders behave like ER Positive/HER2 Negative Responders

We have demonstrated that response to letrozole involves a decrease in proliferation genes and an increase in immune, stromal and ECM remodelling genes. Just under half of the ER+/HER2+ tumours studied (8/17; 47%) did have a good response to letrozole therapy. Analysis of gene expression changes in these tumours shows a very similar profile to the ER+/HER2- Responders. *(Figure 4.7A and Figure 4.8*). This would suggest that HER2 signalling is not present in these HER2+ tumours and that endocrine therapy is effective in these patients.

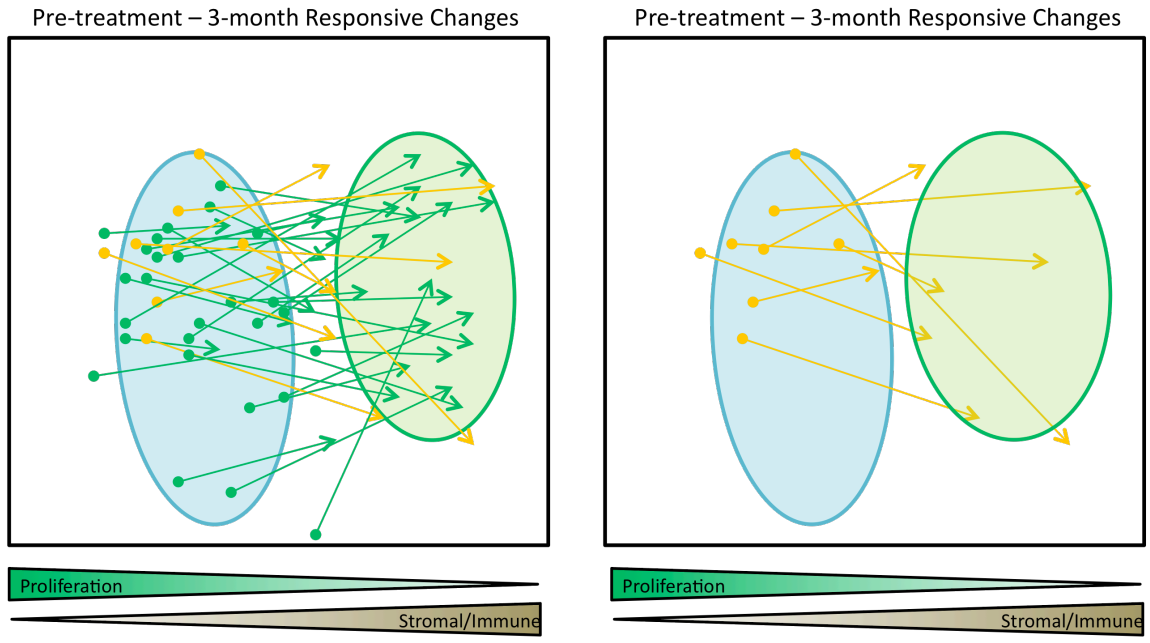


Figure 4.9: Multidimensional scaling (MDS) plot: Trajectory of changed genes from baseline samples (circles) and 3 month on-treatment samples (arrow heads). Left plot demonstrates trajectory of movement of all responding tumours (green: ER+/HER2- R; yellow: ER+/HER+ R). Right plot shows all ER+/ HER2+ responding tumours.

4.10 ER Positive/ HER2 Positive Tumours and Neoadjuvant Letrozole

To further investigate the role of HER2 in endocrine resistance, analyses were focussed on the ER+/ HER2+ tumours and the ER+/ HER2- non responding tumours .

To further assess the way in which the ER+/ HER2+ tumours responded to letrozole therapy, response was further broken down into tumours which were consistently 'non responders' (red); tumours which showed 'partial response then progression' (orange); and tumours which were 'quick stable responders' (green). ER+/ HER2 negative non responding tumours (blue) were investigated to determine whether the pathways of resistance were different between the ER+/ HER2+ and ER+/ HER2- non responding tumours. *(Figure 4.10)*

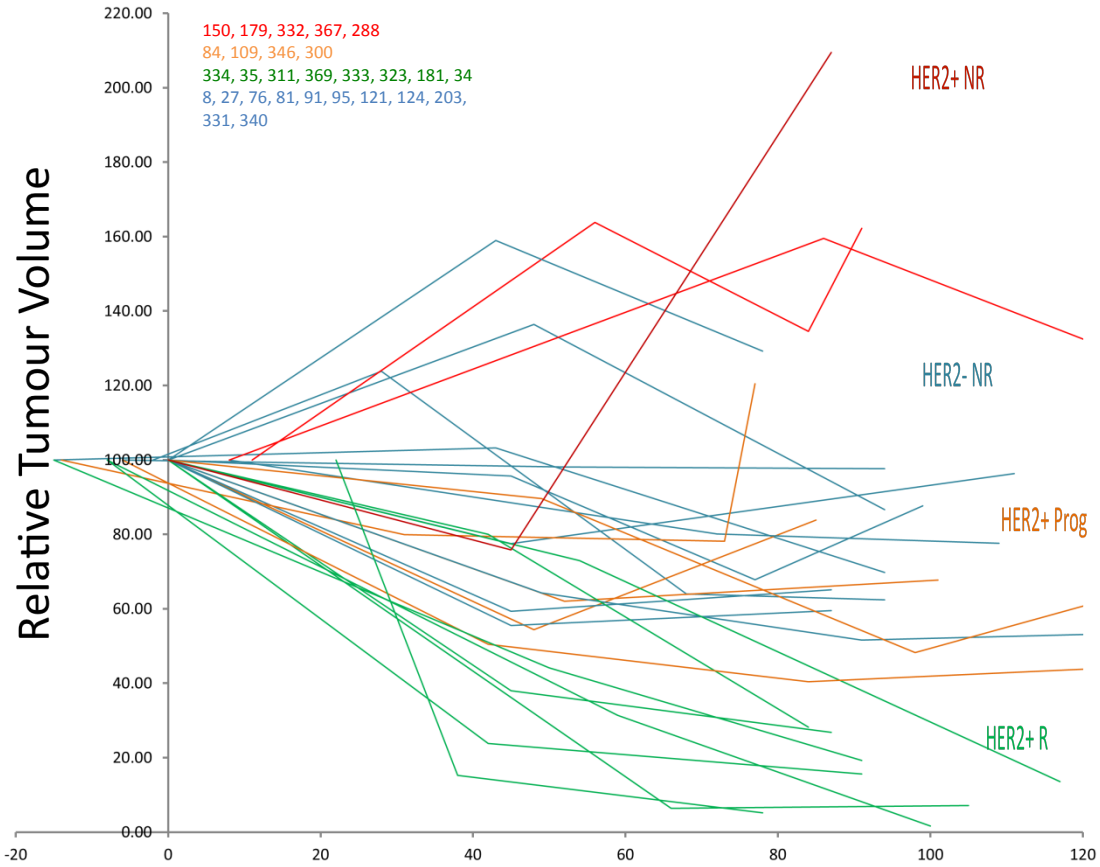


Figure 4.10: Clinical response groups. Change in tumour volume allows classification of response, red: ER+ /HER2+ Non Responders (NR); orange: ER+/ HER2+ Progressors (P) (partial response and then progression); green: ER+/ HER2+ Responders (R); blue: ER+/ HER2- Non Responders (NR).

4.11 Effect of endocrine therapy on proliferation, in ER+/ HER2+ tumours

Using a published list of proliferation genes whose expression has been shown to decrease with endocrine therapy[309] (*Appedndix 4*), it can be seen that following 3 months of neoadjuvant letrozole, expression of these proliferation genes remains higher in the ER+/ HER2+ non responding tumours.

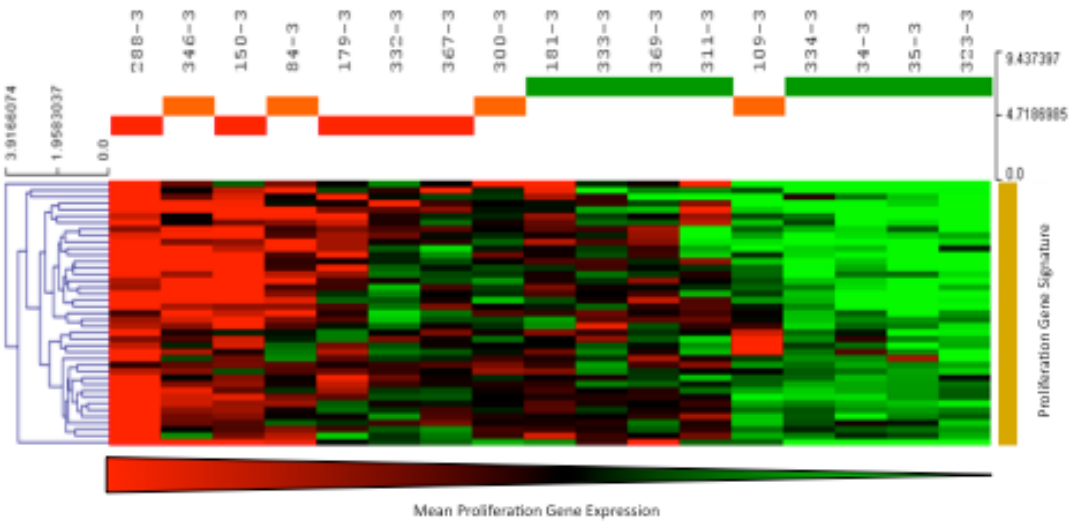


Figure 4.11: Heatmap showing proliferation gene expression in all ER+/ HER2+ tumours after 3 months letrozole. Red: Non Responders; Orange: Progressors; Green: Responders.

It can be seen that the ER+/ HER2+ Responders fall to the right of the heatmap, where there is a low level of expression of proliferation genes. The ER+/ HER2+ Non Responders fall to the left of the heatmap and can be seen to have high levels of expression of proliferation genes, indicative of letrozole resistance after 3 months of neoadjuvant therapy.

4.12 Active HER2 signalling via the MAPK signalling pathway, and estrogen signalling, pre-treatment

Using published data of genes found to be involved in MAPK signalling[289] (*Appendix 5*), and ER signalling[310] (*Appendix 6)*, it can be seen at the pre-treatment timepoint that ER+/ HER2+ tumours which will be resistant to letrozole are characterized by active MAPK signalling and lower ER activity.

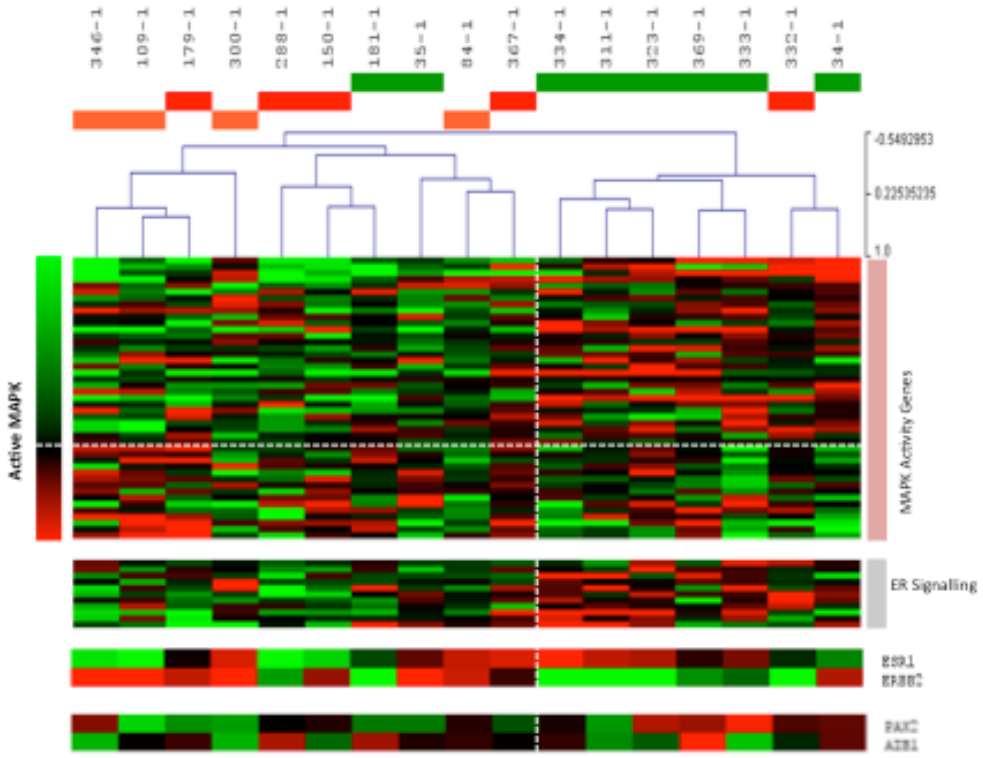


Figure 4.12: Heatmap showing expression of up regulated and down regulated genes indicating active or inactive MAPK signalling, and ER signalling for all ER+/ HER2+ samples at baseline. Expression levels of *ESR1, ERBB2,* PAX2 and *AIB1* also demonstrated on the heatmap.

The heatmap (*Figure 4.12*) demonstrates clustering of the tumours at baseline into active (left) and inactive MAPK signalling. It can be seen that the large majority of clinical responders demonstrate inactive MAPK signalling at the pre-treatment biopsy. This is also associated with a higher level of expression of ER signalling genes.

The vast majority of non responding tumours (8/9) show active signalling at the pre-treatment level. Furthermore, this is associated with a lower level of expression of ER signalling genes.

The pre-treatment gene expression level of *ESR1* and *ERBB2* for each of the ER+/ HER2+ tumours can also be seen (*Figure 4.12*). A lower expression of *ESR1* can be seen to the left of the heatmap where the majority of the non responders are, whereas a higher level can be seen to the right of the heatmap in the responding tumours. A higher expression of *ERBB2* is seen in the non responding tumours to the left of the heatmap, compared to a lower level of expression in the responders to the right.

The heatmap also demonstrates pre-treatment expression levels of *PAX2* and the ER coactivator *AIB1,* genes which have been shown to compete for binding and regulation of *ERBB2* transcription.[353] There is a higher level of expression of *PAX2* in the responding tumours which is consistent with previous data that showed PAX2 to be associated with better sensitivity to endocrine therapy (*Figure 4.12*). There was no significant difference in expression of *AIB1* between the 2 groups.

4.13 Active HER2 signalling via the MAPK signalling pathway, and estrogen signalling, after 3 months neoadjuvant letrozole

After 3 months of neoadjuvant letrozole therapy there is considerable change in the MAPK signalling activity. At this time point the tumour samples do not cluster into distinct responding and non responding arms (*Figure 4.13*).

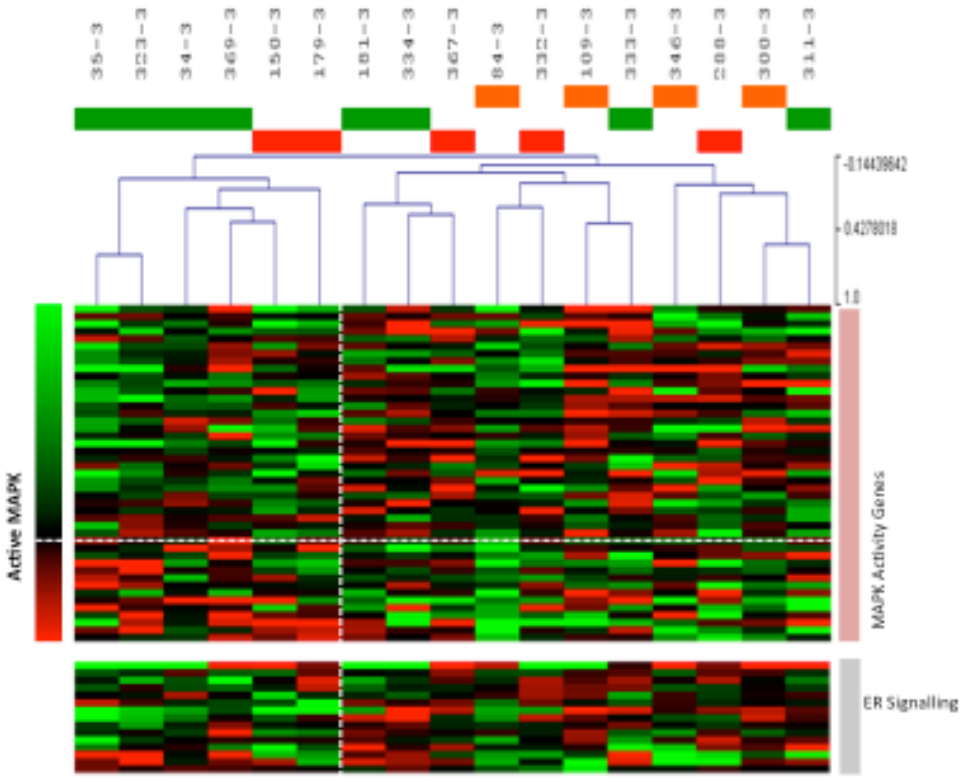


Figure 4.13: Heatmap showing expression of up regulated and down regulated genes indicating active or inactive MAPK signalling, and ER signalling for all ER+/ HER2+ samples at baseline.

It can be seen that ER signalling remains lower in the ER+/ HER2+ non responding tumours after 3 months neoadjuvant letrozole. At this time point ER signalling is still active in the ER+/ HER2+ responders. This pattern of ER signalling activity after neoadjuvant letrozole is consistent with findings at pre-treatment biopsy in the responding and non responding groups (*Figure 4.12*).

4.14 Immunohistochemistry, ERK and phosphorylated ERK

Monoclonal antibodies for ERK and phosphorylated ERK (pERK) were used to stain formalin fixed, paraffin embedded slides. Staining of ERK and pERK was consistent with gene expression levels, however tissue was only available from a small cohort of the samples, with very little available from pre-treatment tissue sections *(Figures 4.14A and 4.14B)*.

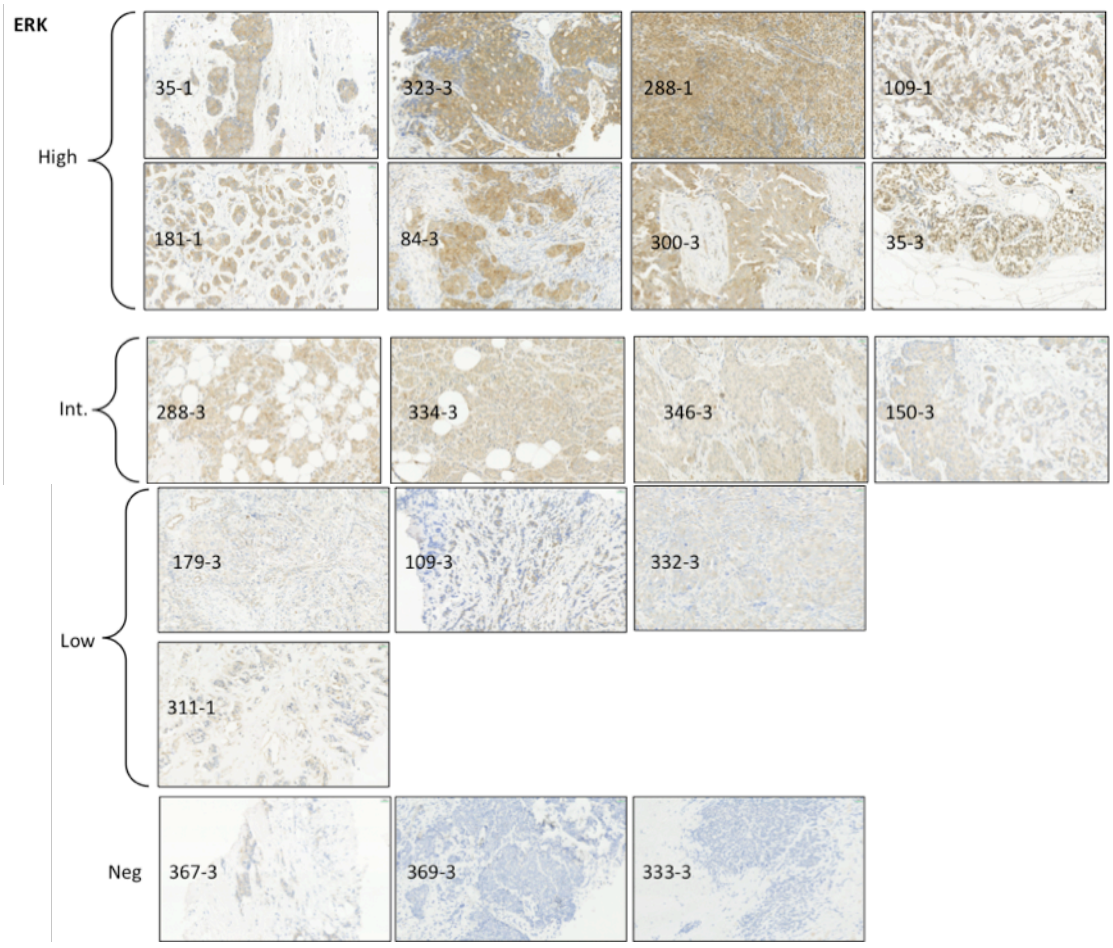


Figure 4.14A: Representative examples of paraffin embedded breast carcinoma tissue biopsies. Biopsies taken at pre-treatment (-1) and after 3 months treatment with neoadjuvant letrozole (-3). Samples analyzed by immunohistochemistry for ERK. Slides categorised into high, intermediate , low and negative according to reactivity to ERK antibody.

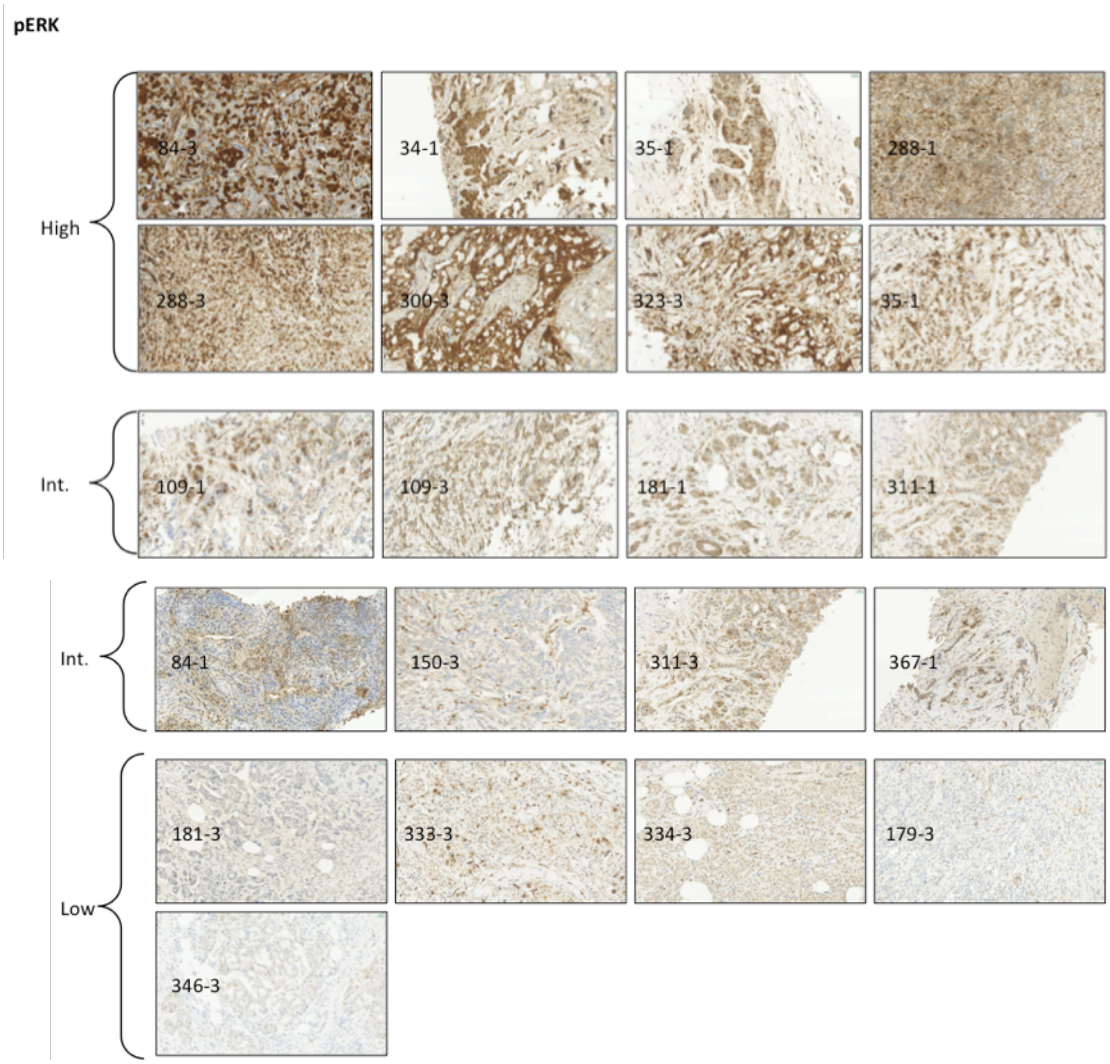


Figure 4.14B: Representative examples of formalin fixed, paraffin embedded breast carcinoma tissue sections. Biopsies taken at pre-treatment (-1) and after 3 months treatment with neoadjuvant letrozole (-3). Samples analyzed by immunohistochemistry for phosphorylated ERK. Slides categorised into high, intermediate , low and negative according to reactivity to ERK antibody.

4.15 Active HER2 signalling via the PI3K signalling pathway, pre-treatment

The phosphatidylinositol 3' -kinase(PI3K)-Akt signalling pathway is activated by many types of cellular stimuli or toxic insults and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival. The binding of growth factors to their receptor tyrosine kinase (RTK) or G protein-coupled receptors (GPCR) stimulates class Ia and Ib PI3K isoforms, respectively. PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane. PIP3 in turn serves as a second messenger that helps to activate Akt. Once active, Akt can control key cellular processes by phosphorylating substrates involved in apoptosis, protein synthesis, metabolism, and cell cycle. Using genes from the Kegg database PI3K signalling pathway *(Appendix 7)*, heatmaps were created for ER+/ HER2+ responders and non responders at the pre-treatment timepoint.

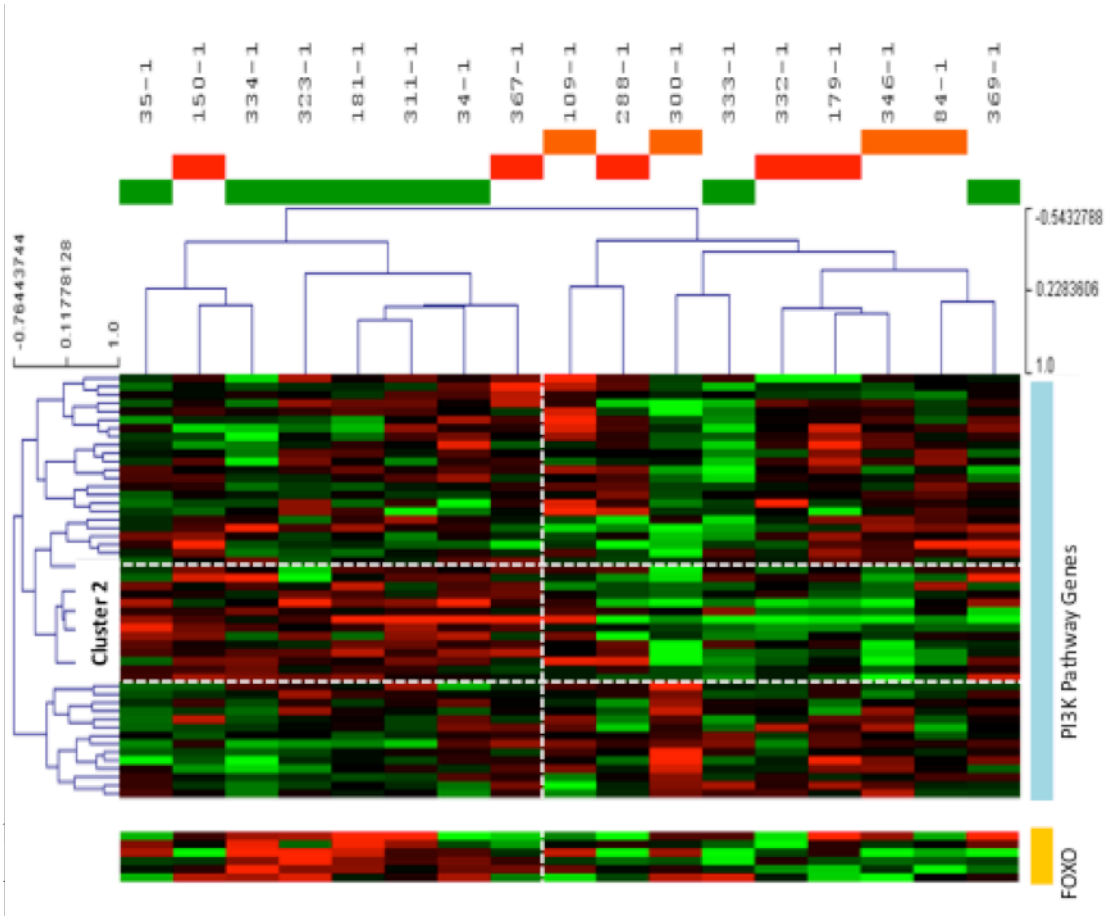


Figure 4.15A: Heatmap showing expression of genes involved in PI3K signalling and an inverse correlation in expression of FOXO target genes, for each ER+/ HER2+ tumour at pre-treatment.

At the pre-treatment timepoint, the responders and non responders cluster seperately. It would appear that there is a distinct group of genes (cluster 2 on heatmap, *Figure 4.15*) which when

highly expressed indicate inactive PI3K signalling and when expressed at low levels would indicate active PI3K signalling. Cluster 2 represents the genes *AKT3, NFKBIA, SHC1, PIK3R1, SRF, GJA1, PDGFRA, FOS, PTEN, JUN, PDPK1, FOXO1, IGF1* and *CDKN1B*. Using this cluster of genes to represent activity of PI3K signalling, it can be seen that at baseline ER+/ HER+ Responders have no evidence of active PI3K signalling. The ER+/ HER+ Non Responders have evidence of active PI3K signalling, indicating that this signalling pathway could play an important role in resistance to endocrine therapy in these ER+/ HER2+ tumours.

PI3K pathway activity negatively regulates forkhead box-O (FOXO) transcription factor activity, and so FOXO gene expression is inversely correlated with PI3K activity. Expression of FOXO target genes[311] (*Appendix 8*) showed an inverse correlation with PI3K expression. ER+/ HER2+ responding tumours had high expression of FOXO target genes at baseline, whilst ER+/ HER2+ non responding tumours demonstrated a low level of expression of FOXO target genes at baseline (*Figure 4.15*).

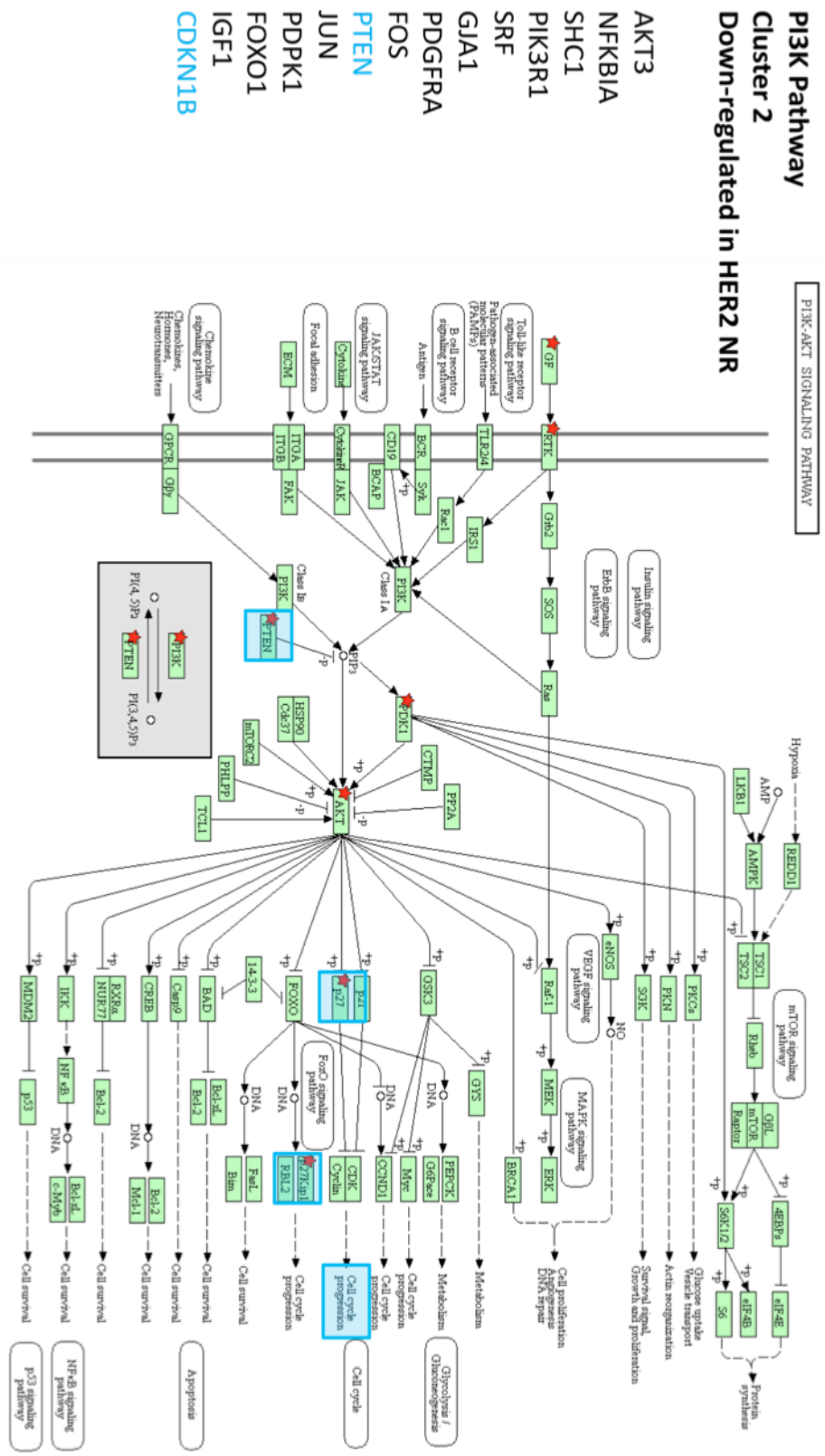


Figure 4.15B: The phosphatidylinositol 3' -kinase(PI3K)-Akt signalling pathway is activated by many types of cellular stimuli or toxic insults and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival. Figure from www.kegg.jp/kegg/xml/IGML.[307]

4.16 Active HER2 signalling via the PI3K signalling pathway, after 3 neoadjuvant letrozole

In non responding ER+/ HER2+ tumours, PI3K pathway activity remains high, once again with an inverse correlation of low activity in FOXO target genes (*Figure 4.16*).

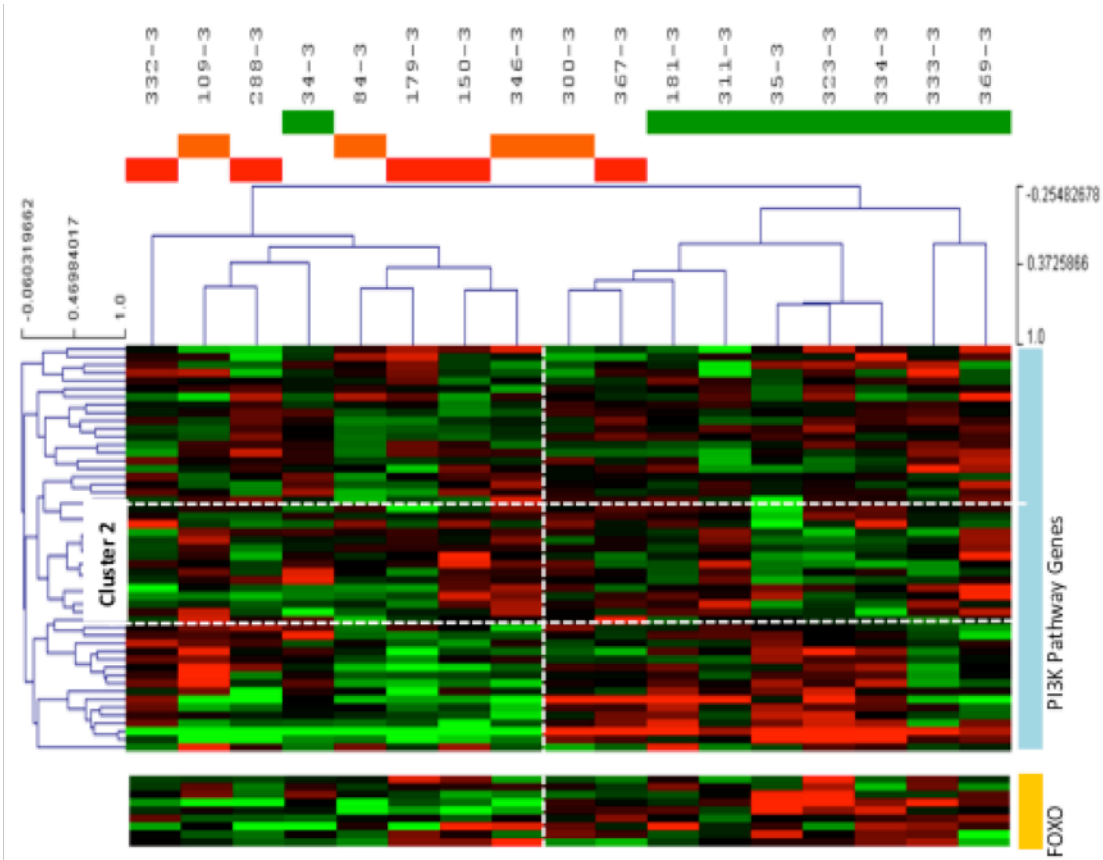


Figure 4.16: Heatmap showing expression of genes involved in PI3K signalling and the inverse correlation in expression of FOXO target genes for each ER+/ HER2+ tumour after 3 months neoadjuvant letrozole therapy.

4.17 Validation with treatment naive ER+/ HER2+ cohort

To validate our findings we identified a further 13 patients with ER+/ HER2+ disease who had primary surgical excision of their tumour, without neoadjuvant letrozole. These patients were not part of the neoadjuvant letrozole study and as such do not have clinical response paramaters as measured by change in tumour volume. Follow up clinical data for recurrence is not yet available for these patients. Microarrays were generated for these 13 ER+/ HER2+ tumour samples and could be used to validate our findings at the pretreatment timepoint.

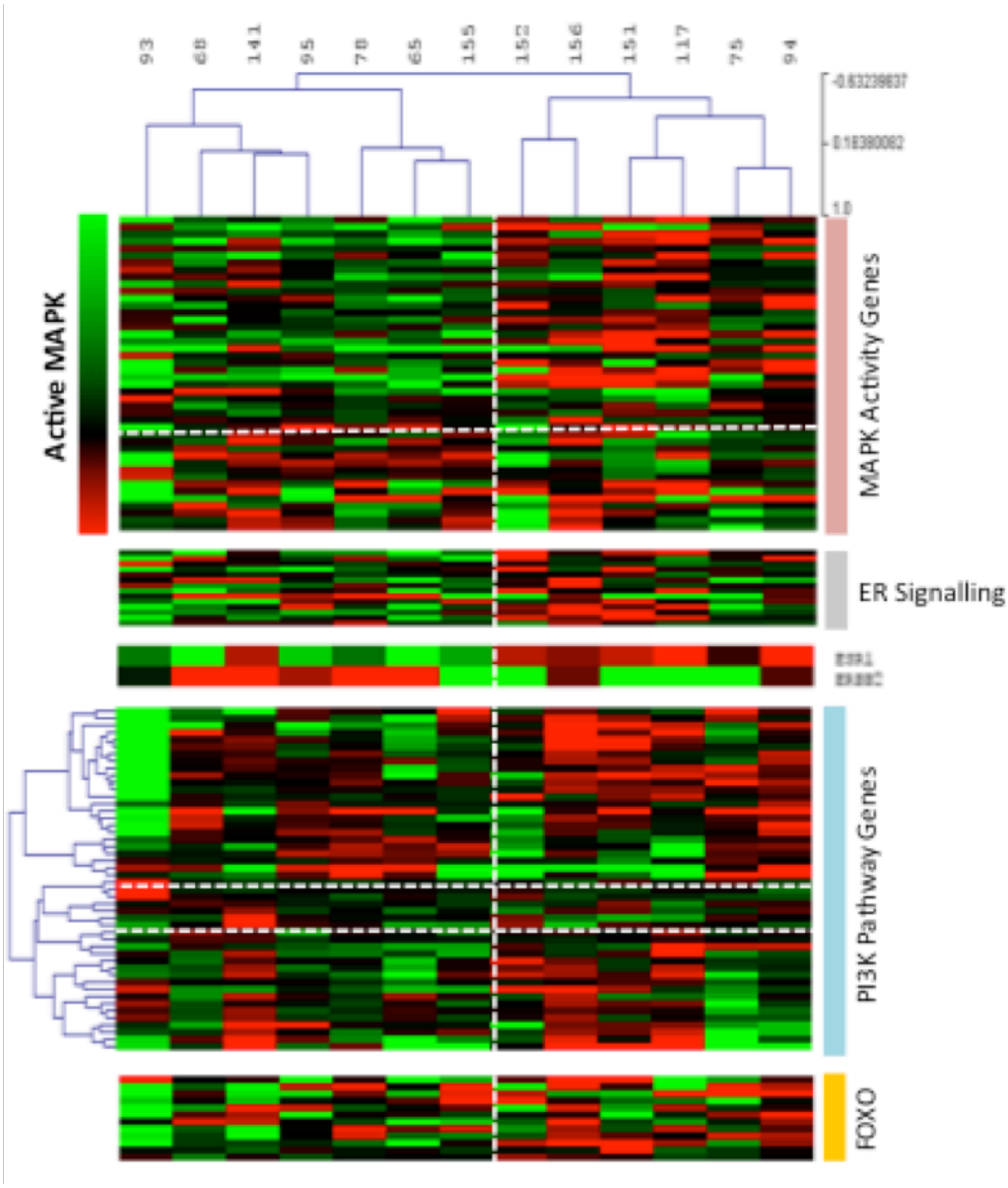


Figure 4.17: Heatmap of treatment naïve cohort, 13 ER+/ HER2+ tumour samples. MAPK activity genes, ER signalling, ESR1, ERBB2, PI3K pathway genes, and FOXO genes shown

The left side of the heatmap (*Figure 4.17*) shows a group of ER+/ HER2+ tumours characterized by active MAPK and PI3K signalling suggesting active HER2 signalling. There is a lower level of ER signalling as well as a lower expression of *ESR1* and a higher level of *ERBB2* expression. Whilst there is no clinical data available yet for these patients, the findings would suggest that this group of patients would be more likely to develop resistance to adjuvant endocrine therapy.

The right side of the heatmap (*Figure 4.17*) demonstrates a group of tumours that would be more likely to have a sustained response to adjuvant endocrine therapy. These tumours samples have inactive MAPK and PI3K signalling pathways, a higher ER signalling activity and higher expression of *ESR1* with lower expression of *ERBB2.*

Patient ID	Response to Letrozole	HER2 Status	Proliferation Gene Level		HER2 IHC		PI3K Gene Level		MAPK Gene Level		ERK IHC		pERK IHC	
			Baseline	3M	Baseline/Clinical	3M	Baseline	3M	Baseline	3M	Baseline	3M	Baseline	3M
8	Non-responder	Neg	High	Low	Negative	N/A	Inactive	Active	Inactive	Inactive	N/A	N/A	N/A	N/A
27	Non-responder	Neg	High	Low	Negative	N/A	Active	Active	Inactive	Inactive	N/A	N/A	N/A	N/A
76	Non-responder	Neg	Low	High	Negative	N/A	Active	Active	Active	Active	N/A	N/A	N/A	N/A
81	Non-responder	Neg	Intermediate	Intermediate	Negative	N/A	Active	Active	Inactive	Active	N/A	N/A	N/A	N/A
95	Non-responder	Neg	Low	Intermediate	Negative	N/A	Active	Active	Active	Inactive	N/A	N/A	N/A	N/A
121	Non-responder	Neg	Low	High	Negative	N/A	Inactive	Active	Active	Active	N/A	N/A	N/A	N/A
124	Non-responder	Neg	High	Low	Negative	N/A	Inactive	Active	Active	Inactive	N/A	N/A	N/A	N/A
91	Non-responder	Neg	High	High	Negative	N/A	Active	Active	Inactive	Inactive	N/A	N/A	N/A	N/A
203	Non-responder	Neg	Low	Low	Negative	N/A	Inactive	Active	Inactive	Inactive	N/A	N/A	N/A	N/A
331	Non-responder	Neg	Low	High	Negative	N/A	Inactive	Active	Active	Inactive	N/A	N/A	N/A	N/A
340	Non-responder	Neg	Low	Low	Negative	N/A	Inactive	Inactive	Inactive	Inactive	N/A	N/A	N/A	N/A
150	Non-responder	Pos	Intermediate	High	3+	3+	Active	Active	Active	Active	N/A	Int	N/A	Int
179	Non-responder	Pos	Low	High	3+	3+	Active	Active	Active	Active	N/A	Low	N/A	Low
332	Non-responder	Pos	Intermediate	Intermediate	3+	3+	Active	Active	Inactive	Inactive	N/A	Low	N/A	N/A
367	Non-responder	Pos	Intermediate	Intermediate	2+ (FISH+)	2+	Inactive	Inactive	Active	Inactive	N/A	Neg	N/A	Int
288	Non-responder	Pos	High	High	2+ (FISH+)	2+	Active	Active	Active	Inactive	N/A	Int	High	High
84	Non-responder	Pos	Intermediate	High	3+	3+	Active	Active	Active	Active	High	High	Int	High
109	Non-responder	Pos	High	Low	3+	3+	Inactive	Active	Active	Inactive	Low	High	Int	Int
346	Non-responder	Pos	High	High	3+	3+	Active	Active	Active	Inactive	N/A	Int	N/A	Low
300	Non-responder	Pos	Low	Intermediate	3+	3+	Active	Inactive	Active	Inactive	N/A	High	N/A	High
334	Responder	Pos	Intermediate	Low	3+	2+	Inactive	Inactive	Inactive	Inactive	N/A	Int	N/A	Low
35	Responder	Pos	Intermediate	Low	3+	3+	Inactive	Inactive	Active	Active	High	High	High	High
311	Responder	Pos	Low	Intermediate	2+ (FISH+)	2+	Inactive	Inactive	Inactive	Inactive	Low	N/A	Int	N/A
369	Responder	Pos	High	Intermediate	2+ (FISH+)	2+	Active	Inactive	Inactive	Inactive	N/A	Neg	N/A	N/A
333	Responder	Pos	High	Intermediate	3+	3+	Active	Inactive	Inactive	Inactive	N/A	Neg	N/A	Low
323	Responder	Pos	Low	Low	3+	3+	Inactive	Inactive	Inactive	Active	N/A	High	N/A	High
181	Responder	Pos	Low	High	3+	Negative	Inactive	Inactive	Active	Inactive	High	N/A	Int	Low
34	Responder	Pos	High	Low	3+	N/A	Inactive	Inactive	Inactive	Inactive	N/A	N/A	High	N/A

Table 4.1: Summary: Proliferation genes; MAPK signalling (IHC and gene level); PI3K signalling genes.

4.18 Signalling pathways in ER+/ HER2 Negative tumours, Non Responders

Microarray data was available for 11 ER+/ HER2- non responding tumours in patients who received 3 months of neoadjuvant letrozole. These tumour samples were investigated for proliferation gene activity (*Figure 4.18A*), MAPK gene activity (*Figure 4.18B*), and PI3K signalling activity (*Figure 4.18C*).

Proliferation in ER+/ HER2- Non Responders

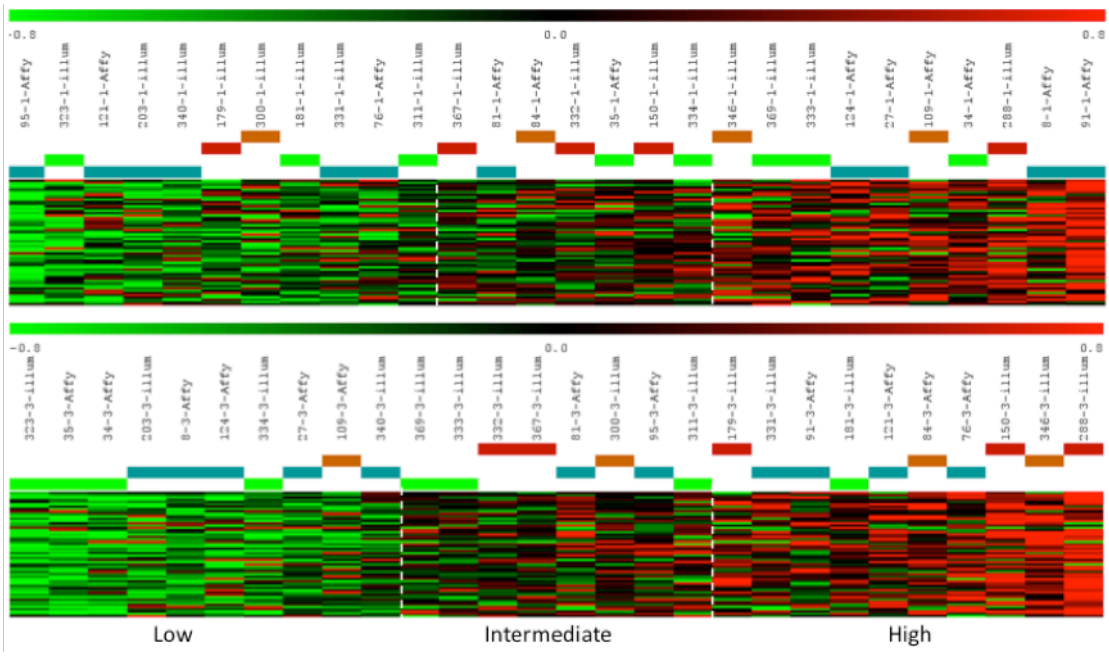


Figure 4.18A: Heatmap representing the same proliferation genes to ER+/ HER2+ NR (red),;P (orange); R (green) and ER+/ HER2- NR (blue) at baseline and 3 month timepoints.

When the same set of proliferation genes (*Appendix 4*) was applied to the ER+/ HER2- non responders (*Figure 4.18A*), most of these tumours had a low expression of proliferation genes at baseline and after 3 months neoadjuvant letrozole therapy.

MAPK Signalling in ER+/ HER2- Non Responders

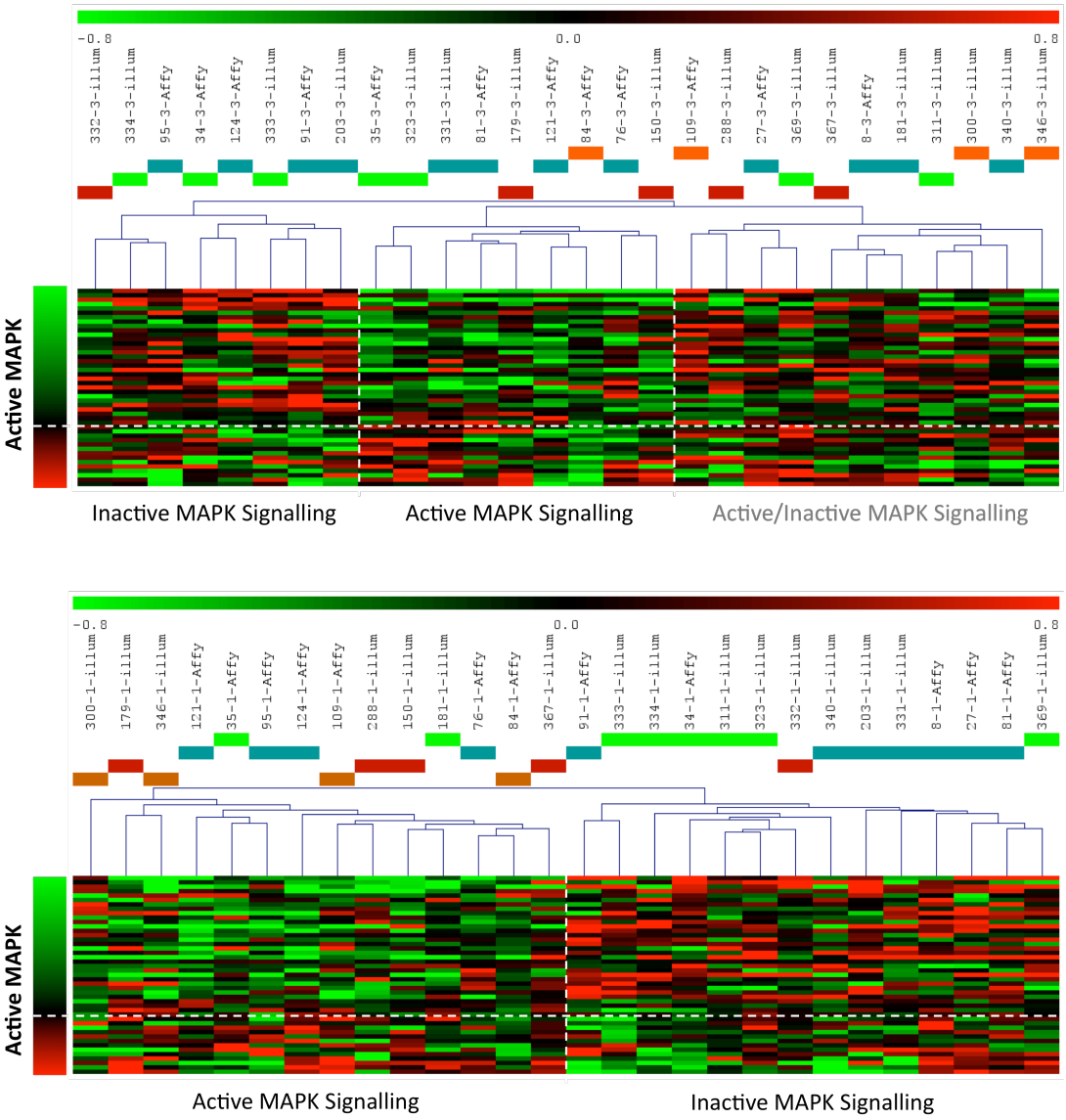


Figure 4.18B: Heatmap showing expression of up regulated and down regulated genes indicating active or inactive MAPK signalling, for all ER+/ HER2+ samples (NR, P, R) and ER+/ HER2- NR at pre-treatment and 3 month intervals. Colour bar at left indicates expression profile associated with activation of MAPK signalling.

When the same set of MAPK activity genes (*Appendix 6*) was applied to ER+/ HER2- Non Responders at baseline and at 3 months there was a mixed picture with 4/9 of the ER+/ HER2- NR tumours showing evidence of active MAPK signalling at baseline (*Figure 4.18B)*. 2 of these tumours (samples 76 and 121) continued to show active MAPK signalling after 3

months treatment, whilst 2 tumours which had inactive signalling at baseline developed evidence of MAPK signalling at 3 months (samples 81 and 331). Whilst there is demonstration of active MAPK signalling in the ER+/ HER2- non responding tumour at both the pre-treatment and 3 month timepoints, the clustering is much less consistent than that seen with ER+/ HER2+ Responding and Non Responding tumours.

PI3K Signalling in ER+/ HER2- Non Responders

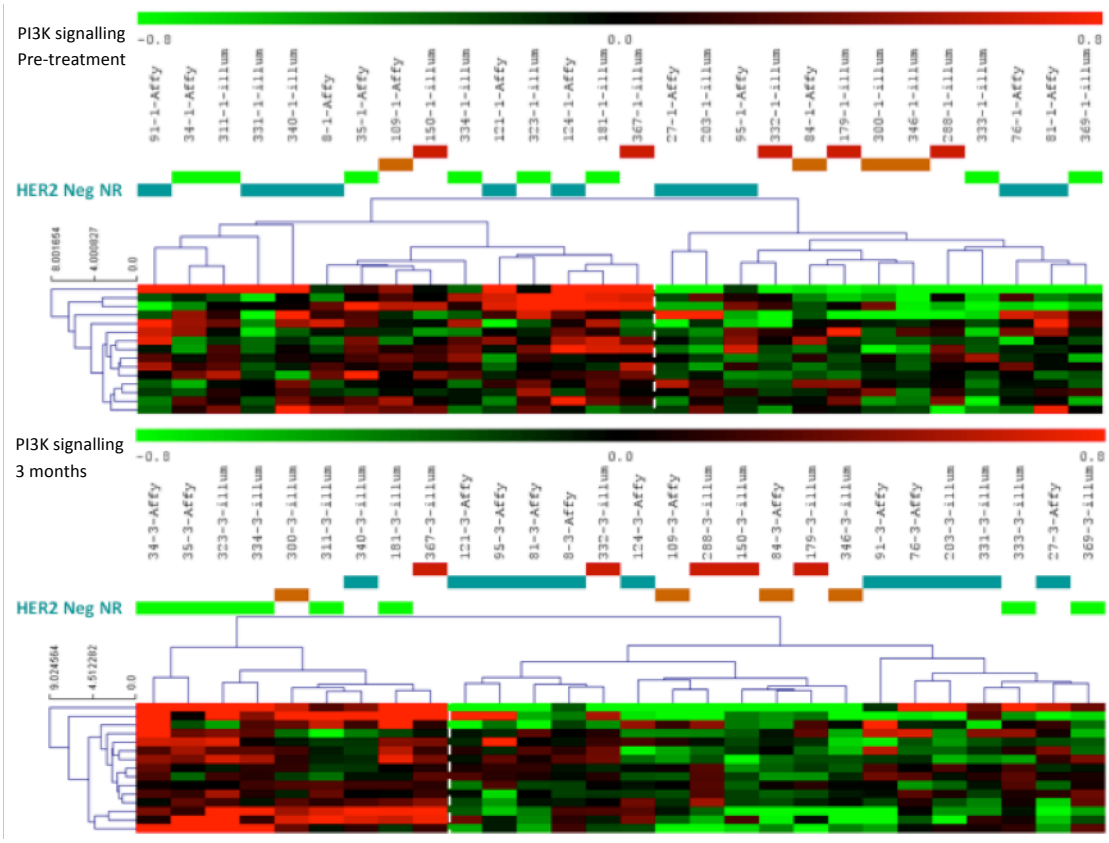


Figure 4.18C: Heatmap of 'Cluster2' genes at pre-treatment and 3 month timepoints for ER+/ HER2+ NR (red), P (orange), R (green) and ER+/ HER2- NR (blue).

Using the same PI3K signalling genes (*Appendix 7*) it can be seen at the pre-treatment level that many of the ER+/ HER2- Non Responders do not show evidence of active PI3K signalling. After 3 months neoadjuvant letrozole, the majority of ER+/ HER2- NR do show evidence of active PI3K signalling.

Whilst there are similar changes in these PI3K signalling genes in both the HER2 positive and HER2 negative Non Responding groups after 3 months neoadjuvant letrozole, at the pre-treatment level the majority of the HER2- tumours do not exhibit PI3K signalling, whereas

the majority of the HER2+ at this pre-treatment level do already show evidence of PI3K signalling. This suggests that there is a difference in PI3K signalling between the ER+/ HER2+ and the ER2+/ HER2- subgroups, which could be determined before treatment with endocrine therapy begins.

4.19 UK wide survey of HER2 testing and HER2 targeted therapies

As part of the work for this thesis a survey of UK surgeons was performed. The survey found that some UK centres do not test for HER2 on initial diagnostic core biopsies. The resulting delay in HER2 positive diagnosis potentially denies patients access to neoadjuvant anti-HER2 therapy. UK surgeons reported that in most units less than 75% of all HER2 results were available for the multidisciplinary meeting after surgery when decisions were made on adjuvant treatment. This figure is remarkably similar to results from a UK audit in 2008. Draft quality assurance guidelines have set a figure of 85% of HER2 test results being available for the preoperative multidisciplinary meeting, but few units currently meet this target. If we fail to achieve this target for HER2 testing it makes if impossible to deliver on the wider range of prognostic and predictive factors that are now in development.

Given the importance of HER2 as a prognostic and treatment related factor, almost all invasive cancers are now tested for the presence of HER2 over-expression. Treatments directed against HER2 have substantially improved the outcome for HER2 positive patients with breast cancer. With this in mind, as part of this study a survey of breast surgeons across the United Kingdom was conducted to ascertain how many breast cancer units were testing their patients for HER2, and how efficient this testing was.

Survey responses were returned from 187 centres across the UK. The survey revealed that only half of patients with invasive cancer had a HER2 result available when treatment was initially discussed. The survey found that some UK centres (12%) did not test for HER2 on initial diagnostic core biopsies. Overall, less than 75% of HER2 results were available for the multidisciplinary team (MDT) meeting, when decisions were made on adjuvant treatment. Draft quality assurance guidelines have set a figure of 85% of HER2 test results being available for the preoperative MDT. Few units currently meet this target. Full results of the survey are shown in *Figure 4.4* and the questionnaire that was sent to surgeons can be seen in *Appendix 9*. The survey results were published in the *British Medical Journal*.[209]

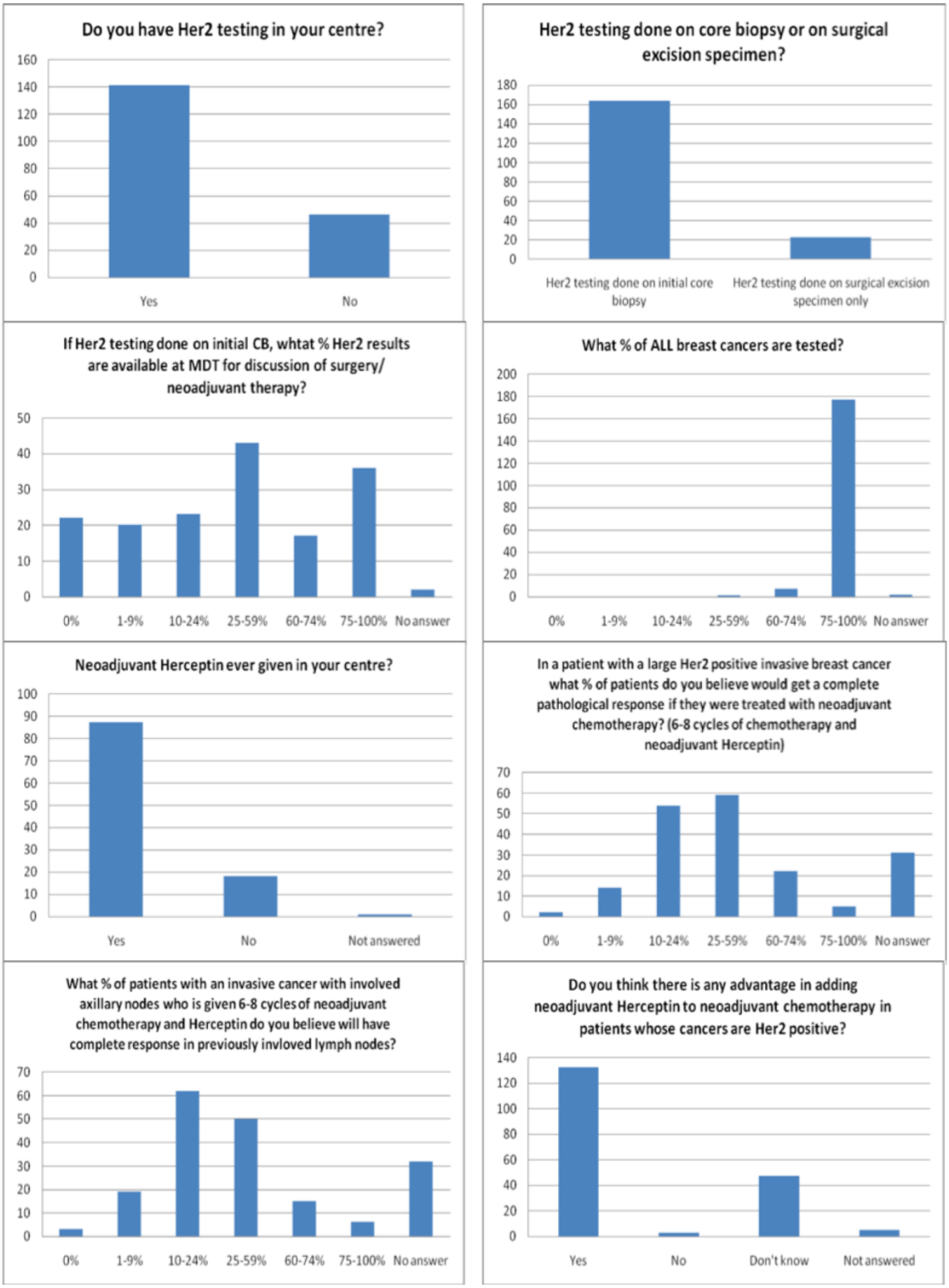


Figure 4.19: Results from 187 UK breast cancer surgeons following a survey on current HER2 testing and treatment practices.

5. Discussion

The ER positive/ HER2 positive subtype accounts for up to 10% of all breast cancers and is an important subtype as these cancers have a worse prognosis than ER+/ HER2 negative breast cancers. This study uses the largest dataset of ER+/ HER2+ matched breast cancer samples before and during therapy to assess the effects of neoadjuvant letrozole on tumours.

Whist there is now considerable preclinical and clinical evidence that ER+/ HER2+ tumours exhibit intrinsic and acquired resistance to endocrine therapy, it remains unclear what is driving this resistance to therapy. Clearly this is a pressing clinical issue as endocrine therapy remains at the forefront of systemic treatment for all women with ER positive disease. However, many patients with ER+/ HER2+ disease do respond well to endocrine therapy. The challenge therefore, is in identifying, early in the process of treatment decision making, who will respond to endocrine therapy, and who might benefit from combined endocrine and HER2 targeted agents.

Over recent years a variety of targeted therapies have evolved which inhibit specific targets and pathways that play key roles in tumour growth and progression. Despite these new developments, endocrine therapies that block ER activity and signalling, remain at the forefront of systemic treatment in women with ER positive disease. Optimal systemic management of breast cancer requires consideration of clinical, pathological and biological parameters. The development of high throughput global gene expression profiling has come into use as a way of defining, at the molecular level, the phenotypes of many kinds of tumours. In 2000 Perou et al described the molecular heterogeneity of breast cancer, as determined by microarray gene expression profiling and defined the ‘intrinsic‘ molecular subtypes of breast cancer, namely luminal A (ER+/ HER2-/ low proliferation), luminal B (ER+/ HER2-/ high proliferation), HER2-like and basal-like (ER-/ HER2-).[78] These subsets were defined mostly by known prognostic parameters (estrogen receptor, HER2 and proliferation) and they exhibit differences in prognosis and treatment sensitivity. There are now several commercially available gene expression assays which are designed to aid the clinician to objectively estimate patient outcome, and to estimate the benefits from systemic adjuvant endocrine therapy and chemotherapy. The available molecular assays have not yet conclusively identified a good prognostic subset of HER2 positive breast cancers that require less aggressive systemic therapy.

Whilst the ER negative/ HER2+ subtype of breast cancers is a relatively well characterised group in terms of tumour biology and behaviour, the ER+/ HER2 + subtype is less so. HER2 positive cancers account for 15-20% of all breast cancers and 50% of these are ER positive.

It has been unclear as to whether ER signalling or HER2 signalling drives resistance to endocrine therapy in the ER+/HER2+ subset.

In postmenopausal women with large, locally advanced, estrogen receptor rich breast cancers, neoadjuvant endocrine therapy is an appealing option as many older women may not be able to tolerate the toxicities of chemotherapy. In these women, neoadjuvant endocrine therapy can be used effectively to shrink tumours, rendering them amenable to breast conserving surgery, as opposed to mastectomy. In tumours suitable for breast conserving surgery, neoadjuvant endocrine therapy can shrink the tumour so that a smaller surgical resection is required, allowing for improved cosmetic outcome. Furthermore, patients with locally advanced breast cancer have a poorer prognosis than patients with early breast cancer, and whilst neoadjuvant treatment was initially dominated by chemotherapy, studies have shown response rates to neoadjuvant treatment with aromatase inhibitors in selected hormone receptor positive patients is comparable to that seen with neoadjuvant chemotherapy.[177] Women with locally advanced breast cancers may also benefit from endocrine therapy in terms of survival, and it allows women to avoid the more unpleasant toxicities associated with chemotherapy. This is all the more important as hormone receptor positive tumours have repeatedly been shown to have lower response rates to neoadjuvant chemotherapy than hormone receptor negative tumours, in terms of pathological complete response.[247,248,249] pCR rates of less than 15% have been reported with neoadjuvant anthracycline-taxane therapy in luminal-type breast cancer.[238]

Whilst systemic treatment of HER2 positive disease has been dominated by chemotherapeutic agents, there is a need to explore whether the combination of HER2 targeted and endocrine therapy in this group of ER+/HER+ patients might firstly tackle the problem of endocrine resistance in these patients, and secondly, whether some of these patients might be treated safely and effectively with this combined therapy, and might avoid the need to use chemotherapy in these women. Given the clear importance of HER2 as a prognostic and treatment related factor, it was felt that as part of this study it would be useful to explore the current practices of HER2 testing conducted by breast surgeons across the UK. Indeed, it was a concerning finding that only half of patients with invasive cancer had HER2 test results available when their treatment plans were initially discussed. This would mean that in the other half of patients who did not have HER2 results available, neoadjuvant HER2 directed therapy could not even be considered.

The neoadjuvant setting allows prompt testing and evaluation of therapies and provides reliable results to inform and direct the design of adjuvant trials. The POETIC trial is a phase

III, multicentre trial for postmenopausal women with ER/ PR positive invasive breast cancer to determine whether 2 weeks perioperative aromatase inhibitor (AI) therapy before and after surgery improves outcome compared with standard adjuvant alone. It is the UK's largest perioperative trial, with recruitement of 4,486 patients from 130 UK sites over a 5.5 year period. Whilst results showed no evidence of improved clinical outcome with perioperative aromatase inhibitor therapy, the study did provide evidence that measuring Ki67 levels at baseline and at 2 weeks of therapy offered significant and independent prognostic information. For patients with high baseline Ki67 values (10% or greater) at both baseline and after 2 weeks of perioperative AI therapy, the 5 year absolute risk for recurrence was 19.6% compared with 8.9% for those with high baseline but low 2-week Ki67 levels, and 4.5% for for those with low levels at both time points.

It is clear that this neoadjuvant approach is becoming increasingly important in the investigation of endocrine resistance, and has been an effective way of looking at the ER+/ HER2+ group of breast cancers in this study.

The objectives of this study were 3 fold:

1. To investigate which ER+/ HER2+ breast cancers respond to letrozole.
2. To compare the mechanisms of resistance to endocrine therapy in ER+/ HER2+ and ER+ / HER2- breast cancers.
3. To determine which cancers should be considered for combined endocrine and anti-HER2 treatment.

While HER2 undeniably plays an important role in resistance to endocrine therapy, half of the ER+/ HER2+ patients in this dataset did respond well to neoadjuvant letrozole. There are now several commercially available gene-expression assays that are designed to aid the clinician to objectively estimate patient outcome and to estimate response to systemic therapy. The available molecular assays have not yet conclusively identified a good prognostic subset of HER2+ breast cancers that might require less aggressive systemic therapy.

ER+/ HER2+ Responding tumours behave more like ER+/ HER2- tumours

A group of ER+/ HER2+ endocrine sensitive cancers has been identified, which appear to have a less aggressive phenotype and behave biologically more like ER+/ HER2- tumours. These ER+/ HER2+ tumours had a clinical response to neoadjuvant letrozole, equal to that of the ER+/ HER2- endocrine sensitive tumours. These tumours demonstrate their sensitivity to

neoadjuvant letrozole with an overall down regulation of expression of proliferation associated genes *(Figure 4.11)*. Furthermore, the ER+/ HER2+ responding tumours did not demonstrate evidence of MAPK or PI3K signalling at the pre-treatment level.

It is likely that in these ER+/ HER2+ endocrine sensitive tumours, estrogen rather than HER2 is driving the growth of these tumours. This finding complements results from 2 previous phase III adjuvant trials performed in the metastatic setting. In the randomised Phase III TAn-DEM trial of patients with ER+/ HER2+ metastatic breast cancer, there was a doubling of progression-free survival time with the addition of trastuzumab to anastrozole alone.[297] Johnston *el al.* also showed a significantly increased PFS for a combination of letrozole plus lapatinib compared with letrozole alone.[298] Interestingly, in the aromatase inhibitor only arm in both of these trials approximately 20% of ER+/ HER2+ metastatic breast cancer patients had no progression at 1 year. These tumours may represent a good prognostic subset of HER2+ breast cancers that might require less aggressive systemic therapy. The challenge remains in accurately selecting the ER+/ HER2+ patients who are resistant to endocrine therapy and who are likely to benefit from combined endocrine and HER2 targeted therapy.

Expression of *ERBB2* in Responding and Non Responding tumours

Despite all ER+/ HER2+ tumours being IHC 3+ or IHC 2+ FISH positive, the level of expression of the *ERBB2* gene was significantly higher in the ER+/ HER2+ non responding group responding ER+/ HER2+ group (p=0.005, *Figures 4.2* and *4.12*).

At the pretreatment level *ERBB2* expression was the same in both the ER+/ HER2- responding and non responding groups. However, in the ER+/ HER2- non responding tumours there was an increased expression of *ERBB2* during the 3 month neoadjuvant treatment period *(Figure 4.3)*. This suggests that even in HER2- tumours HER2 signalling could play a role in endocrine therapy resistance.

ER+/ HER2+ Non Responding tumours demonstrated less estrogen signalling activity than ER+/ HER2+ Responding tumours

Whilst all tumours involved in this study were estrogen rich (Allred score ≥6), the expression level of the *ESR1* gene was significantly lower in the ER+/ HER2+ non responding group than in the ER+/ HER2+ responding group *(Figures 4.4A* and *4.12)* .

It has been suggested that conventional quantification of nuclear ER IHC staining is not sufficiently specific in detecting functional ER pathway activity. According to pathology guidelines, ER activity in a breast cancer tissue sample is inferred from the presence of positive ER staining, with a minimum of 1% of ER positive tumour nuclei as a threshold level. To quantify a measure of ER pathway activation, one group has created an ER pathway gene model.[310] Functional activity of the ER signalling pathway was determined by mRNA expression data of ER transcriptional target genes, which were interpreted in a weighted manner, to calculate a probability of pathway activation. Using this ER pathway gene model it can be seen that the ER+/ HER2+ responding tumours exhibit higher ER signalling activity than the non responding tumours at the pre-treatment level *(Figure 4.12)*.

Studies have shown that expression of a transcription factor known as PAX2 is associated with better sensitivity to endocrine therapy due to its role in the ER mediated repression of HER2. One study has reported that the response of cells to tamoxifen is regulated by competition between the ER coactivator AIB1 and PAX2 binding to the *cis*-regulatory elements in intron 4 of HER2. They showed that a decrease in expression of PAX2 in tamoxifen resistant cells correlated with an increase in HER2 expression.[353] Furthermore, IHC staining of tamoxifen treated, ER positive breast cancers showed that PAX2 expression in the abscence of AIB1 correlated with recurrence free survival and a low rate of HER2 expresion.[354] Expression of *PAX2* and *AIB1* was measured in ER+/ HER2+ responding and non responding tumours at baseline *(Figure 4.12)*. It can be seen that there was a higher expression of *PAX2* in responding tumours than non responding tumours, in keeping with previous findings that PAX2 is associated with better sensitivity to endocrine therapy. The level of expression of *AIB1* was not different between the responding and non responding groups, a result which is difficult to intreprit in this small sample size.

Immune response and immune cell signatures

Previous studies in patients receiving neoadjuvant aromatase inhibitor treatment, have shown suppressed expression of genes associated with proliferation an estrogenic signalling, and an increased expression of genes involved with stomal remodelling, cell adhesion, and immune response.[354,355,356] In this study, tumours that had a good clinical response to neoadjuvant letrozole, showed significant up regulation of genes involved in immune response *(Figures 4.6A & B)*. This was apparent in both ER+/ HER2+ responding tumours and ER+/ HER2- responding tumours, once again providing evidence that this group of endocrine sensitive ER+/ HER2+ tumours appear to behave more like ER+/ HER2- tumours.

The ER+/ HER2- tumours that were resistant to endocrine therapy did show similar changes in up regulation of stromal/ immune response genes and down regulation of proliferation genes, although not to the same extent and it is this that is likely to account for the endocrine resistance seen in these tumours. Indeed, it may be that with sustained endocrine therapy that these tumours would have shown improved clinical response.

The clinical relevance of the immune system in cancer has been demonstrated by the growing field of immune therapies, where they have made a significant impact in the fields of lung cancer and melanoma.[312,313] However, despite the positive response to immune therapy experienced by a subset of patients, the identification of biomarkers to determine which patients will benefit from these agents remains a challenge. Using immune cell gene expression signatures,[308] pre-treatment ER+/ HER2+ samples were investigated to determine whether the responding tumours were more like a particular individual immune cell type than the ER+/ HER2+ non responding tumours. Interpretation of the results was mixed, but many of the ER+/ HER2+ responding tumours had a stronger 'All T-cell gene signature' at baseline *Figure 4.8A)*. Analyses were also performed for CD8+ T-cells, T regulatory cells, B-cells, and Dendritic cell gene signatures there did not appear to be any consistent separation between the ER+/ HER2+ responding and non responding tumours.

Estrogen response is associated with reduced proliferation

Using a published proliferation gene set,[309] non response to 3 months of neoadjuvant letrozole therapy in ER+/ HER2+ tumours was associated with an up regulation of proliferation genes, whilst there was a down regulation of proliferation genes in the ER+/ HER2+ responders *(Figure 4.11)*. This group of ER+/ HER2+ endocrine resistant tumours is likely to be driven by active HER2 signalling and would benefit from the addition of an anti-HER2 targeted agent.

Analysis of the ER+/ HER2- endocrine sensitive tumours showed a clear association between good response and up regulation of stromal and immune response genes, and down regulation of proliferation genes. During the first 14 days of neoadjuvant letrozole there was a significant down regulation of genes known to be involved in the cell cycle (eg AURKA and ASPM, *Figure 4.6A*). Interestingly, a very similar molecular response was observed in the ER+/ HER2+ responding tumours, with up-regulation of stromal and immune response genes and down regulation of proliferation genes. Thus providing more evidence that there is a less

aggressive group of ER+/ HER2+ tumours that behave biologically more like ER+/ HER2- tumours.

HER2 and the Mitogen-Activated Protein Kinase (MAPK) and Phosphoinositide 3-Kinase (PI3K) signalling pathways

Overexpression of the HER2 receptor via amplification of the *ERBB2* gene, results in ligand independent homo-dimerization and constitutive signalling primarily through the phosphoinositide 3-kinase (PI3K) cascade.[314 315] Mutational activation of the PI3K pathway is known to mediate resistance to HER2 targeted therapies in both pre-clinical models and through retrospective analysis of clinical data.[316] Consequently, many small molecules targeting components of the PI3K cascades, including PI3K, AKT, and mTOR inhibitors, are currently undergoing clinical trials in combination with HER2 therapy.[317]

The mitogen activated protein kinase (MAPK) signalling cascade is another pathway hyper-activated in a large number of cancers. Whilst the pathway is not known to play a critical role in HER2 amplified cancers, the dual inhibition of PI3K and MAPK cascades can result in synergistic effects on cell proliferation and apoptosis in multiple cancer models,[318] including HER2 positive breast cancer.[319, 320] This suggests a potential role of MAPK signalling in the growth and survival of HER2+ cancers.

It was hypothesised that both the PI3K and MAPK signalling pathways can potentiate HER2 activity and that together these signalling pathways can cause resistance to endocrine therapy

in ER+/ HER2+ breast cancers.

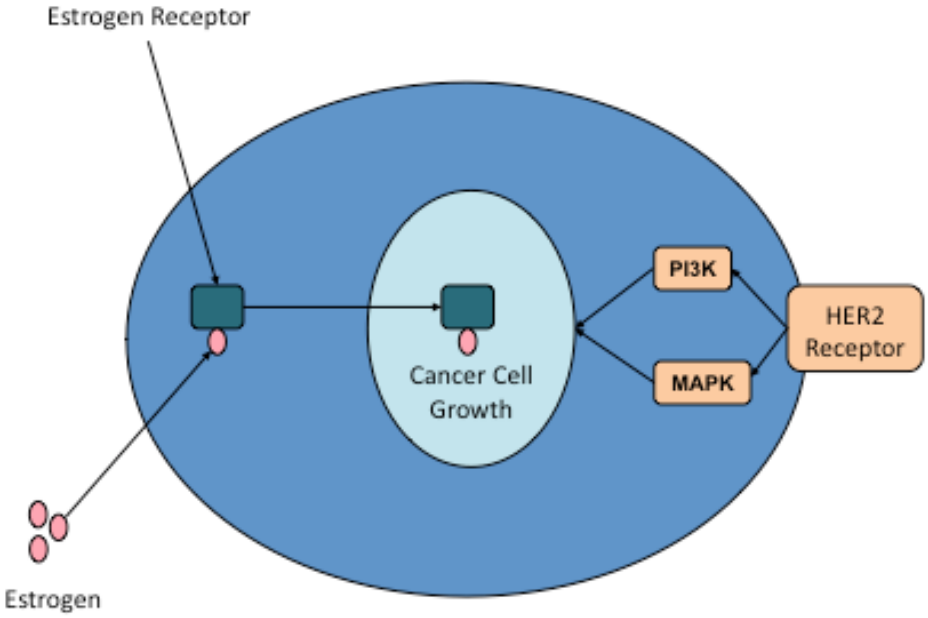


Figure 5.1: HER2 activity depends on 2 oncogenic signalling pathways, PI3K and MAPK and together these signalling pathways cause endocrine resistance in ER+/ HER2+ breast cancers.

MAPK signalling and endocrine resistance

The activation of mitogen-activated protein kinase (MAPK, originally called ERK, extracellular signal-regulated kinase) in breast cancer is associated with ER negative breast cancer, and it is proposed that this MAPK up regulation is associated with estrogen withdrawal.[321] Creighton et al hypothesized that aberrant growth factor signalling resulting in hyperactivation of MAPK would induce a gene expression profile reflective of the hyperactive MAPK and that this would affect breast cancer behaviour.[289] In their study, they attempted to define a 'hyperactive MAPK signature' set of genes whose expression is altered in breast cancer cell lines as well as tumours with high MAPK activity. Using ERα MCF-7 cells engineered for over expression of different growth factor signalling pathways, they developed models to mimic the MAPK hyperactivation seen in ERα negative breast cancers. They then examined gene expression patterns of ERα positive tumour profiles and found a subset of ERα positive, MAPK positive tumour profiles which had significant similarities to the ERBB2 specific mRNA signature.

When this 44 gene signature was applied to the ER+/ HER2+ breast cancers, it could be seen at the pre-treatment level that the majority of ER+/ HER2+ responding tumours did not have evidence of MAPK signalling. At the pre-treatment level, the majority (8/9 ER+/ HER2+ NR samples) of the ER+/ HER2+ non responding tumour samples did show evidence of active MAPK signalling *(Figure 4.12)*. This findings support the hypothesis that active MAPK signalling plays an important role in endocrine resistance in ER+/ HER2+ breast cancer. This finding is clinically important as these ER+/ HER2+ cancers will derive no benefit from letrozole and would benefit instead from a HER2 and/ or a MAPK targeted agent.

PI3K Signalling and endocrine resistance

The phosphatidylinositol 3-kinase (PI3K) pathway is on of the main cellular growth factor signalling pathways, frequently hyperactivated in cancer.[322] This signalling pathway has been associated with resistance to endocrine therapy, HER2 directed therapy and cytotoxic therapy in breast cancer.[323, 324, 325] The PI3K/AKT/mTOR pathway is an intracellular signalling pathway which is important in regulating the cell cycle. In many cancers, this pathway is overactive, causing reduced apoptosis and allowing proliferation.

It is reported that the *PIK3CA* gene is the second most frequently mutated oncogene, and *PTEN* is among the most frequently mutated tumour suppressor genes in cancers.[330,331] The significant role of PI3K activation in tumour cell biology has led to several efforts to target PI3K and/ or downstream kinases such as AKT and mammaian target of rapamycin (mTOR) in cancer. However, clinical data shows limited single-agent activity of inhibitors targeting PI3K, AKT or mTOR.

Using genes from the KEGG (Kyoto Encyclopedia of Genes and Genomes) database PI3K-AKT signalling pathway, gene expression levels for ER+/ HER2+ responding and non responding tumours at baseline and after months neoadjuvant letrozole were displayed on heatmaps *(Figure 4.15* and *Figure 4.16)*. There was a distinct cluster of 14 genes (*AKT3, NFKBIA, SHC1, PIK3R1, SRF, GJA1, PDGFRA, FOS, PTEN, JUN, PDPK1, FOXO1, IGF1* and *CDKN1B)* which at a low expression level would indicate active PI3K signalling.

The mechanism behind this activity is thought to be 2-fold. Firstly, there is down-regulation of *CDKN1B* which encodes for $p27^{Kip1}$, a protein which belongs to the Cip/Kip family of cylcin-dependent kinase (Cdk) inhibitor proteins, which is a potent inhibitor of cyclin-dependent kinases. $p27^{Kip1}$ binds to and prevents the activation of cyclin-CDK complex's, and thus

controls the cell cycle progression at G1. $p27^{Kip1}$ is often referred to as a cell cycle inhibitor protein because its major function is to stop or slow down the cell division cycle.

Figure 5.2: In order to enter S phase, cells must activate CDK4/6 and CDK2. These kinases are expressed throuout the cell cycle, but are only activated upon complex formation with their corresponding cyclins. During early G1 phase, mitogenic signals trigger activation of the CDK4/6-cyclin D complex, which partially deactivates Rb by phosphorylation. Cyclin A facilitates progression through S and G2 phase. Upstream inhibitors, including members of the Kip families, inhibit the mitogenic action of CDKs. Small molecule CDK4/6 inhibitors act primarily by blocking Rb phosphorylation and thus inducing G1 cell cycle arrest.

The second mechanism behind activation of PI3K signalling seen in ER+/ HER2+ non responding tumours is the low expression of *PTEN*. PTEN is a natural inhibitor of the PI3K/AKT pathway, it regulates PI3K signalling by dephosphorylating the lipid signalling intermediate PIP_3. This dephosphorylation is important because it results in inhibition of the AKT signalling pathway. PTEN is one of the most commonly lost tumour suppressors in the cancer pathway and its inactivation is thought to be responsible for a variety of human cancers.[326,327,328] A network involving the mutual dependence of PTEN and p53 has been suggested. Specifically, PTEN may protect p53 from Mdm2-mediated degradation, whereas p53 can enhance the transcription of PTEN.[329]

At both the pre-treatment and 3 month treatment timepoints the ER+/ HER2+ responding and ER+/ HER2+ non responding tumours clustered separately, indicating inactive and active PI3K signalling respectively.

Current clinical data suggests that despite selection of potentially responsive patients based on PI3K pathway mutation analysis, only a subpopulation of patients respond adequately to PI3K pathway inhibitors.[332,334] To improve prediction of response to PI3K inhibitor therapy, a test that can reliably measure functional PI3K pathway activity in cancer samples would be valuable. Forhead box-O (FOXO) transcription transcription factors are negatively regulated by the PI3K pathway and can in principle be used as an inverse correlation for PI3K pathway activity.[311,336,337] A FOXO activity model has been developed as a method for quantitative assessment of functional PI3K pathway acitivity in tissue samples, and to improve prediction of response to drugs that target the receptor tyrosine kinase/ PI3K/ AKT pathway.[311] This model of FOXO gene acitiviy was used to determine further evidence of PI3K activity in the letrozole resistant ER+/ HER2+ tumour samples. At both the pre-treatment and 3 month timepoints there was reduced expression (and inverse correlation with PI3K activity) in ER+/ HER2+ Non Responding tumours *(Figure 4.15* and *Figure 4.16)*.

The PI3K signalling pathway is active at both the pre-treatment and 3 month timepoints, providing evidence that this pathway plays an important role in endocrine resistance in ER+/ HER2+ breast cancers.

Letrozole resistance in ER+/ HER2 negative tumours

11 patients with ER+/ HER2- tumours were resistant to neoadjuvant letrozole. Unlike the ER+/ HER2+ non responding group, these tumours did not show a higher level of expression of proliferation genes at baseline, and did not have an increased expression of these genes throughout the 3 month treatment time period. There was some MAPK activity demonstrated in these tumours at both the pre-treatment and 3 month timepoints, this activity was much less evident than that seen in the ER+/ HER2+ non responding tumours. Whilst there was no PI3K signalling seen in the ER+/ HER2- non responding tumours at the pre-treatment level, most of these tumours did demonstrate active PI3K signalling after 3 months neoadjuvant endocrine therapy.

Combating Endocrine Resistance

Estrogen responsiveness may be lost by upregulating proliferation/ survival signal transduction pathways, like upstream signalling transmembrane growth factor receptors such as HER2 and downstream intracellular signalling such as the MAPK or PI3K/ AKT/ mTOR

signalling pathways. In patients with ER+/ HER2+ breast cancers, modulation of these pathways in combination with endocrine therapy, may circumvent resistance mechanisms. Multiple inhibitors of the PI3K/Akt/mTOR pathway are in preclinical development or are already in clinical trials. The mTOR inhibitor everolimus and EGFR inhibitor gefitinib can reverse the PI3/ AKT/ mTOR mediated resistance to endocrine therapy with used in combination with endocrine therapy.[337] Everolimus is now in wide use in combination with exemestane (a steroidal aromatase inihibitor) after being shown to improve PFS in second or third line treatment of patients with ER positive metastatic breast cancer.[339] Benefit has also been reported from the addition of fulvestrant (a selective estrogen receptor degrador) to everolimus in tamoxifen resistant breast cancer.[340]

In preclinical studies, the mTOR inhibitor Temsirolimus inhibited proliferation of breast cancer cell lines that were estrogen dependent and overexpressed the HER2 receptor, suggesting that temsirolimus might be a useful treatment for ER+/ HER2+ breast cancers.[341] In a randomised phase II study of postmenopausal women with locally advanced or metastatic ER positive breast cancer, who had been heavily 'pretreated', addition of temsirolimus to letrozole showed antitumour activity and a generally tolerable safety profile.[342]

Neratanib is an irreversible small-molecule tyrosine kinase inhibitor of HER1, HER2, and HER4. The US FDA approved 1 year of extended adjuvant neratinib after chemotherapy and a year of trastuzumab for HER2 positive breast cancer based on the results of the ExteNET trial.[343] In the 5 year follow up analysis, it can be seen that most benefit was found in the hormone receptor (HR) positive population. Patients with HR positive disease were treated with concurrent endocrine therapy (either tamoxifen or an aromatase inhibitor) and it has been speculated that neratinib works in part by a mechanism other than HER2 inhibition. As well as inhibition of the HER receptors, it is also known to inhibit the MAPK pathway. [344] It may be that this agent can block 'cross-talk' down-stream signalling and stimulation of growth via the estrogen receptor, and indeed may only work with concurrent endocrine therapy. Of note is that neratinib is not currently approved for use in breast cancer treatment in the UK.

The cyclin dependent kinases (CDKs) play an important role in regulating cell cycle progression. CDK4 and CDK6, activated by cyclin D, facilitate the hyperphosphorylation of retinoblastoma protein (pRb), which can lead to the cell cycle transition from G1 phase to S phase. This critical Rb checkpoint has been demonstrated to be associated with endocrine resistance in breast cancer. Recently, several high-quality clinical randomized controlled trials have identified that CDK 4/6 inhibitors have a great safety and efficacy, and can be used in

combination with letrozole or fulvestrant for women with advanced breast cancer which has progressed while receiving endocrine therapy.[345] The PALOMA-1[346] and PALOMA-2[347] trials were designed to assess the safety and efficacy of the combination of palbociclib and letrozole as a first-line therapy for postmenopausal women with ER positive/ HER2 negative, advanced breast cancer. The results showed that patients in the pablbociclib-letrozole group had a longer median PFS (>10months) than those in the letrozole only group. PALOMA-3 studied the combination of palbociclib and fulvesterant as a second line treatment for women with ER positive/ HER2 negative metastatic breast cancer and progression after prior endocrine therapy. Adding palbociclib to fulvestrant led to a significant improvement in median PFS from 3.8 months to 9.2 months. In addition to plabociclib, 2 other highly selective CDK4/6 inhibitors, ribociclib and abeciclib, are currently been in clinical development. Ribociclib has been tested as a first line therapy in the MONALEESA-2 trial, which reported that addition of this therapy to letrozole also prolonged duration of PFS.[348] The MONARCH-1 and -2 trials studied abeciclib, in MONARCH-1 this agent was given as monotherapy to women who had disease progression after endocrine therapy and chemotherapy, and in MONARCH-2 it was given in addition to fulvestrant. Results have shown significantly improved PFS (of 16.4 months) and overall response rate with abeciclib plus fulvestrant versus placebo versus fulvestrant.[349,350] Related preclinical studies have shown that HER2+ breast cancer cell lines are sensitive to CDK4/6 inhibitors, indicating that HER2+ breast cancer patients may also benefit from treatment with CDK4/6 inhibitors.[351] In contrast, one preclinical study of breast cancer cell lines, has shown there was no significant effect of CDK4/6 inhibitors on the triple negative subtype of breast cancer, suggesting that these agents play a role in HER2 signalling.[352] Whilst more clinical data is needed, this suggests a role for dual targeting of the HER2 and CDK4/6 pathways.

6. Conclusions and Future Perspectives

The results presented in this thesis suggest there are 2 distinct groups of ER+/ HER2+ cancers whose response to endocrine therapy depends on whether tumour growth is driven by estrogen or by HER2. This finding is clinically important as some ER+/ HER2+ cancers will derive no benefit from letrozole and would benefit instead from a HER2 and/ or a MAPK or PI3K targeted agent.

Many of the ER+/ HER2+ tumours which had good clinical response to endocrine therapy showed down regulation of proliferation genes and inactive MAPK and PI3K signalling pathways.

This study has shown that different signalling pathways are active and responsible for resistance to endocrine therapy in tumours that are ER+/ HER2+. Whilst it seems clear that HER2 plays an important role in resistance to endocrine therapy, many of the ER+/ HER2+ tumours were sensitive to endocrine therapy. This would suggest that HER2 positivity alone is not responsible for resistance to endocrine therapy and that this resistance relies in part on active MAPK signalling, active PI3K signalling and active HER2 signalling.

Defining which ER+/HER2+ patients will not respond to endocrine therapy is an important clinical need, as it is likely that these patients will benefit from anti HER2 therapy. Furthermore, identifying a subset of ER+/HER2+ positive cancers which have a less aggressive phenotype could mean that these patients could be treated safely and effectively with combined endocrine and HER2 targeted therapies, without the need for chemotherapy. Data from available molecular assays have so far not definitively identified such a group.

In this study of postmenopausal women with large or locally advanced ER rich/ HER2 positive patients, who have been treated with 3 months of neoadjuvant letrozole, patients could be divided in to two clear response groups. Analysis of gene expression data showed different molecular profiles between these 2 groups at baseline, and differences in gene expression changes throughout treatment, both in terms of the functional molecular pathways that change over time and activation of MAPK and PI3K signalling pathways.

Activation of both the MAPK and PI3K signalling pathways appears to be an important driver of endocrine resistance in some ER+/ HER2+ tumours. This may represent a subgroup of ER+/ HER2+ tumours that would benefit from MAPK and PI3K targeted therapies.

Whilst this study uses the largest dataset of ER+/ HER2+ matched breast cancer samples before and during therapy, the numbers are small and more are needed to consolidate the findings from this research.

More recently in the Edinburgh breast research unit an immunohistochemistry based assay 'EA2Clin', has been developed. It measures the level of pre-treatment IL6ST and on-treatment MCM4 to assess proliferation. This assay has been shown to identify responders and non responders to endocrine therapy, and to predict recurrence free survival and breast cancer specific overall survival. Preliminary data suggests that IL6ST can be used as a biomarker to identify ER+/ HER2+ breast cancers which will respond to endocrine therapy alone and those with a poor clinical response who will benefit from HER2 targeted therapies.

7. References

1. Reeves GK, Pirie K, Beral V, et al. Cancer incidence and mortality in relation to body mass index in the Million Women Study: cohort study. *BMJ*. 2007; 335(7630): 1134.

2. Parkin DM, Boyd L. Cancers attributable to overweight and obesity in the UK in 2010. *Br J Cancer.* 2011; 105(S2): S34-S37.

3. Breast Cancer (C50), Average Number of New Cases per Year and Age-Specific Incidence rates per 100, 000 population, females, UK, 2013-2015. cruk.org/cancerstats.

4. Stratton MR, Rahman N. The emerging landscape of breast cancer susceptibility. *Nature Genetics.* 2008; 40(1): 17-22.

5. Takkouche B, Regueira-Mendez C, Etminan M.Breast cancer and use of nonsteroidal anti-inflammatory drugs: a meta-analysis. *J Natl Cancer Inst*. 2008; 100:1439-47.

6. Hankinson SE, Hunter DJ. Breast Cancer. In: Adami H, Hunter D, Trichopoulos D, eds. Textbook of Cancer Epidemiology. New York. Oxford University Press; 2002:301-37.

7. Henderson BE and Feigelson HS. Hormonal carcinogenesis. *Carcinogenesis*. 2000; 22: 427–433.

8. Titus-Ernstoff L, Longnecker MP, Newcomb PA, et al. Menstrual factors in relation to breast cancer risk. *Cancer Epidemiol Biomarkers Prev*. 1998; 7: 783–789.

9. Pike MC, Henderson BE, Casagrande JT, et al. Oral contraceptive use and early abortion as risk factors for breast cancer in young women. *Br J Cancer*. 1981; 43(1): 72-76.

10. Pathak DR. Dual effect of first full term pregnancy on breast cancer risk: empirical evidence and postulated underlying biology. *Cancer Causes Control*. 2002; 13: 295–298.

11. MacMahon B, Cole P, Lin TM, et al. Age at first birth and breast cancer risk. *Bull World Health Organ*. 1970; 43: 209–221.

12. Pike MC, Krailo MD, Henderson BE, et al. Hormonal risk factors, breast tissue age and the age-incidence of breast cancer. *Nature*. 1983; 303: 767– 770.

13. Bernstein L. Epidemiology of endocrine-related risk factors for breast cancer. *J Mammary Gland Biol Neoplasia*. 2002; 7: 3–15.

14. Kelsey JL, Gammon MD,and John EM. Reproductive factors and breast cancer. *Epidemiol Rev.* 1993; 15: 36–47.

15. Kvale G and Heuch I. Menstrual factors and breast cancer risk. *Cancer*. 1988; 62; 1625–1631.

16. Tworoger SS & Hankinson SE. Prolactin and breast cancer etiology: an epidemiologic perspective. *Journal of Mammary Gland Biology and Neoplasia*. 2008; 13: 41–43.

17. Boyle P, Boniol M, Koechlin A et al. Diabetes and breast cancer risk: a meta-analysis. *Br J Cancer*. 2012; 107(9): 1608-1017.

18. Peyrat JP, Bonneterre J, Hecquet B et al. Plasma insulin-like growth facto-1 (IGF-1) concentrations in human breast cancer. *Eur J Cancer*. 1993; 29A(4): 492-497.

19. Beaber EF, Buist DSM, Barlow WE, Malone KE, Reed SD and Li CI. Recent oral contraceptive use by formulation and breast cancer risk among women 20 to 49 years of age. *Cancer Res.* 2014; 74(15): 4079-4089.

20. Chlebowski RT, Hendrix SL, Langer RD et al. Iinfluence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: The Women's Health Initiative Randomised Trial. *JAMA*. 2003: 289(24); 3243-3253.

21. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer Epidemiol Biomarkers Prev*. 2006; 15:1159-6.9

22. Tamimi RM, Byrne C, Colditz GA, et al. Endogenous hormone levels, mammographic density, and subsequent risk of breast cancer in postmenopausal women. *J Natl Cancer Inst*. 2007; 99:1178-87.

23. Luo J, Margolis KL, Wactawski-Wende J, et al.Association of active and passive smoking with risk of breast cancer among postmenopausal women: a prospective cohort study. *BMJ* 2011; 342:d1016.

24. Key TJ, Appleby PN, Reeves GK, et al. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *J Natl Cancer Inst*. 2003; 95: 1218-26.

25. Deapen D, Liu L, Perkins C, et al. Rapidly rising breast cancer incidence rates among Asian-American women. *International Journal of Cancer*. 2002; 99: 747-50.

26. Boyd NF, Stone J, Vogt KN, et al. Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature. *Br J Cancer* .2003; 89(9); 1672-85.

27. Thiebaut AC, Kipnis V, Chang SC. Dietary fat and post menopausal breast cancer in the National Institutes of Health -AARP Diet and Health Study Cohort. *J Natl Cancer Inst*. 2007; 99:451-62.

28. Bingham SA, Luben R, Welch A, et al. Are imprecise methods obscuring a relation between fat and breast cancer? *Lancet.* 2003; 362:212-14.

29. Aune D, Chan DS, Greenwood DC, et al. Dietary fiber and breast cancer risk: a systematic review and meta-analysis of prospective studies. *Ann Oncol*. 2012; 23(6):1394-402.

30. Aune D, Chan DS, Vieira AR, et al. Fruits, vegetables and breast cancer risk: a systematic review and meta-analysis of prospective studies. *Breast Cancer Res Treat*. 2012; 134(2):479-93.

31. Key J, Hodgson S, Omar RZ. Meta-analysis of studies of alcohol and breast cancer with consideration of the methodological issues. *Cancer Causes Control*. 2006; 17:759-70.

32. Parkin DM. Cancers attributable to consumption of alcohol in the UK in 2010. *Br J Cancer*. 2011; 105 (S2):S14-S18.

33. Monninkhof EM, Elias SG, Vlems FA. Physical activity and breast cancer: a systematic review. *Epidemiology*. 2007; 18(1):137-57.

34. Chan MF, Dowsett M, Folkerd E, et al. Usual physical activity and endogenous sex hormones in postmenopausal women: the European prospective investigation into cancer-norfolk population study. *Cancer Epidemiol Biomarkers Prev*. 2007; 16:900-5.

35. Parkin DM. Cancers attributable to inadequate physical exercise in the UK in 2010. *Br J Cancer*. 2011; 105 (S2): S38-S41.

36. Schernhammer ES, Ankinson SE. Urinary melatonin levels and postmenopausal breast cancer risk in the nurses' health study cohort. *Cancer Epidemiol Biomarkers Prev* 2009; 18:74-9.

37. Parkin DM. Cancers attributable to occupational exposures in the UK in 2010. *Br J Cancer*. 2011; 105 (S2): S70-S72.

38. Travis RC, Balkwill A, Fensom GK, et al. Night shift work and breast cancer incidence: Three prospective studies and meta-analysis of published studies. *J Natl Cancer Inst*. 2016: 108(12); 169-178.

39. Xue F, Michels KB. Intrauterine factors and risk of breast cancer: a systemativic review and meta-analysis of current evidence. *Lancet Oncol*. 2007; 8: 1088-100.

40. van den Brandt PA, Spiegelmann D, Yaun SS, et al. Pooled analysis of prospective cohort studies on height, weight and breast cancer risk. *Am J Epidemiol*. 2000; 152: 514-27.

41. Hankinson SE, Hunter DJ. Breast Cancer. In: Adami H, Hunter D, Trichopoulos D, eds. Textbook of Cancer Epidemiology. New York. Oxford University Press; 2002: 301-37.

42. Alm El-Din MA, Hughes KS, Finkelstein DM, et al. Breast cancer after treatments of Hodgkin's lymphoma: Risk factors that really matter. *Int J Radiat Oncol Biol Phy*. 2008; 72(5): 1291-7.

43. John EM, Phipps AI, Knight JA, et al. Medical radiation exposure and breast cancer risk: findings from the Breast Cancer Family Registry. *Int J Cancer.* 2007; 121: 386-94.

44. Parkin DM, Darby SC. Cancers attributable to ionising radiation exposure in the UK in 2010. *Br J Cancer*. 2011; 105 (S2): S57-S65.

45. Boice JD, Harvey EB, Blettner M, et al. Cancer in the contralateral breast after radoptherapy for breast cancer. *N Engl J Med*. 1992: 326; 781-785.

46. Berrington de González A. Estimates of the potential risk of radiation-related cancer from screening in the UK. *J Med Screen*. 2011;18(4): 163-164.

47. Takkouche B, Regueira-Mendez C, Etminan M. Breast cancer and use of nonsteroidal anti-inflammatory drugs: a meta-analysis. *J Natl Cancer Inst*. 2008; 100: 1439-47.

48. Hudson AG, Gierach GL, Modugno F, et al. Nonsteroidal anti-inflammatory drug use and serum total estradiol in postmenopausal women. *Cancer Epidemiol Biomarkers Prev*. 2008: 17: 680-7.

49. Largent JA, Bernstein L, Horn-Ross PL, et al. Hypertension, antihypertensive medication use, and breast cancer risk in the California Teachers Study cohort. *Cancer Causes Control*. 2010; 21(10): 1615-24.

50. Titus-Ernstoff L, Hatch EE, Hoover RN, et al.Long-term cancer risk in women given diethylstilbestrol (DES) during pregnancy. *Br J Cancer*. 2001; 84(1): 126-33.

51. Boyle P, Boniol M, Koechlin A, et al. Diabetes and breast cancer risk: a meta-analysis. *Br J Cancer.* 2012 Oct 23; 107(9): 1608-17.

52. Monami M, Dicembrini I, Mannucci E. Thiazolidinediones and cancer: results of a meta-analysis of randomized clinical trials. *Acta Diabetol*. 2013; 51(1): 91-101.

53. Hartmann LC, Sellers TA, Frost MH, et al. Benign breast disease and the risk of breast cancer. *N Engl J Med*. 2005; 353: 229-37.

54. Kerlikowske K, Molinaro A, Cha I, et al.Characteristics associated with recurrence among women with ductal carcinoma insitu treatted by lumpectomy. *J Natl Cancer Inst*. 2003; 95: 1692-702.

55. Volk N, Pompe-Kim V.Second primary cancers in breast cancer patients in Slovenia. *Cancer Causes Control*. 1997; 8: 764-70.

56. Rubino C, Arriagada R, Delaloge S, et al. Relation of risk of contralateral breast cancer to the interval since the first primary tumour. *Br J Cancer*. 2010; 102(1): 213-9.

57. Independent UK panel on breast cancer screening. The benefits and harms of breast cancer screening: an independent review. *Lancet*. 2012; 380(9855): 1778-86.

58. Thompson D & Easton DF. Cancer incidence in BRCA1 mutation carriers. *J. Natl. Cancer Inst.* 2002; 94: 1358-1365.

59. The Breast Cancer Linkage Consortium. Cancer risks in BRCA2 mutation carriers. *J. Natl. Cancer Inst.* 1999; 91: 1310-1316.

60. Anglian Breast Cancer Study Group. Prevalence and penetrance of BRCA1 and BRCA2 mutations in a population-based series of breast cancer cases. *Br. J. Cancer.* 2000; 83: 1301-1308.

61. Peto J. Prevalence of BRCA1 and BRCA2 gene mutations in patients with early-onset breast cancer. *J. Natl. Cancer Inst.* 1999; 91: 943-949.

62. Thompson D. & Easton D. The genetic epidemiology of breast cancer genes. *J. Mammary Gland Biol. Neoplasia*. 2004; 9: 221-236.

63. Antoniou AC & Easton D.F. Models of genetic susceptibility to breast cancer. *Oncogene.* 2006; 25: 5898-5905.

64. Easton DF, Pooley KA, Ponder BAJ. Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature.* 2007; 447: 1087-1093.

65. Hunter DJ, Kraft P, Jacobs KB et al. A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. *Nat. Genet*. 2007; 39: 870-874.

66. Stacey SN, Manolescu A, Sulem P et al. Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer. *Nat. Genet*. 2007; 39: 865-869.

67. Sainsbury JR, Anderson TJ, Morgan DA, et al. ABC of breast diseases: Breast cancer. *BMJ.* 2000; 309(6962): 1150-3.

68. Anderson, WF, Schairer C, Chen BE, et al. Epidemiology of inflammatory breast cancer (IBC). *Breast Dis*. 2005; 22: 9-23.

69. Independent UK Panel on Breast Cancer Screening. The benefits and harms of breast cancer screening: an independent review. *Lancet.* 2012; 380: 1778–86.

70. Rosen PP, Braun DW, Kinne DE. The clinical significance of pre-invasive breast cancer. *Cancer* 1980; 46: 919–25.

71. Seth A, Kitching R, Landberg G, et al. Gene expression profiling of ductal carcinomas in situ and invasive breast tumours. *Anticancer Res*. 2003; 23: 2043–51.

72. Saphner T, Tormey DC, Gray R. Annual hazard rates of recurrence for breast cancer after primary therapy. *J Clin Oncol.* 1996; 14: 2738-46.

73. Edge SB and Compton CC. The American Joint Committee on Cancer: the 7th Edition of the AJCC Cancer Staging Manual and the Future of TNM. *Ann Surg Oncol*. 2010; 17: 1471-1474.

74. Awada A, Bozovic Spasojevic I, and Chow L. New therapies in HER2-positive breast cancer: a major step towards a cure of the disease? *Cancer Treat Rev*. 2012. 38(5): 494-504.

75. Goncalves A, et al.Triple-negative breast cancer: histoclinical and molecular features, therapeutic management and perspectives. *Bull Cancer*. 2013; 100(5): 453-64.

76. Sonnenblick A, Fumagalli D, Sotiriou C, Piccart M. Is the differentiation into molecular subtypes of breast cancer important for staging, local and systemic therapy, and follow up? *Canc Treat Rev*. 2014; 40: 1089-1095.

77. Yerushalmi R, Woods R, Ravdin PM, Hayes MM, Gelmon KA. Ki67 in breast cancer: prognostic and predictive potential. *Lancet Oncol*. 2010; 11:174–183.

78. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000; 406(6797): 747-752.

79. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B & Senn HJ Panel members. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. *Annals of Oncology*. 2013; 24: 2206-2223.

80. Eroles P, Bosch A, Perez-Fidalgo JA, and Lluch A. Molecular biology in breast cancer: Intrinsic subtypes and signalling pathways. *Canc Treat Rev*. 2012; 38: 698-707.

81. Kennecke H, Yerushalmi R, Woods R, et al. Metastatic behaviour of breast cancer subtypes. *J Clin Oncol*. 2010; 28(20): 3271–7.

82. Cheang MC, Chia SK, Voduc D, et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J Natl Cancer Inst*. 2009; 101(10): 736–50.

83. Parker JS, Prat A, Cheang MCU, et al. Breast cancer molecular subtypes predict response to anthracycline/ taxane-based chemotherapy. *Cancer Res*. 2009; 69 (24 Suppl. 3).

84. Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. *Mol Oncol.*2011;5(1):5–23.

85. Bosch A, Eroles P, Zaragoza R, Vina JR, Lluch A. Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. *Cancer Treat Rev.* 2010; 36(3): 206–15.

86. Livasy CA, Karaca G, Nanda R, et al. Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. *Mod Pathol*. 2006; 19(2): 264–71.

87. Nielsen TO, Hsu FD, Jensen K, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res*. 2004; 10(16): 5367–74.

88. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA*. 2001; 98(19): 10869–74.

89. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA*. 2003; 100(14): 8418–23.

90. Dent R, Trudeau M, Pritchard KI, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*. 2007; 13(15): 4429–34.

91. Weigelt B, Mackay A, A'Hern R, et al. Breast cancer molecular profiling with single sample predictors: a retrospective analysis. *Lancet Oncol*. 2010;11(4): 339–49.

92. Parker JS, Prat A, Mullins M, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. *J Clin Oncol*. 2009;27(8):1160–7.

93. Prat A, Parker JS, Karginova O, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res*. 2010; 12(5): R68.

94. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, and Senn HJ Panel members. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of

the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer. *Annals of Oncology*. 2011; 22(8): 1736-47.

95. Goscin CP, Berman CG, Clark RA. Magnetic Resonance Imaging of the Breast. *Cancer Control.* 2001: 8(5).

96. Thomas, St J, Kerr GR, Jack WJL et al. histological grading of invasive breast carcinoma- a simplification of existing methods in a large conservation series with long-term follow-up. *Histopathology*. 2009; 54:724-731.

97. Ellis IO, et al. Pathological prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up. *Histopathology*. 1992; 20(6): 479-89.

98. Elston, CW and Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*. 1991; 19(5): 403-10.

99. Elston CW, Ellis IO, and Pinder SE. Pathological prognostic factors in breast cancer. *Crit Rev Oncol Hematol.* 1999; 31(3): 209-23.

100. Shah S, Chen B. Testing for HER2 in breast cancer: a continuing evolution. *Patholog Res Inst.* 2010; 2011: 903202.

101. Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch Pathol Lab Med.* 2007; 131: 18-43.

102. Press MF, Slamon DJ, Flom KJ, et al. Evaluation of HER-2/neu gene amplification and overexpresssion: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens. *J Clin Oncol*. 2002; 20: 3095-105.

103. Press MF, Bernstein L, Thomas PA, et al. HER2/neu gene amplification characterised by fluorescence in situ hybridisation: poor prognosis in node negative breast carcinomas. *J Clin Oncol.* 1997; 15: 2894-904.

104. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001; 2: 127-37.

105. Giuliano AE, Edge SB, Hortobagyi GN. Eighth edition of the AJCC cancer staging manual: breast cancer. *Ann Surg Oncol.* 2018; 25(7): 1783-1785.

106. Van 't Veer LJ, Dai H, Van't Veer MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002; 415(6871): 530-6.

107. Woodward WA, Strom EA, Tucker SL, et al. Changes in the 2003 American Joint Committee on Cancer staging for breast cancer dramatically affect stage-specific survival. *J Clin Oncol.* 2003; 21: 3244-3248.

108. Recht A, Gray R, Davidson NE, et al. Locoregional failure10 years after mastectomy and adjuvant chemotherapy with or without tamoxifen without irradiation: experience of the Eastern Cooperative Oncology Group. *J Clin Oncol*. 1999; 17: 1689-1700.

109. Guiliano AE, Kirgan DM, Guenther JM, Morton DL. Lympha- tic mapping and sentinel lymphadenectomy for breast cancer. *Ann Surg*. 1994; 220:391–401.

110. Fleissig A, Fallowfield LJ, Langridge CI, et al. Post-operative arm morbidity and quality of life:results of the ALMANAC randomised trial comparing sentinel node biopsy with standard axillary treatment in the management of patients with early breast cancer. *Breast Cancer Res Treat.* 2006; 95(3): 279-293.

111. Jemal A, Siegel R, Xu J,Ward E. Cancer statistics, 2010. *CA Cancer J Clin*. 2010; 60(5): 277-300.

112. Albertini JJ, Lyman GH, Cox C, et al. Lymphatic mapping and sentinel node biopsy in the patient with breast cancer. *JAMA*. 1996; 276: 1818–1822.

113. Veronesie U, Paganelli G, Galimberti V, et al. Sentinel-node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph-nodes. *Lancet*. 1997; 349: 1864–1867.

114. Rao, R, Euhus D, Mayo HG et al. Axillary node interventions in breast cancer: a systematic review. *JAMA*. 2013; 310(13): 1385-1394.

115. Krag DN, Anderson SJ, Julian TB, et al. Sentinel-lymph-node resection compared with conventional axillary-lymph-node dissection in clinically node-negative patients with breast cancer: overall survival findings from the NSABP B-32 randomised phase 3 trial. *Lancet Oncol.* 2010; 11(10): 927-933.

116. Naik AM, Fey J, Gemignani M, et al. The risk of axillary relapse after sentinel lymph node biopsy for breast cancer is comparable with that of axillary lymph node dissection: a follow-up study of 4008 procedures. *Ann Surg*. 2004; 240: 462-468.

117. Rutgers EJ. Sentinel node biopsy: interpretation and management of patients with immunohistochemistry-positive sentinel nodes and those with micrometastases. *J Clin Oncol.* 2008; 26: 698-702

118. Bergkvist L, de Boniface J, Jönsson PE, et al. Axillary recurrence rate after negative sentinel node biopsy in breast cancer: three-year follow-up of the Swed- ish Multicenter Cohort Study. *Ann Surg*. 2008; 247: 150-156

119. Veronesi U, Orecchia R, Zurrida S, et al. Avoiding axillary dissection in breast cancer surgery: a randomized trial to assess the role of axillary radiotherapy. *Ann Oncol.* 2005; 16: 383-388.

120. Ploeg IM, Nieweg OE, van Rijk MC, et al. Axillary recurrence after a tumour-negative sentinel node biopsy in breast cancer patients: A systematic review and meta-analysis of the literature. *Eur J Surg Oncol*. 2008; 34: 1277-1284.

121. Veronesi U, et al. Prognostic Significance of number and level of axillary node metastases in breast cancer. *Breast*. 1993; 3: 224-228.

122. Singletary SE, Allred C, Ashley P, et al. Revision of the American Joint Committee on Cancer staging system for breastcancer. *J Clin Oncol*. 2002; 20(17): 3628-36.

123. Olivotto IA, Chua B, Allen SJ,et al. Long-term survival of patients with supraclavicular metastases at diagnosis of breast cancer. *J Clin Oncol*. 2003; 21(5): 851-4.

124. Leone BA, Romera A, Rabinovich MJ, et al. Stage IV breast cancer: clinical course and survival of patients with osseous versus extraosseous metastases at initial diagnosis. The GOCS (Grupo Oncologico Cooperativo del Sur) experience. *Am J Clin Oncol*. 1988; 11(6): 618-22.

125. Silverstein MJ, Lagios MD, Recht A, et al. Image-detected breast cancer: state of the art diagnosis and treatment. *J Am Coll Surg.* 2005; 201: 586–97.

126. Anderson WF, Chen BE, Jatoi I, Rosenberg PS. Effects of estrogen receptor expression and histopathology on annual hazard rates of death from breast cancer. *Breast Cancer Res Treatment.* 2006; 100: 121-126.

127. Pan H, Gray R, Braybrooke J, et al. for the EBCTCG. 20 year risks of breast cancer recurrence after stopping endocrine therapy at 5 years. *N Engl J Med*. 2017; 377: 1836-1846.

128. Jatoi I, Anderson WF, Jeong JH, Redmond CK. Breast cancer adjuvant therapy: time to consider its time-dependent effects. *J Clin Oncol.* 2011; 29:2301-2304.

129. Hess KR, Pusztai L, Buzdar AU, Hortobagyi GN. Estrogen receptors and distinct patterns of breast cancer relapse. *Breast Cancer Res Treat.* 2003; 78: 105-118.

130. Masuda H, Zang D, Bartholomeusz C et al. Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Res Treat*. 2012; 136(2): 331-45.

131. Sjogren S, Inganas M, Lindgren A, Holmberg L, Bergh J. Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. *J Clin Oncol*. 1998; 16:462-9.

132. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overex- presses HER2. *New Engl J Med.* 2001; 344: 783-92.

133. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med*. 2005; 353: 1673- 84.

134. Verma S, Miles D, Gianni L, et al. Trastuzumab emtansine for HER2-positive advanced breast cancer. *New Engl J Med.* 2012; 367: 1783-91.

135. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. *Cancer*. 2007; 109: 1721-8.

136. O'Shaughnessy J, Osborne C, Pippen JE, et al. Iniparib plus chemotherapy in metastatic triple-negative breast cancer. *N Engl J Med*. 2011; 364: 205-14.

137. Bouzubar N, Walker KG, Griffiths K, et al. Ki67 immunostaining in primary breast cancer: pathological and clinical associations. *Br J Cancer.* 1989; 59(6): 943-7.

138. Gerdes J, Schwab U, Lemke H, et al. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer*. 1983; 31(1): 13-20.

139. Gerdes J, Lemke H, Baisch H, et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunology*. 1984; 133(4): 1710-5.

140. Spyratos F, Ferrero-Pous, Trassard M et al. Correlation between MIB-1 and other proliferation markers: clinical implications of the MIB-1 cutoff value. *Cancer*. 2002; 94(8): 2151-9.

141. Urruticoechea A, Smith IE, and Dowsett M. Proliferation marker Ki-67 in early breast cancer. *J Clin Oncol*. 2005; 23(28): 7212-20.

142. Van Diest PJ, Van der Wall E, and Baak JP. Prognostic value of proliferation in invasive breast cancer: a review. *J Clin Pathol*. 2004; 57(7): 675-81.

143. De Azambuja, E, Cardoso F, de Castro G et al. Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients. *Br J Cancer*. 2007; 96(10): 1504-13.

144. Goldhirsch A, Wood WC, Gelber RG et al. Meeting highlights: updated international expert consensus on the primary therapy of early breast cancer. *J Clin Oncol*. 2003. 21(17): 3357-65.

145. Polley MYC, Leung SCY, McShane LM, et al, on behalf of the International Ki67 in Breast Cancer Working Group of the Breast International Group and North American Breast Cancer Group. An international Ki67 reproducibility study. *J Natl Cancer Inst*. 2013; 105: 1897-1906.

146. Balslev I, Axelsson CK, Zedeler K, et al. The Nottingham Prognostic Index applied to 9,149 patients from the studies of the Danish Breast Cancer Cooperative Group (DBCG). *Breast Cancer Res Treat*. 1994; 32(3): 281–290.

147. D'Eredita G, Giardina C, Martellotta M, et al. Prognostic factors in breast cancer: the predictive value of the Nottingham Prognostic Index in patients with a long-term follow-up that were treated in a single institution. *Eur J Cancer*. 2001; 37(5): 591–596.

148. Goldhirsch A, Ingle JN, Gelber RD, et al. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer. *Ann Oncol*. 2009; 20(8): 1319–1329.

149. Carlson RW, Brown E, Burstein HJ, et al. NCCN Task Force Report: adjuvant therapy for breast cancer. *J Natl Compr Canc Network*. 2006; 4(Suppl 1): S1–S26.

150. Ravdin PM, Siminoff LA, Davis GJ, et al. Computer program to assist in making decisions about adjuvant therapy for women with early breast cancer. *J Clin Oncol*. 2001; 19(4): 980–991.

151. Blamey RW, Ellis IO, Pinder SE, et al. Survival of invasive breast cancer according to the Nottingham Prognostic Index in cases diagnosed in 1990-1999. *Eur J Cancer*. 2007; 43(10): 1548-55.

152. Van de Vijver MJ, He YE, Van de Vijver LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med.* 2002; 347(25): 1999-2009.

153. Mook S, Schmidt MK, Viale G, et al. The 70-gene prognosis-signature predicts disease outcome in breast cancer patients with 1–3 positive lymph nodes in an independent validation study. *Breast Cancer Res Treat*. 2009; 116: 295–302.

154. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med*. 2002; 347:1 999–2009.

155. Cardosa F, van't Veer LJ, Bogaerts J et al. 70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer. *N Engl J Med*. 2016; 375: 717-29.

156. Hudis CA, Dickler, M. Increasing Precision in Adjuvant Therapy for Breast Cancer. *N Engl J Med*. 2016; 375: 790-91.

157. Prosigna [Package Insert]. Seattle, WA: NanoString Technologies, Inc; 2013.

158. Nielsen T, Wallden B, Schaper C, et al. Analytical validation of the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay and nCounter Analysis System using formalin-fixed paraffin-embedded breast tumor specimens. *BMC Cancer*. 2014; 14: 177-191.

159. Cuzick J, Dowsett M, Pineda S, et al. Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the genomic health recurrence score in early breast cancer. *J Clin Oncol*. 2011; 29: 4273–8.

160. Sparano JA, Gray RJ, Makower DF, et al. Adjuvant chemotherapy guided by a 21-gene expression assay in breast cancer. *N Engl J Med*. 2018; 379; 111-121.

161. Campbell HE, Taylor MA, Harris AL, Gray AM. An investigation into the performance of the Adjuvant! Online prognostic programme in early breast cancer for a cohort of patients in the United Kingdom. *Br J Cancer*. 2009; 101:1074-1084.

162. Wishart GC, Azzato EM, Greenberg DC, et al. PREDICT: a new UK prognostic model that predicts survival following surgery for invasive breast cancer. *Breast Cancer Res*. 2010; 12(R1): 1186-1196.

163. Wishart GC, Bajdik CD, Dicks E, et al. PREDICT Plus: development and validation of a prognostic model for early breast cancer that includes HER2. *Br J Cancer*. 2012; 107(5): 800-7.

164. Wishart GC, Rakha E, Green A, et al. Inclusion of KI67 significantly improves performance of the PREDICT prognostication and prediction model for early breast cancer. *BMC Cancer*. 2014; 14: 908.

165. Blichert-Toft M, Nielsen M, During M, et al. Long-term results of breast conserving surgery vs. mastectomy for early stage invasive breast cancer: 20-year follow-up of the Danish randomized DBCG-82TM protocol. *Acta Oncol*. 2008; 47: 672–81.

166. Fisher B, Anderson S, Bryant J, et al. Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. *N Engl J Med*. 2002; 347: 1233–41.

167. van Dongen JA, Voogd AC, Fentiman IS, et al. Long-term results of a randomized trial comparing breast-conserving therapy with mastectomy: European Organization for Research and Treatment of Cancer 10801 trial. *J Natl Cancer Inst*. 2000; 92: 1143–50.

168. Jacobs, L. Positive margins: The challenge continues for breast surgeons. *Annals of Surgical Oncology*. 2009; 15(5): 1271-1272.

169. Komoike Y, Akiyama F, Iino Y, et al. Ipsilateral breast tumor recurrence (IBTR) after breast conserving treatment for early breast cancer: risk factors and impact on distant metastases. *Cancer*. 2006; 106: 35–41.

170. Nottage MK, Kopciuk KA, Tzontcheva A, et al. Analysis of incidence and prognostic factors for ipsilateral breast tumour recurrence and its impact on disease-specific survival of women with node-negative breast cancer: a prospective cohort study. *Breast Cancer Res*. 2006; 8: R44.

171. Bijker N, Peterse JL, Duchateau L, et al. Risk factors for recurrence and metastasis after breast-conserving therapy for ductal carcinoma-in-situ: analysis of European Organization for Research and Treatment of Cancer Trial 10853. *J Clin Oncol*. 2001; 19: 2263–71.

172. Singletary SE. Surgical margins in patients with early-stage breast cancer treated with breast conservation therapy. *Am J Surg. 2002*; 184(5): 383-93

173. Pleijhuis RG, Graafland M, de Vrie J, et al. Obtaining adequate surgical margins in breast conserving therapy for patients with early stage breast cancer: current modalities and future directions. *Annals of Surgical Oncology*. 2009; 16: 2717-2730.

174. Miller WR, White S, Dixon JM et al. Proliferation, steroid receptors and clinical/pathological response in breast cancer treated with letrozole. *Br J Cancer*. 2006; 94(7): 1051-6.

175. Peintinger F, Keurer HM, McGuire SC et al. Residual specimen cellularity after neoadjuvant chemotherapy for breast cancer. *Br J Surg.* 2008; 95(4): 433-7.

176. Sasano H, Suzuki T, and Moriya T. Pathology of breast cancer following neoadjuvant therapy, in Endocrine Therapy in Breast Cancer, W.R. Miller and J.M. Ingle, Editors. 2002, Marcel Dekker: New York: 213–222.

177. Semiglazov, VF, Semiglazov VV, Dashyan GA et al. Phase 2 randomized trial of primary endocrine therapy versus chemotherapy in postmenopausal patients with estrogen receptor-positive breast cancer. *Cancer*. 2007; 110(2):244-54.

178. Straver ME, Meijnen P, van Tienhoven G et al. Role of axillary clearance after a tumor-positive sentinel node in the administration of adjuvant therapy in early breast cancer. *J Clin Oncol.* 2010; 28: 731–737.

179. De Boer M, Deurzen CHN, van Dijck JAM, et al. Micrometastases or isolated tumor cells and the outcome of breast cancer. *N Engl J Med*. 2009; 361: 653–663.

180. Donker M, van Tienhoven G, Straver MG, et al. Radiotherapy or surgery of the axilla after a positive sentinel node in breast cancer (EORTC 10981-22023 AMAROS): a randomised, multicentre, open-label, phase 3 non-inferiority trial.Comparison of the sentinel node procedure between patients with multifocal and unifocal breast cancer in the EORTC 10981–22023 AMAROS trial: identification
rate and nodal outcome. *Eur. J. CanThe Lancet Oncology*cer. 20143; 15(12)49: 1303-1310.2093–2100

181. Giuliano AE, McCall B, Beitsch P, et al. Locoregional recurrence after sentinel lymph node dissectionwith or without axillary dissection in patients with sentinel lymph node metastases: The American College of Surgeons Oncology Group Z0011 Randomised Trial. Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial. *Ann Surg* JAMA. 20101; 252305: 426569–433.575

182. Improving Outcomes for Breast Cancer: Research Evidence for the Manual Update, 2002, National Institute for Clinical Evidence: York.

183. Breast Cancer Services. National Overview, 2003, NHS Quality Improvement Scotland: Edinburgh.

184. Clarke M, Collins R, Darby S, et al. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet*. 2005; 366(9503): 2087- 106.

185. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. *Lancet*. 2011; 378(9804): 1707-16.

186. James ML, Lehman M, Hider PN, et al. Fraction size in radiation treatment for breast conservation in early breast cancer. *Cochrane Database of Systematic Reviews*. 2010; Issue 11.

187. Vaidya jS jD, Tobias jS, Bulsara M, et al. Targeted intraoperative radiotherapy versus whole breast radiotherapy for breast cancer (TARGIT-A trial): an international, prospective, randomised, non-inferiority phase 3 trial. *Lancet*. 2010; 376(9735): 91-102.

188. Scottish Intercollegiate Guidelines Network. Treatment of primary breast cancer: A national clinical guideline. 2013.

189. Early Breast Cancer Trialists' Collaborative Group. Favourable and Unfavourable Effects on Longterm Survival of Radiotherapy for Early Breast Cancer; An Overview of the Randomised Trials. *Lancet*. 2000; 355(9217): 1757-70.

190. Rastogi P, Anderson SJ, Bear HD, et al. Preoperative chemotherapy: updates of National Surgical Adjuvant Breast and Bowel Project Protocols B-18 and B-27. *J Clin Oncol*. 2008; 26: 778–85.

191. Seo JH, Kim YH and Kim JS. Meta-analysis of pre-operative aromatase inhibitor versus tamoxifen in postmenopausal woman with hormone receptor-positive breast cancer. *Cancer Chemother Pharmacol*. 2009; 63(2): 261–6.

192. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100 000 women in 123 randomised trials. *Lancet*. 2012; 379: 432–44.

193. Citron ML, Berry DA, Cirrincione C, et al. Randomized trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: first report of Intergroup Trial C9741/Cancer and Leukemia Group B Trial 9741. *J Clin Oncol.* 2003; 21: 1431–39.

194. Harbeck N, Gnant M. Breast Cancer. *Lancet*. 2016; 16: 31891-31898.

195. Biganzoli L, Aapro M, Loibl S, Wildiers H, Brain E. Taxanes in the treatment of breast cancer: have we better defined their role in older patients? A position paper from a SIOG Task Force. *Cancer Treat Rev*. 2016; 43: 19–26.

196. Ando M, Yamauchi H, Aogi K, et al. Randomized phase II study of weekly paclitaxel with and without carboplatin followed by cyclophosphamide/epirubicin/5-fluorouracil as neoadjuvant chemotherapy for stage II/IIIA breast cancer without HER2 overexpression. *Breast Cancer Res Treat*. 2014; 145: 401–09.

197. von Minckwitz G, Schneeweiss A, Loibl S, et al. Neoadjuvant carboplatin in patients with triple-negative and HER2-positive early breast cancer (GeparSixto; GBG 66): a randomised phase 2 trial. *Lancet Oncol*. 2014; 15: 747–56.

198. Sikov WM, Berry DA, Perou CM, et al. Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete response rates in stage II to III triple-negative breast cancer: CALGB 40603 (Alliance). *J Clin Oncol*. 2015; 33: 13–21.

199. von Minckwitz G, Loibl S, Schneeweiss A, et al. Early survival analysis of the randomized phase II trial investigating the addition of carboplatin to neoadjuvant therapy for triple-negative and HER2-positive early breast cancer (GeparSixto). Thirty-Eighth Annual CTRC-AACR San Antonio Breast Cancer Symposium; San Antonio, TX; Dec 8–12, 2015; S2–04.

200. Sledge GW, Neuberg D, Bernardo P, et al. Phase III trial of doxorubicin, paclitaxel, and the combination of doxorubicin and paclitaxel as front-line chemotherapy for metastatic breast cancer: an intergroup trial (E1193). *J Clin Oncol*. 2003; 21: 588–92.

201. Slamon D, Eiermann W, Robert N, et al; Breast Cancer International Research Group. Adjuvant trastuzumab in HER2-positive breast cancer. *N Engl J Med*. 2011;365(14):1273–1283.

202. Goldhirsch A, Gelber RD, Piccart-Gebhart MJ, et al; Herceptin Adjuvant (HERA) Trial Study Team. 2 years versus 1 year of adjuvant trastuzumab for HER2-positive breast cancer (HERA): an open-label, randomised controlled trial. *Lancet*. 2013;382(9897):1021–1028.

203. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001; 344(11): 783–792.

204. Thery JC, Spano JP, Azria D, Raymond E, Penault Llorca F. Resistance to human epidermal growth factor receptor type 2-targeted therapies. *Eur J Cancer*. 2014; 50(5): 892–901.

205. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med*. 2005; 353: 1673-84.

206. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med*. 2005; 353: 1659-72.

207. Gianni L, Pienkowski T, Im YH, et al. Neoadjuvant pertuzumab (P) and trastuzumab (H): antitumor and safety analysis of a randomized phase II study (NeoSphere) [abstract]. San Antonio Breast Cancer Symposium. 2010;S3-2.

208. Storniolo A, Pegram MD, Overmoyer B, et al. Phase I dose escalation and pharmacokinetic study of lapatinib in combination with trastuzumab in patients with advanced ErbB2-positive breast cancer. *J Clin Oncol*. 2008; 26: 3317-23.

209. Dixon JM, Wilson VL, Verrill M, Symmans WF. HER2 testing in patients with breast cancer. *BMJ*. 2012; 344: 3958-3959.

210. Perez EA, Romond EH, Suman VJ, et al. Trastuzumab plus adjuvant chemotherapy for human epidermal growth factor receptor 2-positive breast cancer: planned joint analysis of overall survival from NSABP B-31 and NCCTG N9831. *J Clin Oncol*. 2014; 32: 3744–52.

211. Slamon DJ, Eiermann W, Robert NJ, et al, on behalf of the BCIRG-006 Investigators. Ten year follow-up of BCIRG-006 comparing doxorubicin plus cyclophosphamide followed by docetaxel (AC→T) with doxorubicin plus cyclophosphamide followed by docetaxel and trastuzumab (AC→TH) with docetaxel, carboplatin and trastuzumab (TCH) in HER2+ early breast cancer. San Antonio Breast Cancer Symposium 2015; San Antonio, TX, USA; Dec 8–12, 2015. S5–04 (abstr).

212. Piccart-Gebhart M, Holmes E, Baselga J, et al. Adjuvant lapatinib and trastuzumab for early human epidermal growth factor receptor 2-positive breast cancer: results from the randomized phase III adjuvant lapatinib and/or trastuzumab treatment optimization trial. *J Clin Oncol.* 2016; 34: 1034–42.

213. Blackwell KL, Burstein HJ, Storniolo AM, et al. Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *J Clin Oncol*. 2010; 28(7): 1124–1130.

214. Blackwell KL, Burstein HJ, Storniolo AM, et al. Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *J Clin Oncol*. 2010; 28(7):1124–1130.

215. Swain SM, Baselga J, Kim SB, et al; CLEOPATRA Study Group. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. *N Engl J Med*. 2015; 372(8): 724–734.

216. Blackwell KL, Miles D, Gianni D, et al. Primary results from EMILIA, a phase III study of trastuzumab emtansine (T-DM1) versus capecitabine (X) and lapatinib (L) in HER2-positive locally advanced or metastatic breast cancer (MBC) previously treated with trastuzumab (T) and a taxane [abstract]. J Clin Oncol. 2012; 30(suppl): LBA1.

217. Perez EA, Barrios C, Eiermann W et al. Trastuzumab Emtansine With or Without Pertuzumab Versus Trastuzumab Plus Taxane for Human Epidermal Growth Factor Receptor 2–Positive, Advanced Breast Cancer: Primary Results From the Phase III MARIANNE Study. *J Clin Oncol*. 2017; 35: 141-148.

218. de Azambuja E, Holmes AP, Piccart-Gebhart M, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol*. 2014;15(10):1137–1146.

219. Baselga J, Bradbury I, Eidtmann H, et al. NeoALTTO Study Team. Lapatinib with trastuzumab for HER2- positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet*. 2012; 379(9816): 633-640.

220. Gianni L, Pienkowski T, Im YH, et al. Efficacy and safety of neoadjuvantpertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *Lancet Oncol*. 2012; 13(1): 25-32.

221. Harbeck N, Gluz O, Christgen M et al. De-Escalation Strategies in Human Epidermal Growth Factor Receptor (HER2)–Positive Early Breast Cancer (BC): Final Analysis of the West German Study Group Adjuvant Dynamic Marker-Adjusted Personalized Therapy Trial Optimizing Risk Assessment and Therapy Response Prediction in Early BC HER2- and Hormone Receptor- Positive Phase II Randomised Trial- Efficacy, Safety and Predictive Markers for 12 weeks of Neoadjuvant Trastuzumab Emtansine With or Without Endocrine Therapy (ET) Versus Trastuzumab plus ET. *J Clin Oncol*. 2017; 35:3030-3038.

222. Budzar AU. Endocrine Therapies in Breast Cancer. New York: Oxford University Press, 2007: 29-36.

223. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty- year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst.* 2007; 99: 283-290.

224. Hammond ME, Hayes DF, Dowsett M, et al. American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J Clin Oncol*. 2010; 28: 2784-2795.

225. Cohen I, Rosen DJ, Shapira J, et al. Endometrial changes with tamoxifen: comparison between tamoxifen- treated and nontreated asymptomatic, postmenopausal breast cancer patients. *Gynecol Oncol*. 1994; 52: 185–190.

226. Cheng WF, Lin HH, Torng PL, et al. Comparison of endometrial changes among symptomatic tamoxifen-treated and nontreated premenopausal and postmenopausal breast cancer patients. *Gynecol Oncol*. 1997; 66: 233–237.

227. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet*. 2003; 361: 296–300.

228. Dowsett M, Nicholas RIM Pietras RJ. Biological characteristics of the pure antiestrogen fulvestrant: overcoming endocrine resistance. *Breast Cancer Res Treat*. 2005; 93(S1): S11-18.

229. Nelson LR, Bulun SE. Estrogen production and action. *J Am Acad Dermatol*. 2001; 45 (suppl 3): 116-124.

230. Evans CT, Ledesma DB, Schulz TZ et al. Isolation and characterization of a complementary DNA specific for human aromatase-system cytochrome P-450 mRNA. *Proc Natl Acad Sci USA*. 1986; 83: 6387–6391.

231. Lonning PE. Pharmacology of new aromatase inhibitors. *Breast*. 1996; 5: 202–208.

232. Geisler J, King N, Anker G et al. In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients. *Clin Cancer Res.* 1998; 4: 2089–2093.

233. Goss PE, Strasser K. Aromatase inhibitors in the treatment and preven- tion of breast cancer. *J Clin Oncol*. 2001; 19: 881–894.

234. Miller WR. Background and Development of Aromatase Inhibitors in Aromatase Inhibitors. F. BJA, Editor. 2008. Berghauser Verlag AG, Switzerland: Berlin, Germany.

235. Fabian CJ. The what, why and how of aromatase inhibitors: hormonal agents for treatment and prevention of breast cancer. *Int J Clin Pract*. 2007; 61(12): 2051-2063.

236. Francis PA, Pagani O, Fleming GF, et al. Tailoring adjuvant endocrine therapy for premenopausal breast cancer. *N Engl J Med*. 2018; 379: 122-137.

237. Regan MM, Pagani O, Fleming GF, et al. Adjuvant treatment of premenopausal women with endocrine-responsive early breast cancer: design of the TEXT and SOFT trials. *Breast*. 2013; 22:1094-1100.

238. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet*. 2005; 365: 1687–1717.

239. Osborne CK, Schiff R. Growth factor receptor crosstalk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer. *Breast*. 2003; 12: 362–367.

240. Davies C, Godwin J, Gray R, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet*. 2011; 378(9793): 771–84.

241. Pagani O, Regan MM, Walley BA, et al. Adjuvant exemestane with ovarian suppression in premenopausal breast cancer. *N Engl J Med*. 2014; 371(2): 107–18.

242. Davies C, Godwin J, Gray R, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 50 years after diagnosis of estrogen receptor-positive breaat cancer: ATLAS, a randomised trial. *Lancet*. 2013; 381: 805-816.

243. Goss PE, Ingle AN, Martino S, et al. Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. J *Natl Cancer Inst.* 2005; 97(17): 1262-71.

244. Goss PE, Ingle KI, Pritchard et al. Extending Aromatase-Inhibitor Adjuvant Therapy to 10 Years. *N Engl J Med*. 2016; 375: 209-219.

245. Webber V, Dixon JM. Role of endocrine therapy in ER+/ HER2+ breast cancers. *Breast Cancer Management*. 2014; 3(1): 103-111.

246. Semiglazov VF, Semiglazov V, Ivanov A, et al. The relative efficacy of neoadjuvant endocrine therapy vs chemotherapy in postimenopausal women with ER- positive breast cancer. *Journal of Clinical Oncology*. 2004; 22: 519.

247. Colleoni M, Viale G, Zahrieh D, et al. Chemotherapy is more effective in patients with breast cancer not expressing steroid hormone receptors: a study of preoperative treatment. *Clinical Cancer Research.* 2004; 10(19): 6622-6628.

248. Faneyte IF, Schrama JG, Peterse JL. Breast cancer response to neoadjuvant chemotherapy: Predictive markers and relation with outcome. *Br J Cancer*. 2003; 88: 406-412.

249. Buzdar AU, Valero V, Theriault RL, et al. Pathological complete response to chemotherapy is related to hormone receptor status. *Breast Cancer Res Treat*. 2003; 82: S69 (suppl1, abstr 302).

250. Dixon JM, Renshaw L, Bellany C, et al. Efficacy of anastrozole as neoadjuvant therapy in postmenopausal women with large operable breast cancers: reductions in tumour volume. *Breast*. 1999; 8: 215.

251. Haddad FC and Goetz MD. Landscape of neoadjuvant therapy for breast cancer. *Ann Surg Oncol*. 2015; 22: 1408-1415.

252. Yeo B, Dowsett M. Neoadjuvant endocrine therapy: Patient selection, treatment duration and surrogate endpoints. *Breast*. 2015; 24(2): S78-S83.

253. von Minckwitz G, Untch M, Blohmer JU, et al. Definition and impact of pathological complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. *J Clin Oncol*. 2012; 30: 1796-1804.

254. Dixon JM, et al. Lessons from the use of aromatase inhibitors in the neoadjuvant setting. *Endocr Relat Cancer*. 1999; 6(2): 227-30.

255. Eiermann W, Paepke S, Appfelstaedt et al. Preoperative treatment of postmenopausal breast cancer patients with letrozole: a randomized double-blind multicenter study. *Ann Oncol*. 2001; 12: 1527-1532.

256. Olson JA, Gildy BV, Unzeitig G, et al. Neoadjuvant aromatase inhibitor therapy permits breast conservation in postmenopausal women with large, estrogen receptor (ER)-rich breast cancer facing mastectomy: Results from the AmericanCollege of Surgeons Oncology Group(ACOSOG) Z1031Trial. Society of Surgical Oncology65th Annual Cancer Symposium. Abstract 3. Presented March 23, 2012.

257. Dixon JM, Love CDB, Renshaw L, et al. Lessons from the use of aromatase inhibitors in the neoadjuvant setting. *Endocrine Related Cancer*. 1999; 6: 227-230.

258. Taylor KT, Sims AH, Liang Liang, et al. Dynamic changes in gene expression in vivo predict prognosis of tamoxifen-treated patient with breast cancer. *Breast Cancer Research*. 2010; 12:R39.

259. Sims AH, Bartlett JM: Approaches towards expression profiling the response to treatment. *Breast Cancer Research*. 2008; 10:115.

260. Miller WR, Larionov AA, Renshaw L, et al. Changes in breast cancer transcriptional profiles after treatment with the aromatase inhibitor letrozole. *Pharmacogenet Genomics.* 2007; 17: 813-826.

261. Sabine VS, Sims AH, Macaskill EJ, et al. Gene expression profiling of response to mTOR inhibitor everolimus in pre-operatively treated post-menopausal women with estrogen receptor-positive breast cancer. *Breast Cancer Res Treat*. 2010; 122: 419-428.

262. Smith IE, Johnson L, Dowsett M, et al. Trial of perioperative endocrine therapy: individualizing care (POETIC). *J Clin Onc*. 2011; 2011(43): 120-123.

263. Ellis MJ. Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1 and/ or ErbB-2 positive, estrogen receptor positive primary breast cancer: evidence from a phase III randomized trial. *J Clin Oncol*. 2001; 19(18): 3808–16.

264. Smith IE, Dowsett M, Ebbs SR, et al. Neo- adjuvant treatment of postmenopausal breast cancer with anastrozole, tamoxifen, or both in combination: the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) multicenter double-blind randomized trial. *J Clin Oncol*. 2005; 23: 5108-5116.

265. Cataliotti L et al. Comparison of anastrozole versus tamoxifen as preoperative therapy in postmenopausal women with hormone receptor-positive breast cancer: the Pre-Operative "Arimidex" Compared to Tamoxifen (PROACT) trial. *Cancer*. 2006; 106(10): 2095–2103.

266. Masuda N. Neoadjuvant anastrozole versus tamoxifen in patients receiving goserelin for premenopausal breast cancer (STAGE): a double-blind, randomised phase 3 trial. *Lancet Oncol.* 2012; 13(4): 345–52.

267. Dixon JM, Renshaw L, Macaskill EJ et al. Increase in response rate by prolonged treatment with neoadjuvant letrozole. *Breast Cancer Res Treat*. 2009; 113(1): 145–51.

268. Carpenter R, Doughty JC, Cordiner C et al. Optimum duration of neoadjuvant letrozole to permit breast conserving surgery. *Breast Cancer Res Treat*. 2014; 144(3): 569–76.

269. Semiglazov VF, Semiglazov VV, Dashyan GA, et al. Phase 2 randomized trial of primary endocrine therapy versus chemotherapy in postmenopausal patients with estrogen receptor-positive breast cancer. *Cancer*. 2007; 110(2): 244–54.

270. Alba E, Calvo L, Albanell J, et al. Chemotherapy (CT) and hormone therapy (HT) as neoadjuvant treatment in luminal breast cancer patients: results from the GEICAM/2006-03, a multicenter, randomized, phase-II study. *Ann Oncol*. 2012; 23(12): 3069–74.

271. Palmieri C, Cleator S, Kilburn LS, et al. NEOCENT: a randomised feasibility and translational study comparing neoadjuvant endocrine therapy with chemotherapy in ER-rich postmenopausal primary breast cancer. *Breast Cancer Res Treat*. 2014; 148(3): 581–90.

272. Beatson GT. On the Treatment of Inoperable Cases of Carcinoma of the Mamma: Suggestions for a New Method of Treatment, with Illustrative Cases. *Lancet*. 1896; 2: 104-7.

273. Anderson E. The role of estrogen and progesterone receptors in human mammary development and tumorigenesis. *Breast Cancer Res*. 2002; 4(5): 197-201.

274. Laron Z, Pauli R, Pertzelan A. Clinical evidence on the role of estrogens in the development of the breasts. *Proc R Soc Edinburgh B1*. 1989; 95:13-22.

275. Key TJA, Pike MC. The role of estrogens and progestagens in the epidemiology and prevention of breast cancer. *Eur J Cancer Clin Oncol*. 1984, 24:29-43.

276. Humphreys R, Lydon J, O'Malley B, Rosen J. Use of the PRKO mice to study the role of progesterone in mammary gland development. *J Mammary Gland Biol Neoplasia*. 1997; 2: 343- 354.

277. Ross RK, Paganini-Hill A, Wan PC, Pike MC. Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. *J Natl Cancer Inst*. 2000; 92: 328-332.

278. Miller WR. Endocrine treatment for breast cancers: biological rationale and current progress. *J Steroid Biochem Mol Biol*. 1990; 37(4): 467-80.

279. Vegeto E, Shahbaz MM, Wen DX, et al. Human progesterone receptor A form is a cell and promoter-specific repressor of human progesterone receptor B function. *Mol Endocrinol.* 1993; 7:1244-1255.

280. Clarke R, Howell A, Potten C, Anderson E. Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res*. 1997; 57: 4987-4991.

281. Speirs V, Skliris GP, Burdall SE, Carder PJ. Distinct expression patterns of ERα and ERβ in normal human mammary gland. *J Clin Pathol*. 2002; 55: 371-374.

282. Dowsett M. Biology of Estrogen-Dependent Breast Cancer. Aromatase Inhibitors for the Treatment of Breast Cancer, ed. E. MJ. 2008, New York: CMP Healthcare Media, Oncology Publishing Group.

283. Riggins RB, Zwart A, Nehra R, Clarke R. The nuclear factor κB inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells. *Mol. Cancer Ther*. 2005; 4: 33–41.

284. Bouker KB, Skaar TC, Fernandez DR, et al. Interferon regulatory factor-1 mediates the proapoptotic but not cell cycle arrest effects of the steroidal antiestrogen ICI 182,780 (Faslodex, fulvestrant). *Cancer Res*. 2004; 64: 4030–4039.

285. Gomez BP, Riggins RB, Shajahan AN,et al. Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. *FASEB J*. 2007; 21: 4013–4027.

286. Ali S, Metzger D, Bornert JM, Chambon P. Modulation of transcriptional activation by ligand-dependent phosphorylation of the human estrogen receptor A/B region. *EMBO*. 1993; 12(3): 1153-1160.

287. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst*. 2003; 95(5): 353-361.

288. Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signalling to the estrogen receptor. *Mol Cell Biol*. 2000; 20(14): 5041-5047.

289. Creighton CJ, Hilger AM, Murthy S, et al. Activation of mitogen-activated protein kinase in estrogen receptor alpha-positive breast cancer cells in vitro induces an in vivo molecular phenotype of estrogen receptor alpha-negative human breast tumors. *Cancer Res.* 2006; 66(7): 3903-3911.

290. Xia W, Bacus S Hegde P, et al. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc Natl Acad Sci USA*. 2006: 103(20): 7795-7800.

291. Oh AS, Lorant LA, Holloway JN, et al. Hyperactivation of MAPK induces loss of ER alpha expression in breast cancer cells. *Mol Endocrinol*. 2001; 15(8): 1344-1359.

292. Creighton CJ, Massarweh S, Huang S, et al. Development of resistance to targeted therapies transforms the clinically associated molecular profile subtype of breast tumor xenografts. *Cancer Res*. 2008; 68(18): 7493-7501.

293. Benz C, Scott GK, Sarup JC, et al. Estrogen-dependent tamoxifen resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat*. 1992; 24: 85-95.

294. Nicholson RI, McClelland RA, Robertson JFR, Gee JMW. Involvement of steroid hormone and growth factor cross-talk in endocrine response in breast cancer. *Endocr Relat Cancer.* 1999; 6: 373-387.

295. Nicholson RI, Hutcheson IR, Knowlden JM, et al. Nonendocrine pathways and endocrine resistance: observations with antiestrogens and signal transduction inhibitors in combination. *Clin Cancer Res*. 2004; 10(1Pt2): 346S-54S.

296. Yarden Y. Biology of HER2 and its importance in breast cancer. *Oncology*; 2001: 61(S2): 1-13.

297. Kaufman B, Mackey JR, Clemens MR, et al. Trastuzumab Plus Anastrozole Versus Anastrozole Alone for the Treatment of Postmenopausal Women With Human Epidermal Growth Factor Receptor 2–Positive, Hormone Receptor–Positive Metastatic Breast Cancer: Results From the Randomized Phase III TAnDEM Study. *J Clin Oncol*. 2009; 27(33): 5529-5537.

298. Johnston S, Pippen J Jr, Pivot X, et al. Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. *J Clin Oncol*. 2009; 27(33): 5538-5546.

299. Huober J, Fasching PA, Barsoum M, et al. Higher efficacy of letrozole in combination with trastuzumab compared to letrozolemonotherapy as first-line treatment in patients with HER2 positive, hormone-receptor- positive metastatic breast cancer - results of the eLEcTRA trial. *Breast*. 2012; 21(1): 27-33.

300. Johnston SR, Martin LA, Head J, Smith I, Dowsett M. Aromatase inhibitors: combinations with fulvestrant or signal transduction inhibitors as a strategy to overcome endocrine resistance. *J Steroid Biochem Mol Biol*. 2005; 95: 173-181.

301. Leary AF, Drury S, Detre S,et al. Lapatinib restores hormone sensitivity with differential effects on estrogen receptor signalling in cell models of human epidermal growth factor receptor2-negative breast cancer with acquired endocrine resistance. *Clin Cancer Res*. 2010; 16(5): 1486-1497.

302. Sabnis G, Schayowitz A, Goloubeva O, Macedo L, Brodie A. Trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen. *Cancer Res*. 2009; 69(4): 1416-1428.

303. O'Regan RM, Osipo C, Ariazi E, et al. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res.* 2006; 12(7 Pt 1): 2255-63.

304. Wang YC, Morrison G, Gillihan R, et al. Different mechanisms for resistance to trastruzumab versus lapatinib in HER2-positive breast cancers role of estrogen receptor and HER2 reactivation. *Breast Cancer Res*. 2011; 13(6): R121.

305. Chang JCN, Forero-Torres A, Nanda R et al. BCRC 006: a multicentre phase II study of neoadjuvant lapatinib and trastuzumab in patients with HER2-overexpressing breast cancer. *J Clin Oncol*. 2011: 29(Suppl)(Abstract 505).

306. Mittendorf EA, Wu Y, Scaltriti M, et al. Loss of HER2amplificationfollowingtrastuzumab-basedneoadjuvantsystemictherapy and survival outcomes. *Clin Cancer Res.* 2009; 15(23): 7381-7388.

307. www.kegg.jp/kegg/xml/IGML

308. Lyons YA, Sherry YW, Willem WO et al. Immune cell profiling in cancer: molecular approaches to cell-specific identification. *NPJ Precision Oncology*. 2017; 26:1698-017.

309. Turnbull AK, Arthur LM, Renshaw L et al. Accurate prediction and validation of response to endocrine therapy in breast cancer. *J Clin Oncol.* 2015; 33(20): 2270-2278.

310. Verhaegh W, van Ooijen H, Inda MA, et al. Selection of Personalized Patient Therapy through the Use of Knowledge-Based Computational Models That Identify Tumor-Driving Signal Transduction Pathways. *Cancer Res*. 2014; 74(11): 2936-2945.

311. Van Ooijen H, Hornsveld M, Dam-de Veen C, et al. Assessment of functional phosphatidylinositol 3-kinase pathway activity in cancer tissue using forkhead box-o target gene expression in a knowledge-based computational model. *Am J Pahology.* 2018; 188((): 1956-1972.

312. Borghaei H, Paz-Ares L, Horn L et al. Nivolumab versus docetaxel in advanced nonsquamous non- small-cell lung cancer. *N Engl J Med*. 373, 1627–1639 (2015).

313. Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 364, 2517–2526 (2011).

314. Faber AC, Li D, Song Y, et al. Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition. *Proc Natl Acad Sci USA*. 2009; 106: 19503–19508.

315. Sos ML, Fischer S, Ullrich R, et al. Identifying genotype-dependent efficacy of single and combined PI3K- and MAPK-pathway inhibition in cancer. *Proc Natl Acad Sci USA*. 2009; 106: 18351–18356.

316. Miller TW, Rexer BN, Garrett JT, Arteaga CL. Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer. *Breast Cancer Res*. 2011; 13: 224.

317. Lauring J, Park BH, Wolff AC. The phosphoinositide-3-kinase-Akt-mTOR pathway as a therapeutic target in breast cancer. *J Natl Compr Cancer Netw*. 2013; 11: 670–678.

318. Jin N, Jiang T, Rosen DM, Nelkin BD, Ball DW. Synergistic action of a RAF inhibitor and a dual PI3K/mTOR inhibitor in thyroid cancer. *Clin Cancer Res*. 2011; 17: 6482–6489.

319. Serra V, Scaltriti M, Prudkin L, et al. PI3K inhibition results in enhanced HER signalling and acquired ERK dependency in HER2-overexpressing breast cancer. *Oncogene*. 2011; 30: 2547–2557.

320. Kirouac DC, Du JY, Lahdenranta J, et al. Computational Modeling of ERBB2-Amplified Breast Cancer Identifies Combined ErbB2/3 Blockade as Superior to the Combination of MEK and AKT Inhibitors. *Sci Signal*. 2013; 6: 68.

321. Santen RJ, Song RX, McPherson R, et al. The role of mitogen-activated protein (MAP) kinase in breast cancer. *J Steroid Biochem Mol Biol*. 2002; 80: 239–56.

322. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW: Cancer genome landscapes. Science 2013, 339: 1546e1558

323. Nahta R. Pharmacological strategies to overcome HER2 cross-talk and trastuzumab resistance. *Curr Med Chem*. 2012; 19: 1065–1075

324. Paplomata E, Zelnak A, O'Regan R. Everolimus: side effect profile and management of toxicities in breast cancer. *Breast Cancer Res Treat*. 2013; 140: 453–462.

325. O'Regan R, Ozguroglu M, Andre F, et al. Phase III, randomized, double-blind, placebo-controlled multicenter trial of daily everolimus plus weekly trastuzumab and vinorelbine in trastuzumab- resistant, advanced breast cancer (BOLERO-3). *J Clin Oncol*. 2013; 31(Suppl.): abstract 505.

326. Alimonti A, et al. Subtle variations in Pten dose determine cancer susceptibility. *Nat Genet*. 2010; 42: 454–458.

327. Carracedo A, Pandolfi PP. The PTEN-PI3K pathway: of feedbacks and crosstalks. *Oncogene*. 2008; 27: 5527–5541.

328. Chen Z, Trotman LC, Shaffer D, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*. 2005; 436 (7051): 725–30.

329. Stambolic V, MacPherson D, Sas D, et al. Regulation of PTEN transcription by p53. *Mol Cell*. 2001; 8: 317–325.

330. Chaudhary S, Krishna BM, Mishra SK. A novel *FOXA1/ ESR1* interacting pathway: A study of Oncomine™ breast cancer microarrays. *Oncology letters.* 2017; 14: 1247-1264.

331. Samuels Y and Ericson K. Oncogenic PI3K and its role in cancer. *Curr Opin Oncol.* 2006; 18: 77-82.

332. Song MS, Salmena L, and Pandolfi. The functions and regulation of the PTEN tumour suppressor. *Nature Rev Mo Cell Biol.* 2012; 13: 283- 296.

333. Rodon J, Dienstmann R, Serra V, Tabernero J. Development of PI3K inhibitors: lessons learned from early clinical trials. *Nat Rev Clin Oncol.* 2013; 10: 143-153.

334. Kwiatkowski DJ, Wagle N. mTOR inhibitors in cancer: what can we learn from exceptional responses? *E Bio Medicine.* 2015; 2: 2-4 .

335. Eijkelenboom A, Burgering BM. FOXOs: signalling integrators for homeostasis maintenance. *Nat Rev Mol Cell Biol.* 2013; 14: 83-97.

336. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell.* 1999; 96: 857-868.

337. Kim HJ, Lee SY, Kim CY, et al. Subcellular localization of FOXO3a as a potential biomarker of response to combined treatment with inhibitors of PI3K and autophagy in PIK3CA-mutant cancer cells. *Oncotarget.* 2017; 8: 6608-6622.

338. Osborne CK, Neven P, Dirix LY, et al. Gfitinib or placebo in combination with tamoxifen in patients with hormone receptor positive metastatic breast cancer: a randomized phase II study. *Clin Canc Res.* 2011; 17(5): 1147-1159.

339. Baselga J, Campone M, Piccart M, et al. Everolimus in postmenopausal hormone receptor positive advanced breast cancer. *N Engl J Med.* 2012; 366(6): 520-529.

340. Massarweh S, Romond E, Black EP, et al. A phase II study of combined fulvestratnt and everolimus in patients with metastatic estrogen receptor (ER) positive breast cancer after aromatase inhibitor failure. *Breast Cancer Res and Treat.* 2014; 143(2): 325-332.

341. Yu K, Toral-Barza L, Discafani C, et al. mTOR, a novel target in breast cancer: The effect of CCI-779, an mTOR inhibitor, in pre- clinical models of breast cancer. *Endocr Relat Cancer.* 2001; 8: 249-258.

342. Chan S, Johnston S, Mross L, et al. Phase II Study of Temsirolimus (CCI-779), a Novel Inhibitor of mTOR, in Heavily Pretreated Patients With Locally Advanced or Metastatic Breast Cancer. *J Clin Oncol.* 2005; 23(23): 5314-5322.

343. Martin M, Holmes FA, Ejlertsen B, et al. Neratinib after trastuzumab-based adjuvant therapy in HER2 positive breast cancer (ExteNET): 5-year analysis of a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* 2017; 18(12) 1688-1700.

344. Rugo H, Chien AJ. HER2-positive breast cancer: Is more treatment better? *Lancet Oncol.* 2016; 17: 268-270.

345. Ding W, Li Z, Wany C, et al. The CDK4/6 inhibitor in HER- positive advanced breast cancer: A systematic review and meta-analysis. *Medicine.* 2018; 97(20): 10746-10755.

346. Finn RS, Crown JP, Lang I, et al. The cyclin dependent kinase 4/6 inhibitor paclociclib in combination with letrozole versus letrozole alone as first line treatment of oestrogen receptor positive, HER2 negative, advanced breast cancer (PALOMA-1/TRIO-18): a ranomised phase 2 study. *Lancet Oncol.* 2015; 16(1) 25-35.

347. Fin RS, Marin MM, Hope S, et al. Palbociclib and letrozole in advanced breast cancer. *N Engl J Med.* 2016; 375: 1925-1936.

348. Hortobagyi GN, Stemmer SM, Burris HA, et al. Ribociclib as first-line therapy for HR-positive, advanced breast cancer. *N Engl J Med.* 2016; 375: 1738–48.

349. Dickler MN, Tolaney SM, Rugo HS, et al. MONARCH1: results from a phase II study of abemaciclib, a CDK4 and CDK6 inhibitor, as monotherapy, in patients with HR+/HER2- breast cancer, after chemotherapy for advanced disease. *J Clin Oncol.* 2016; 34 (15S): 510-522.

350. Sledge GWJr, Toi M, Neven P, et al. MONARCH 2: abemaciclib in combination with fulvestrant in women with HR+/HER2- advanced breast cancer who had progressed while receiving endocrine therapy. *J Clin Oncol.* 2017; 35: 2875–84-95.

351. Goel S, Wang Q, Watt AC, et al. Overcoming therapeutic resistance in HER2-positive breast cancers with CDK4/6 inhibitors. *Cancer Cell.* 2016; 29: 255–69.

352. Finn RS, Dering J, Conklin D, et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. *Breast Cancer Res.* 2009; 11(5): 77-90.

353. Hurtado A, Holmes KA, Geistlinger TR, et al. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. *Nature*. 2008; 456 (7222): 663–666.

354. Beauchemin D, Lacombe C, van Themsche C. PAX2 is activated by estradiol in breast cancer cells of the luminal subgroup selectively, to confer a low invasive phenotype. *Molecular Cancer*. 2011; 10: 148-158.

8. **Appendicies:** *Appendix 1:* Ethics committee approval; 2001/8/80 and 2001/8/81.

Lothian NHS Board

Deaconess House
148 Pleasance
Edinburgh
EH8 9RS
Telephone 0131 536 9000
Fax 0131 536 9009
www.nhslothian.scot.nhs.uk

NHS Lothian

13 February 2007

Mr J. M. Dixon
Consultant Surgeon and Senior Lecturer in Surgery
Lothian University Hospitals Division
Edinburgh Breast Unit
Western General Hospital
Crewe Road South, Edinburgh
EH4 2XR

Dear Mr Dixon

Full title of study: **Edinburgh Breast Unit and Edinburgh Cancer Research Centre tissue and body fluids collection with linkage to pathological and clinical data**

REC reference number: **06/S1103/65**

Thank you for your letter of 5 February 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered on behalf of the Committee by the Chair, Dr Christine West.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		06 December 2006
Investigator CV		
Protocol	1.1	04 December 2006
Covering Letter	2 - with changes	05 February 2007
Covering Letter	1 - with original submission	06 December 2006
Letter of invitation to participant	1.1	04 December 2006
GP/Consultant Information Sheets	1.0	04 December 2006
Participant Information Sheet	1.1	04 December 2006
Participant Consent Form	1.2	05 February 2007
Response to Request for Further Information		

INVESTOR IN PEOPLE

Lothian NHS Board

Deaconess House
148 Pleasance
Edinburgh
EH8 9RS
Telephone 0131 536 9000
Fax 0131 536 9009
www.nhslothian.scot.nhs.uk

NHS
Lothian

13 February 2007

Mr J. M. Dixon
Consultant Surgeon and Senior Lecturer in Surgery
Lothian University Hospitals Division
Edinburgh Breast Unit
Western General Hospital
Crewe Road South, Edinburgh
EH4 2XR

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC Reference Number	**06/S1103/65**	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

pp **Chair**
Lothian Local Research Ethics Committee 03
Email: elizabeth.harden@lhb.scot.nhs.uk

Enclosures: *Standard approval conditions*
Site approval form

Copy to: Professor Heather Cubie
Lothian University Hospitals Division
Research & Development Office
Queen's Medical Research Institute
47 Little France Crescent, Edinburgh
EH16 4TJ

R&D Department for NHS Lothian

University Hospitals Division

Queen's Medical Research Institute
47 Little France Crescent, Edinburgh, EH16 4TJ

HAC/SM/approval/Dixon/2d/3/3a/3b/3e

7th February 2007

Mr J Mike Dixon
Consultant Surgeon and Senior Lecturer in Surgery
Edinburgh Breast Unit
Western General Hospital
Crewe Road South
Edinburgh
EH4 2 XR

Dear Mr Dixon

MREC No:	**N/A**
CRF No:	**Not yet known**
LREC No:	**06/S1103/65**
R&D ID No:	**2007/W/BU/01**
Title of Research	Edinburgh Breast Unit and Edinburgh Cancer Research Centre tissue and body fluids collection with linkage to pathological and clinical data.
Protocol No/Acronym:	**N/A**

The above project has undergone an assessment of risk to NHS Lothian and review of resource and financial implications. I am satisfied that all the necessary arrangements have been set in place and that all Departments contributing to the project have been informed.

As this is a single site project involving patients, NHS Lothian agrees to act as Sponsor for the study.

Use of Tissue or Samples

- The study involves the use of patient tissue or samples. You must be familiar with NHS Lothian's Tissue Policy and abide by its conditions and also with all regulations in place at the time. Approval is subject to the prevailing legal requirements.
- Approval for the use of tissue is restricted to the protocol associated with this application, but may include additional collaborators within University of Edinburgh. Collaborators who are not named in the original protocol require to be notified to local REC.
- If material is to be transferred to academic collaborators outwith University of Edinburgh or to any commercial entity then a Material Transfer Agreement must be obtained from the R&D Office and signed by all relevant parties prior to transfer of the material. Such collaborations must be fully discussed with the R&D Office and management approval is only effective once this is in place.

NHS Lothian

RESEARCH & DEVELOPMENT OFFICE
Room E1.12

Tel: 0131 242 3330
Fax: 0131 242 3343
Email:
R&DOffice@luht.scot.nhs.uk

Univers

Queen
47 Little

HAC/SM/approval/Dixon/2d/3/3a/3b/3e

- I note that the use of excess diagnostic specimens has been discussed and approved by the Pathologist with delegated authority.
- I note that additional samples will be taken for the study and that this will be done with the patient's explicit consent.
- Where a prospective collection of material is intended, explicit consent must be obtained for storage and use in possible future research projects. A model consent forms along the lines recommended in the MRC Guidelines 'Human Tissue and Biological Samples for Use in Research' should be used and a copy lodged with the R&D Office.

On behalf of the Chief Executive and Medical Director, I am happy to grant management approval from NHS Lothian to allow the project to commence, subject to the approval of the appropriate Research Ethics Committee(s) having also been obtained. You should note that any substantial amendments must be notified to the relevant Research Ethics Committee and to R&D Management with approval being granted from both before the amendments are made.

Please note that under Section A, Q35, NHS Lothian provides indemnity for negligence for NHS and Honorary clinical staff for research associated with their clinical duties. It is not empowered to provide non-negligent indemnity cover for patients. NHS Lothian does not provide indemnity against negligence for healthy volunteer studies. This is the personal responsibility of both NHS and honorary employees and is usually arranged with a medical defence organisation or through the University of Edinburgh.

This letter of approval is your assurance that NHS Lothian is satisfied with your study. As Chief Investigator or local Principal Investigator, you should be fully committed to your responsibilities within the Research Governance Framework for Health and Community Care, an extract of which is attached to this letter.

Yours sincerely

Heather A Cubie

Professor Heather A Cubie
R&D Director

Enc
- Research Governance Certificate ☑ (to be signed and returned)
- NRR authorisation ☑ (to be signed and returned)
- Tissue Policy (if applicable) ☑
- MTA (if applicable) ☐ (to be signed and returned by the recipient)

Copies *Administrators, Research Ethics Committee*
CRF

NHS
Lothian

RESEARCH & DEVELOPMENT OFFICE
Room E1.12

Tel: 0131 242 3330
Fax: 0131 242 3343
Email: R&DOffice@luht.scot.nhs.uk

Appendix 2:

Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) biopsy tissue blocks were cut into 5-10 micrometre thick tissue sections and mounted to labelled microscope slides. Upon commencement of experiments, tissue was dewaxed in an autostainer.

Antigen Retrieval for all markers was performed using sodium citrate solution within a pressure cooker, heated for 10 minutes in a microwave. Slides were subsequently washed in PBST solution on a laboratory shaker for 10 minutes. Tissue was then washed in hydrogen peroxide solution, again for 10 minutes on a laboratory shaker. Two additional 5 minute PBST washes were then performed.

Each slide was assembled with a sequenza, followed by two further PBST washes at 5 minute intervals. DAKO total protein block was added to each slide and allowed to incubate for 30 minutes to prevent non-specific antibody binding.

Primary antibody calculations (in accordance with concentration provided in table) were determined and 150 micro-litres of antibody solution was added to sequenza. Following incubation two PBST washes were undertaken at 5 minute intervals.

Secondary antibody was then added to each sequenza. This was permitted to incubate for 1 hour and was followed with 2 more PBST washes.

DAKO Dab chromogen and substrate buffer was used to localise the secondary antibody, thereby detecting the protein marker. A solution of 1:50 concentration (DAB: substrate buffer) was made and 150 microliters of this solution was added to each sequenza. The DAB was allowed to incubate for exactly 10 minutes.

Following DAB incubation, slides were bathed in distilled water and then counterstained with Hematoxylin in an auto-stainer. Cover slips were added to each slide before they were scanned using Nanozoomer Software to permit evaluation.

Appendix 3:

Immune cell profiling

Enriched genes in immune cells.[Ref 308]

(Figures 4.8A; 4.8B; 4.8C; 4.8D; 4.8E)

All T Cells:

(Figure 4.8A)

CD3G	*GFI1*	*CD3E*	*RORA*	*RASGRP1*	*DUSP16*
CD3D	*GATA3*	*INPP4B*	*MGC1976*	*LRIG1*	*KIAA0748*
TRA	*SH2D1A*	*MAL*	*TCF7*	*DPP4*	*CDR2*
CD6	*TRB*	*NPDC1*	*ZAP70*	*CD3Z*	*STAT4*
CD5	*TNFRSF2*	*ITM2A*	*LEF1*	*PDE4D*	*FLT3LG*
NPDC1	*5 NK4*	*ITK*	*SPOCK2*	*FYN*	
CD28	*TACTILE*	*LCK*	*PRKCQ*	*WWP1*	
CAMK4	*BCL11B*	*NFATC3*	*SATB1*	*LAT*	
IL6ST					

CD8+ T cells:

(Figure 4.8B)

FCGBP	*ZNF145*	*CCL5*	*GZMH*	*GPR56*	*CD8B1*
C1ORF21	*ADRB2*	*GZMC*	*PRF1*	*KLRC1*	*CD8A*
PHEMX	*DUSP2*	*TBX21*	*GNLY*	*S100B*	
KLRG1	*IL2RB*	*CCL4L*	*CST7*	*D12S248E*	

T regulatory cells:

(Figure 4.8C)

AKAP2	*LOC51191*	*HPGD*	*IL10*	*NLN*	*C1ORF186*
ANXA2	*MKP-7*	*PTT1*	*GAB3*	*MS4A3*	*LYZ*
C8FW	*NINJ2*		*NR4A1*	*UCK2*	*SFXN4*
CALM2	*P53DINP1*	*SAT*	*IL1R2*	*TNIP3*	*CYP1B1*
CDH13	*PMAIP1*	*SHMT2*	*ULBP1*	*TNFRSF9*	*SLC7A5*
ENTPD1	*PTPLA*	*TMOD3*	*IL17F*	*IL7R*	*C17ORF58*
EPSTI1	*GPR2*	*TNFRSF6*	*EIF4EBP1*	*NR4A3*	*SLC4A5*
GBP2	*HLA-DMA*	*VIL2*	*RGS16*	*CSGALNA*	*CD52*
GBP5	*HLA-DPA1*	*LRRC32*	*BCAT1*	*CT1*	*KLRB1*
HS3ST3B1	*HLA-DPB1*	*CHAC1*	*VEGFA*	*SLC43A1*	*S100A4*
HSPCA	*HLA-DQB1*	*EGR1*	*GIMAP1*	*ZBTB32*	*POLA1*
ICA1	*HLA-DRA*	*PYCR1*	*GPR44*	*SCFD2*	*SLC7A1*
LAIR2	*HLA-DRB4*	*IL13*	*GPT2*	*HK2*	
LGALS3	*HLA-DRB5*	*C3AR1*	*PSPH*	*C17ORF96*	
		ZBED2			

ATPBD4	*HOMER1*	*CHD6*	*CNPY4*	*TMEM63A*	*EIF2B3*
UBXN8	*ATF3*	*IL1R1*	*TIMM8A*	*MXI1*	*URB2*
CASP8	*AGPAT4*	*LPCAT4*	*CCDC28A*	*MRTO4*	*DDX21*
FAIM3	*CTH*	*CITED2*	*MNAT1*	*SESN2*	*ITGA4*
IL12RB2	*JAKMIP1*	*DUSP4*	*MS4A2*	*C1ORF38*	*RGS2*
MPP7	*TFRC*	*SSBP2*	*GZMB*	*STK38*	*RORC*
ZNF831	*RPL26L1*	*LAYN*	*E2F5*	*SLC6A6*	*IFNAR2*
BNIP3L	*MICAL1*	*ZFAND2B*	*HIBCH*	*ALG14*	*CSDA*
LRP8	*GPRASP1*	*PIK3R1*	*C9ORF91*	*LXN*	*SORL1*
UTRN	*TMX4*	*GCNT4*	*NHP2*	*TNFRSF1*	*LRRC37B*
SOCS3	*FYB*	*PPP3CA*	*CDC25B*	*ACSS1*	*IL10RA*
LY9	*ZNF792*	*TIMP1*	*VDR CSF1*	*UBA7*	*PRKACB*
EVI2B	*WDR4*	*SIRPG*	*GPATCH4*	*MTSS1*	*CBWD1*
SFXN3	*HUWE1*	*CCR3*	*NFKBID*	*PSMA6*	*SMAD3*
TNFRSF4	*CD83*	*PTP4A3*	*TMCO7*	*FAM8A1*	*C1ORF96*
ING4	*TRIB2*	*SERINC5*	*EHBP1*	*RAB31*	*FCER1A*
BLM	*LARP1B*	*MAGOHB*	*FAM173B*	*TNRC6B*	*HSP90AB*
TNFSF11	*SLC38A5*	*GIMAP4*	*FARS2*	*SESN1*	*EMG1*
SLFN5	*AUH*	*KLHL24*	*FLVCR2*	*CEP68*	
CCR8	*CTPS*	*CCNG2*	*NPM3*	*LAPTM5*	
CD244	*CYTH1*	*WDR70*	*PNPO*	*PLAGL2*	
MYO1F	*APOL3*	*MYBL1*	*P2RY8*	*LRRC33*	

B Cells:

(Figure 4.8D)

PSEN2	*SCRN1*	*GM2A*	*ZCCHC7*	*MK2S4*	*NFKBIE*
FZD5	*PTD008*	*HRK*	*KIAA0274*	*APG4A*	*CHD7*
PLEKHF2	*CYB561D2*	*ZNF207*	*SLC22A3*	*DAPP1*	*SP140*
SIDT2	*SCAP2*	*LOC924F5*	*SPI1*	*TNFRSF18*	*WDR34*
MTPN	*MMP11*	*DMXL1*	*AMFR*	*HERPUD1*	*BTNL9*
TUBB6	*ERDJ5*	*CLIC4*	*ANXA4*	*POU2F2*	*ATP6V0A1*
FLJ21127	*TTC7A*	*PRCP*	*EBF*	*ADK*	*UROS*
SMC6L1	*PMAIP1*	*DKFZP434*	*ITPR1*	*CKIP-1*	*HLA-DOA*
KIAA1026	*GARNL4*	*C0328*	*HIST1H2B*	*ACTA2*	*C22ORF13*
NUP88	*TMEPAI*	*STRBP*	*COPS3*	*PARP14*	*IFI27*
GNA12	*SH3BP5*	*PHF16*	*COL14A1*	*MTSS1*	*JMJD2B*
RNTRE	*EDD*	*JUP*	*SAV1*	*DTX1*	*WEE1*
GSTZ1	*UCP4*	*TEAD2*	*APOBEC3*	*DDR1*	*ODC1*
RFX5	*EVI5L*	*PPP3CA*	*B CCNG2*	*RRAS2*	*KIAA0746*
XYLT1	*PIK3C2B*	*CNR2*	*STAG3*	*RNF141*	
GL012	*SMAD3*	*ATP5B*	*LOC34893*	*SYPL*	
EPB41L2	*LHFPL2*	*ABCA1*	*POLD4*	*SSPN*	
LOC51760	*LOC57228*	*BMF*	*TBC1D1*	*CORO1C*	

WDR11	*CORO1A*	*RD4*	*CD86*	*IGHA*	*OSBPL10*
SYNGR2	*PRKCEHC*	*RALGPS2*	*LAF4*	*CYBASC3*	*SLC2A1*
FOXP1	*FLJ25604*	*SEMA4B*	*LOC28366*	*KIAA0125*	*NCF1*
BLR1	*TRIM26*	*BSG IGKC*	*FREB*	*RAM2*	*IGL2*
MYO1E	*FBXO41*	*HLA-DRB6*	*DKFZP586*	*CD83*	*IGLC2*
MYBL2	*GCNT1*	*FUBP1*	*A0522*	*FCRH1*	*SPAP1*
GYLTL1B	*LRMP*	*UNC84B*	*UREB1*	*FLII*	*IGHG3*
SETBP1	*UBE2J1*	*IFNGR2*	*IGLL1*	*SNX10*	*HLA-DMA*
KLF1	*IGKV3D*	*HSPA5*	*HLA-DQA2*	*IGHG1*	*HLA-DMB*
INPPL1	*PCCA*	*HLA-DRB5*	*KIAA1219*	*EPHX1*	*CD79A*
LOC54103	*GLDC*	*MARCKS*	*PLCG2*	*FLJ10979*	*HSPA6*
ZNF154	*CYSLTR1*	*KYNU*	*MARCH-1*	*GTPAP*	*IGHD*
MHC2TA	*BTLA*	*PACAP*	*BCNP1*	*IGLL3*	*KIAA0476*
CD24	*NET5*	*RHOBTB2*	*PNOC*	*E2F5*	*SLC7A7*
ARHGEF3	*HLA-DPA1*	*FLJ12363*	*CD20*	*TNFRSF17*	*NKG7*
BIRC3	*BLNK*	*TMED8*	*PSCD1*	*BLK*	*CD19*
TRIM56	*CD79B*	*FGD2*	*BCL11A*	*FLJ00332*	*SAMD9*
HLA-DPB1	*TM4SF8*	*FBXO10*	*VPS28*	*SNX2*	*LY86*
UVRAG	*TFEB*	*IL4R*	*SWAP70*	*CD200*	*SPIB*
CD38	*CD11ORF2*	*CD1C*	*SYK*	*HLA-DQB1*	*NAPSB*
PEA15	*ADRBK2*	*MGC5084*	*BCL7A*	*TRIO*	*RNASE6*
FLJ10697	*CDKN2A*	*MRPL49*	*NAP1L*	*CYBB*	*IGHM*
TLR10	*RIPK2*	*CTSH*	*TEM6*	*PIK3AP1*	*LY64*
ARHGAP1	*STX7*	*LYN*	*BTK*	*IRF4*	*CD72*
HLA-DRA	*NCF4*	*WASPIP*	*RAB30*	*CD22*	*IRTA2*
SHMT2	*SRGAP2*	*C3ORF6*	*FCGR2B*	*BACE2*	*CD1D*
LGALS9	*SLC2A5*	*EGR1*	*STAT6*	*IGJ*	*HLA-DQA1*
FLJ10853	*CTSZ*	*IGKV15*	*BRDG1*	*PAX5*	*SAS*
CEBPB	*AIM2*	*SPAP1*	*LOC20189*	*RGS13*	*CTSH*
PRICKLE1	*TCF4*	*CHERP*	*ITGB1*	*TCL1A*	*LYN*
LCP21	*TPD52*	*IGHG3*	*CD74*	*MEF2C*	*WASPIP*
CR1	*MAP3K8*	*ADAM28*	*CORO2B*	*C13ORF18*	*C3ORF6*
KLHL14	*MOBKL2B*	*HLA-DMA*	*VPREB3*	*POU2AF1*	*EGR1*
TLR7	*HLA-DOB*	*HLA-DMB*	*HSPC182*	*HHEX*	*FREB*
C20ORF72	*IFIT3*	*PALM2-*	*MGC1561*	*IRF8*	*DKFZP586*
HLA-DQB2	*MGC2403B*	*AKAP2*	*RHOH*	*BANK1*	

Dendritic cells:

(Figure 4.8E)

ACCL5	*CCR7*	*IF127*	*IFIH1*	*MX1*	*ISG20*
CXCL10	*IL15*	*IF144L*	*IFIT1*	*ISG15*	*IRF7*

GBP4	*CCL4*	*TNFRSF9*	*MT2A*	*CCL8*	*CD200*
DUSP5	*TNFA1P6*	*SOD2*	*TRAF1*	*EBI3*	*LAMP3*
NFKB1A	*IFIT3*	*CD38*	*GADD45B*	*IFITM1*	*RGS1*
ATF3	*OASL*	*CD44*	*MT1M*	*MT1B*	*SAT1*
TNFSF10	*GBP1*	*CD80*	*MTIP2*	*MT1E*	
IL6	*HES4*	*CD83*	*BIRC3*	*MT1G*	
IL8	*CYP27B1*	*CD86*	*USP18*	*MT1H*	
IL7R	*RIPK2*	*INDO*	*TUBB2A*	*GADD45A*	

Appendix 4:

Proliferation Gene List[309]

(Figures 4.11A and 4.11B)

CCNB1	*KIF15 RFC4*	*SMARCC1*	*ASPM*	*MCM4*	*TOP2A*
ACTL6A	*H2AFZ*	*UBE2C*	*MCM2*	*EZH2*	*CMNN*
NUSAP1	*HMGB2*	*H2AFX*	*MCM6*	*TMEM97*	*CDC20*
KIF11 ARL3	*MAD2L1*	*SYCP2*	*NDC80*	*NCAPG*	*CENPF*
CEP55	*CENPN*	*CD2AP*	*AURKA*	*CCND1*	*ZWNT*
IGF1R	*RRM1*	*PRC1*	*TPX2*	*TIMELESS*	*DLGAP5*
CCNA2	*SAC3D1*	*PTT3GP*	*CDKN3*	*CHPT1*	*HSPA2*
BUB3	*HIST1H2AC*	*FGFR10P*	*PCNA*		*GINS2*
ZNF281	*DK1 RRM2*	*IL17RB*	*RBBP8*		

Appendix 5:

MAPK Signalling Gene List[289]

(Figures 4.12A 4.12B 4.12C)

HEBP1	*PRDM4*	*TOB1*	*COX6C*	*ELF4*	*ANGPTL4*
PGR	*PEX7*	*CELSR2*	*RET*	*OPTN*	*FTH1*
ESR1	*MRPS30*	*ARMT1*	*KIF5C*	*IRF1*	*SFN*
TCLF5	*MYB*	*KNAJB12*	*BCL2*	*TNFRSF21*	*MAFF*
BCKDK	*KBTBD4*	*PBCD4*	*GREB1*	*ADM*	*PLSCR1*
BCAT2	*FLNB*	*GPD1L*	*SP100*	*RBMS1*	*HAL-C*
SLC9A3	*APTX*	*PRDX3*	*FKBP1A*	*LMNA*	*HLA-E*
HLA-F					

Appendix 6:

ER Signalling Pathway Genes[310]

AP1B1
CA12
CDH26
CELSR2
COL18A1
COX7A2L
CTSD
DSCAM
EBAG9
ERBB2
ESR1
GREB1
HSPB1
IGFBP4
KRT19
MYC
NRIP1
PGR
PISD
PTMA
RARA
SGK3
SOD1
TFF1
TRIM25
WISP2
XBP1

Appendix 7:

PI3K Signalling Gene List

(Figures 4.13A, 4.13C)

BAD	*NFKBIA*	*MAPK14*	*TLR4*	*ADAR*	*JUN*
IGF1	*CSNK2A1*	*SRF*	*IGF1R*	*TSC1*	*GRB2*
EIF2AK2	*MAPK3*	*SOS1*	*PDPK1*	*ILK*	*PTPN11*
RHOA	*TSC2*	*AKT3*	*AKT1*	*PRKCB*	*IRAK1*
PRKCS	*RBL2*	*RPS6KA1*	*PIK3R1*	*CTNNB1*	*EIF4EBP*
CDC42	*PIK3R2*	*FOXO3*	*RASA1*	*MAP2K1*	
TOLLIP	*GRB10*	*PIK3CA*	*PAK1*	*PTK2*	
GSK3B	*RPS6KB1*	*YWHAH*	*FOXO1*	*FOS*	
FKBP1A	*NFKB1*	*RAF1*	*EJF4E*	*CD14*	
MAPK1	*CCND1*	*PDGFRA*	*GJA1*	*PTEN*	
TCL1A	*CDKN1B*	*RAC1*	*SHC1*	*HRAS*	

Cluster 2

IGF1
NFKBIA
CDKN1B
SRF
AKT3
PDGFRA
PDPK1
PIK3R1
FOXO1
GJA1
SHC1
FOS
PTEN
JUN

Appendix 8:

Forkhead Box-O (FOXO) Target Genes:

AGRP
BCL2L11
BCL6
BNIP3
BTG1
BCL6
BNIP3
BTG1
CAT
CAV1
CCND1
CCND2
CCNG2
CDKN1A
CDKN1B
ESR1
FASLG
FOXO32
GADD45A
INSR
MXI1
NOS3
PCK1
POMC
PPARGC1A
PRDX3
RBL2
SOD2
TNFSF10

Appendix 9:

Her2 questionnaire

Questionnaire on HER2 Testing

Please circle your answer for each question

1. What is your grade?

   Consultant

   SpR

   Staff Grade/Ass Specialist

2. How many breast cancers do you treat in one year?

   <50

   50-100

   >100

3. Which of the following best applies to the setting in which you work?

   Specialist breast unit

   Breast unit within a district general hospital

   General surgical unit

4. Where is your main hospital?

……..

5. Do you have HER2 testing in your centre?

   Yes

   No

6. Is HER2 testing done on the initial core biopsy in your centre?

   Yes

   No

   Don’t know

7. If HER 2 testing is done on the initial core biopsy in your centre, approximately what percentage of the cores you take from cancers is tested?

   0

   1-9

   10-24

   25-59

   60-74

   75-100

8. If HER2 testing is done on the core biopsy, what percentage of HER2 results are available when you/or the multidisciplinary team make a decision as to whether the patient should have initial surgery or neoadjuvant therapy?

   0

   1-9

10-24

25-59

60-74

75-100

9. Do you **ONLY** perform HER2 testing on the cancer after it has been removed by surgery?

Yes

No

Don’t know

10. What percentage of ALL breast cancers in your centre are HER2 tested?

0

1-9

10-24

25-59

60-74

75-100

11. Does your centre use SISH or FISH testing alone?

Yes

No

Don’t know

12. Does your centre test with both the Hercept and SISH or FISH tests?

Yes

No

Don’t know

13. In what percentage of patients do you have the full HER2 result (that includes immunohistochemistry and SISH or FISH testing) available when a patient is discussed at a multidisciplinary meeting when decisions are made on adjuvant therapy following a surgical procedure?

0

1-9

10-24

25-59

60-74

75-100

14. Have any of your patients ever received neoadjuvant Herceptin in your centre?

Yes

No

Not sure

15. What percentage of patients with large or locally advanced HER2 positive cancers in your centre who are given neoadjuvant chemotherapy also get neoadjuvant Herceptin?

0

1-9

10-24

25-59

60-74

75-100

16. In a patient with a large HER2 positive invasive breast cancer what percentage of patients do you believe would get a complete pathological response if they were treated with neoadjuvant chemotherapy (6-8 cycles of chemotherapy and neoadjuvant Herceptin?

0

1-9

10-24

25-59

60-74

75-100

17. What percentage of patients with an invasive cancer with involved axillary nodes who is given a 6-8 cycles of neoadjuvant chemotherapy and Herceptin do you believe will have complete response in previously involved lymph nodes?

    0

    1-9

    10-24

    25-59

    60-74

    75-100

18. Do you think there is any advantage in adding neoadjuvant Herceptin to neoadjuvant chemotherapy in patients whose cancers are HER2 positive?

    Yes

    No

    Don’t know

19. Would you like to make any specific comments about HER2 testing in your centre which you think might be valuable for us to know?

    If yes, please detail below: