Targeting within ER positive Early Breast Cancer: patient selection for current therapies and novel therapeutic approaches in a heterogeneous group.

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A thesis submitted for the degree of Doctor of Philosophy to the Faculty of Medicine, University of Glasgow

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August 2012

I am the sole author of this thesis. Unless otherwise acknowledged, all the work presented in this thesis was performed personally. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Esther Jennifer Campbell, August 2012

Dedication

This thesis is dedicated to Alexander and Ru, my gorgeous boys-mummy was not just playing computer games! Your love and (im) patience kept me going.

And Professor Timothy Cooke, a brilliant mentor.

Acknowledgments

I would like to thank Dr. Joanne Edwards, my supervisor for her help, patience, support and guidance. Joanne, thank you, with all of my heart, I have been very fortunate to have the benefit of your supervision, great knowledge and encouragement.

I would also like to thank the Radiation Oncology group 2008-2010, particularly Dr Anthony McCluskey, Dr Annette Sorensen, Lesley Wilson, Anne Marie Clarke and fellow PhD student Mathias Tesson. The guidance you guys gave me was invaluable and some of the laughs unforgettable-thank you so much. Big thanks to Dr Tammy Doig for her expertise in histopathology and her invaluable help, and Dr Liane McGlynn for her guidance and patience during my tentative steps in tissue culture and statistical analysis. Dr Paul Shiels and his student Mr Samer Zino many thanks for collaborating with samples and the friendly advice and support you both always gave. I would also like to thank Dr Zara Mohamed, for undertaking a sister project, without her collaboration, I would still be scoring slides. A thank you to my advisor Dr Elizabeth Mallon, your support was very much appreciated. Thank you to Colin Nixon in department of histology, Beatson for his expertise with the work-up of the NIS antibody. Mr Iain White, a true IT guru, thank you.

My Mum and Dad- Esther and James, thank you both from the bottom of my heart. Without your love, support and grandparenting this would have been unmanageable. Jan and Normski, thank you for taking great care of Ally and Ru and your patience with me. Special mention to Gav, thanks Gav- you are a total star! My friends, particularly Ing, T and Carol for listening to me moan, a lot.

I would like to thank everyone who has helped me at any point over the course of my thesis.

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Abbreviations

AI	Aromatase Inhibitors
ASCO	American Society of Clinical Oncology
ATAC	Arimidex, Tamoxifen, alone or in combination Trial
BIG-1-98	Breast International Group
CAP	College of American Pathologists
CER	Combined Endocrine Receptor
c.c	correlation coefficient
COS	Clinical Outcome Score
CI	confidence Interval
CMF	Cyclophosphamide, Methotrexate and 5-Flourouracil
DCIS	Ductal Carcinome in situ
DFS	Disease Free Survival
E1	Oestrone
E2	Oestradiol
E3	Oestriol
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen Receptor α
ER+	Oestrogen Receptor +
ERE	Oestrogen Response Elements
FISH	Fluorescent In situ Hybridization
GEP	Gene Expression Profile
HR	Hazard Ratio
HER2	Human Epidermal Growth Factor Receptor 2
HSP	Heat shock proteins

JNK	Janus Kinase
IHC	Immunohistochemistry
LBA	Ligand Binding Assay
LBD	Ligand Binding Domain
mRNA	messenger RNA
MINIDACT	Microarray in Node Negative Disease May Avoid ChemoTherapy
MAPK	Mitogen activated protein kinase
NaClO4	perchlorate
NCCN	National Comprehensive Cancer Network
NSABP	National Surgical Adjuvant Breast and Bowel Project
NST	No Special Type (Ductal Carcinoma)
NIS	sodium iodide symporter
PI3K	Phospatidylinositol-3 kinase
PTEN	Phosphatase and tensin homolog
pMAPK	phosphorylated p44/42 MAPK
PgR	Progesterone Receptor (isoforms A and B)
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RS	Recurrence Score
RXR	Retinoid X Receptor
SERM	Selective Oestrogen Receptor modulators
SLC5A5	Solute carrier family 5A5 (NIS)
tRA	all trans Reinoic acid
TAILORx	Trial Assigning IndividuaLized Options for Treatment (Rx)
TMA	Tissue microarray

Summary

Over 1 million women a year are diagnosed with Breast Cancer. The majority, approximately 70% express the oestrogen receptor (ER). ER positive breast cancer has historically been perceived as a 'good cancer', although many women with ER+ breast cancer still succumb to their disease and globally breast cancer is the leading cause of female cancer deaths. The advent of gene expression profiling and the definition of the molecular instrinsic subtypes has defined at least two subtypes of ER positive breast cancers (luminal A and luminal B) that differ markedly in terms of biological behaviour, response to adjuvant therapies and most importantly patient outcome.

The focus of this research is ER+ breast cancer and targeting patient therapy in this heterogeneous group. This work attempts to translate our understanding of the biology of the ER and cell signalling interactions to aid the correct identification of patients for both current therapy and more novel therapeutic approaches.

Following a hypothesis generating pilot study examining whether the level of ER influences response to endocrine therapy, 557 formalin fixed paraffin embedded (FFPE) breast cancer specimens retrieved at time of definitive surgery from early breast cancer patients with available accurate 15 years follow up data were analysed to measure ER, Progesterone receptor (PgR), HER2 and Ki67 expression using immunohistochemistry. Tumour expression of ER, PgR and the combined endocrine receptor (CER), which considers the expression level of both hormone receptors and hypothesised to more accurately quantify endocrine responsiveness by acting as a surrogate marker of a functioning ER signalling pathway, were analysed. The results suggest that in this cohort of ER+ endocrine treated patients CER is a better predictor of endocrine response than either the ER or PgR independently. The CER was thereafter utilised as a surrogate marker of oestrogen receptor signalling pathway to develop a scoring system which included HER2 IHC expression and tumour histological

grade, as surrogate markers of the 3 key pathways (ER signalling, HER2 signalling and proliferation). These were chosen as previous studies comparing various gene prognostic profiles indicate commonality in sampling groups of the genes representing their activation. The scoring system, named the Clinical Outcome Score (COS) was developed to represent a pragmatic equivalent of gene prognostic profiles utilising currently routinely measured tumour markers. We hypothesised that COS as an indicator of tumour biology may aid identification of risk in the very challenging group, ER+/HER2 negative patients with intermediate grade and low disease burden, and may help guide adjuvant therapy decisions particularly the indication for chemotherapy . In this exploratory analysis, the distribution of COS scores (2-10) followed a linear response with a notable separation between low scores (2-4) and high scores (5-10). Importantly, when analysed in combination with tumour burden, low COS may help identify patients with nearly 100% long term survival, however in all analysis high COS was associated with a highly significant poorer outcome in terms of early recurrence, late recurrence and 15 year breast cancer specific survival. This group of high risk ER+ breast cancer patients represent a real challenge (and concern) in the treatment of early breast cancer, as there is increasing evidence that ER+ tumours are relatively chemo-in senstive and the response to chemotherapy agents is limited. As a secondary analysis, within our cohort of ER+/ HER2- endocrine treated patients we retrospectively analysed the benefit of chemotherapy in patients with low and high COS scores and the results indicate lack of benefit in the cohort of patients diagnosed 1995-1998. Investigating novel therapeutic targets focusing on the subtypes of breast cancer, and tumour biology involved in endocrine resistance is now beginning to take precedence in breast cancer research.

Two potential new therapeutic targets in ER+ breast cancer were studied. The first is the sodium iodide symporter, NIS, a transmembrane glycoprotein which has been exploited for the safe delivery of radio-iodide in the treatment of thyroid cancers for many years. NIS is

expressed in many breast cancers, however most breast cancers expressing NIS lack functional uptake as demonstrated by scintography studies and in vivo animal work. In vitro results suggest that the ER is important in NIS regulation and function. In addition MAPK and PI3K-Akt signalling pathways may have a role in NIS regulation- both these pathways are often activated in ER+ breast cancer and known to have extensive crosstalk with the ER. Utilising ER+ and ER negative breast cancer cell lines we examined NIS function following gene delivery with a human NIS (hNIS) transfected plasmid and assessed function and expression of NIS following ER knockdown by siRNA. Our results suggest that the ER phenotype is important but not necessarily the ER *per se*. We examined NIS expression in a mixed ER+ and ER- cohort (n=50) of patient tumour samples using real time RT-PCR, and report high levels of NIS mRNA expression was limited to ER+ breast tumours. Prompting analysis of NIS expression, cellular location and correlations with cell signalling proteins in 300 ER+ breast cancers using IHC . Significant correlations were identified with key members of the PI3K-Akt and MAPK supporting their role in NIS regulation in vivo. Importantly, in both patient cohorts NIS was found to be significantly associated with poor outcome, and we hypothesize that this is an effect of enhanced growth factor signalling and activation of pathways in biologically more aggressive ER+ cancer (ER+/PgR-) may also regulate NIS and suggest future directions of research. Lastly, as a pilot study expression of Src kinase, a non receptor tyrosine kinase implicated in tamoxifen resistance and breast cancer virulence, was analysed by IHC in the ER+ breast cancer patient cohort. Interestingly nuclear Src kinase was found to be associated with improved outcome and hypothesise that Src Kinase expression in breast cancer may have varying roles in the different subtypes of breast cancer, an important consideration as Src Kinase inhibitors are currently in clinical trials. This pilot study formed a hypothesis that was subsequently examined in another student's PhD thesis.

1 Introduction

1.1 Breast Cancer: Incidence, Mortality and trends

Breast Cancer is the most common cancer in the UK, despite the fact it is rare in men. In 2008 there were 48,034 new cases of breast cancer (>99% of these in woman). The estimated lifetime risk of developing a breast cancer for a woman living in the UK is 1 in 8 [1] and the incidence rates of breast cancer are rising.

Breast cancer mortality rates in the UK have fallen dramatically, in 1989 15,626 woman died from breast cancer and this fell to 12,047 in 2008. The reduction in breast cancer mortality rates is likely to have several different causes including screening, increasing specialisation of care and the widespread adoption of tamoxifen treatment since 1992. It is however, still the second most common cause of cancer deaths in woman, accounting for 15% of cancer related deaths in the UK, 2010 [1]. Globally, breast cancer is the most frequently diagnosed cancer, and the leading cause of cancer death in females [2]

1.2 Breast Cancer Diagnosis

The NHS breast screening programme was introduced 1988-1991 and included woman 50-64years. The upper age limit was extended to 70 years in 2000 and this is currently being extended to 47-73 years. In the UK screen detected cancer accounts for over half of all breast cancers diagnosed in patients aged 50-70 and over 30% of all cancers diagnosed [3]. The aim of screening is early detection. Breast cancers that are smaller or non-palpable have a more favourable prognosis.

The majority of symptomatic presentations are with a painless breast lump. Unilateral, focal breast pain only accounts for 5% of breast cancer presentations. Patients may also present with unilateral nipple changes or inflammatory cancers may occasionally present as an acute cellulitis/ abscess.

Patients with suspected breast cancer are assessed at a “one-stop/ triple assessment clinic”. This includes clinical and radiological evaluation in all cases of suspected breast cancer. The final stage of triple assessment is pathological (tissue) assessment and involves taking either a core biopsy or a fine needle aspirate (FNA) of the suspicious area to confirm the diagnosis of cancer. Most clinicians will consult with patients at the end of the one stop clinic and in cases of suspected breast cancer prepare the patient for her awaited conformational / pathological diagnosis.

All hospitals in the UK that run a specialist breast service have a Breast Cancer Multidisciplinary team (MDT). This is a well-established group of experts with a specialist role in the diagnosis, treatment and management of people with breast cancer. The team comprises doctors, nurses and other healthcare professionals who manage the treatment of breast cancers. Key members of the MDT are surgeons, oncologists, radiologists, pathologists, specialist breast nurses and an MDT co-ordinator. The team meets weekly, in confidence, to discuss newly referred patients with a suspected or confirmed diagnosis of breast cancer. The MDT meetings offer a forum for the team members to plan and agree a recommended programme of treatment specific to individual patient needs. This approach ensures that all necessary investigations are carried out as quickly as possible and the best available treatment is offered. Treatment options are then discussed with the patient and their family at a subsequent appointment soon after.

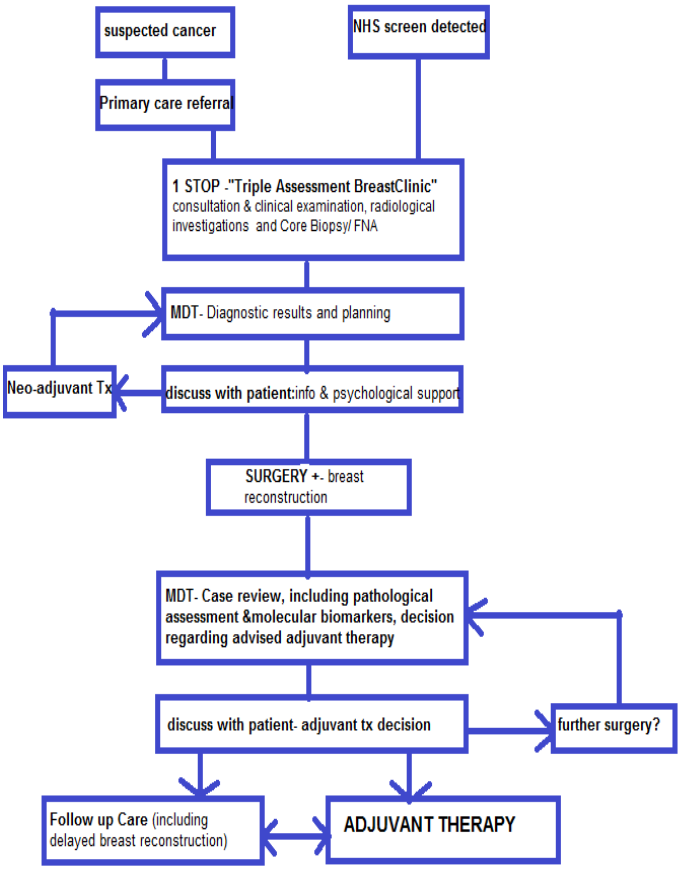


Figure 1-1 Flow Diagram of the Diagnostic and treatment pathway in Early Breast Cancer

1.3 Stage at presentation

The diagnosis of breast cancer includes carcinoma in situ, early breast cancer and advanced breast cancer, stages of the disease that differ in terms of tumour burden (or anatomical extent of disease) and differ markedly in terms of outcome and treatment strategy. The tumour node metastasis (TNM) staging system for breast cancer is an internationally accepted system used to define accurately the disease stage. This is used to guide management and determine prognosis. The tumour node metastasis (TNM) staging system for breast cancer classifies tumours on the basis of the primary tumour type (invasive or in situ) and size (T), the presence or absence of regional lymph node involvement (N), and the presence or absence of distant metastases (M). The overall stage of the tumour (stage 0 through IV) depends upon

the particular combination of T, N, and M characteristics. Periodic revisions are necessary because advanced imaging techniques and treatments evolve and impact survival. Using the TNM staging system, 5 staging groups (0-IV) exist according to the extent the disease has spread at time of presentation. For the purpose of discussing management strategies it is more useful to collapse these into three subgroups:

1. Carcinoma in situ, Stage 0
2. Early breast cancer, Stages I and II
3. Advanced breast cancer, Stages III and IV.

1.3.1 Carcinoma in situ (Stage 0)

Ductal Carcinoma in situ (DCIS) of the breast represents a heterogeneous group of neoplastic lesions confined to the breast ducts and lobules, and on histological examination have not yet invaded the breast stromal tissue. Most cases of DCIS are detected only on imaging studies (most commonly by the presence of mammographic microcalcifications). Its diagnosis has increased dramatically with the introduction of breast cancer screening mammography, and accounts for 25% of screen detected cancers [4].

The aim of treatment for DCIS is to prevent the development of invasive breast cancer. The mainstay of treatment is surgical +/- radiotherapy. Mastectomy achieves excellent long-term survival with a local recurrence rate of less than 1 percent, although in some instances this may be overly aggressive treatment. Patients with a lesion limited to one quadrant or section of the breast are candidates for Breast Conserving therapy (BCT). BCT is followed by adjuvant radiotherapy and has less morbidity but is associated with a higher risk of local recurrence, although similar survival outcome. The role of adjuvant endocrine therapy in DCIS lesions expressing the hormone receptors is debatable, and not universal standard practice.

Lobular carcinoma in situ (LCIS) is a noninvasive lesion that arises from the lobules and terminal ducts of the breast and very uncommon. It almost always represents an incidental finding that is diagnosed on a breast biopsy that is performed for some other reason. LCIS is not identified clinically, mammographically, or by gross pathologic examination, and it is not a precursor lesion for invasive breast cancer. The importance of LCIS is that it is an indicator lesion for the risk of bilateral invasive ductal or lobular carcinoma. For LCIS, postexcision treatment options include careful surveillance, or bilateral prophylactic mastectomy. Some experts favour prevention and advise endocrine therapy, although its value is debatable.

1.3.2 Early Breast Cancer (Stage I-II)

The majority of women with breast cancer present with early stage disease. These breast cancers are amenable to primary local surgical intervention. The goal of therapy is cure, and at 5 years the estimated survival rate for Early Breast Cancer (stage I-II) is 70-95%.

The primary management of early breast cancer involves surgery +/- radiotherapy (loco-regional therapy) and followed in most cases with systemic adjuvant therapy. The term early breast cancer encompasses a very heterogenous group of invasive tumours, with different biological behaviour and pathological assessment is important to adjuvant treatment recommendations. This is discussed in detail below.

1.3.3 Advanced Breast Cancer (Stage III-IV)

Locally Advanced Breast Cancer

Locally advanced breast cancer accounts for 10% of newly diagnosed breast cancers. It includes breast cancers with advanced primary tumours (such as skin and/or chest wall involvement, very large cancers >5cm or inflammatory cancers) and patients with advanced regional lymph node involvement. Most cases of locally advanced breast cancer are visible and palpable on clinical examination, and most are inoperable at first presentation.

Locally advanced breast cancer is best managed with multimodality therapy employing systemic and loco-regional therapy with the aim of long term disease free survival. Neoadjuvant (upfront) systemic therapy, in particular neoadjuvant chemotherapy and biological therapy if indicated, has become the standard approach for patients to enable operability. This is followed by loco-regional treatment after response. 5 years survival rates for locally advanced breast cancer (Stage 3) are 50%.

Metastatic Breast Cancer

Fewer than 10 percent of women present with metastatic disease at the time of diagnosis. However, the majority of women who relapse after definitive therapy for early stage or locally advanced disease will do so with disseminated metastatic disease rather than an isolated local recurrence. The most common sites of distant tumour involvement are bone, liver, and lungs. The primary goals of systemic treatment for metastatic breast cancer are prolongation of survival, alleviation of symptoms, and maintenance or improvement in quality of life. Although metastatic breast cancer is unlikely to be cured, meaningful improvements in survival have been seen, coincident with the introduction of newer systemic therapies. Median overall survival approaches two years, with a range from a few months to many years[5].

1.4 Treatment of Early Breast Cancer

The treatment of early stage breast cancer includes the treatment of loco-regional disease with surgery, radiation therapy, or both, and in most cases adjuvant medical treatment with one or a combination of chemotherapy, endocrine therapy, or biologic therapy.

Loco-regional Treatment

Surgery is considered primary treatment for early breast cancer. The goals of breast cancer surgery include complete resection of the primary tumour with negative margins to reduce

the risk of local recurrences, and pathologic staging of the tumour and draining axillary lymph nodes for providing necessary prognostic information. Several different types of operations are available for the treatment of breast cancer and the decision is based on size and location of tumour relative to the breast, clinical and radiological assessment of draining lymph nodes and patient fitness and informed choice.

Mastectomy is the complete surgical resection of all the ipsilateral breast tissue. A number of types of mastectomy exist. Simple mastectomy preserves the pectoralis muscles and axillary contents, and with the advent of sentinel node biopsy this is more frequently performed. Breast Conservation Treatment (BCT) refers to surgical removal of the tumour (with negative surgical margins- ideally 1cm margin around the lesion) followed by radiotherapy to eradicate any local residual disease. The goals of BCT are to provide a cancer operation equivalent to mastectomy and a cosmetically acceptable breast, with a low rate of recurrence in the treated breast. This is often cosmetically and psychologically preferable to the patient. The National Surgical Adjuvant Breast and Bowel Project's B-06 (NSABP-B06) was a landmark study that established breast-conserving surgery with radiation therapy to be equivalent to modified radical mastectomy [6]. A number of prospective randomised control trials comparing BCT with mastectomy and an overview of all completed trials [7] has shown equivalent survival between the two treatment approaches. Breast reconstruction has grown significantly in popularity and for woman requiring mastectomy confers significant psycho-social benefit. Reconstruction can be performed at the time of primary surgery or as a delayed procedure.

Axillary surgery should be undertaken in all patients with invasive cancer, and in most cases this is combined with breast surgery in one procedure. Spread of metastatic disease to axillary nodes is one of the most significant prognostic indices and is used in decision making regarding appropriate systemic therapy. Up until fairly recently, axillary clearance was

considered the standard of care for all patients diagnosed with invasive breast cancer, however, this carries a high rate of surgical morbidity. Clearance is still undertaken in patients with clinically or radiologically involved nodes, but in patients without clinical or radiological diagnosis of axillary node involvement it is now standard practice to perform 'minimal axillary surgery' designed to stage the axilla such as sentinel lymph node (SLN) biopsy or axillary sample, because it offers accuracy equivalent to that of axillary lymph node dissection with less morbidity.

The purpose of external beam radiation therapy is to eradicate local subclinical residual disease and minimise local recurrence rates. It is standard practice following all breast conservation surgery. Adjuvant chest wall radiotherapy following mastectomy is indicated in patients with high risk of loco-regional recurrence this is usually when the resection margins are positive, the tumours are large or there is substantial axillary nodal involvement (≥4 nodes +). External beam radiotherapy to the axilla is currently not indicated in patients with lymph node negative disease. Following minimal axillary surgery, radiotherapy to axilla may be undertaken when nodes subsequently demonstrate microscopic invasive cancer on pathological examination. Following axillary lymph node dissection in patients with ≥4 + nodes the supraclavicular field should be irradiated, in cases with 1-3+ nodes and other poor prognostic factors additional irradiation of the supra clavicular field may be offered.

1.5 Adjuvant Medical Therapy in Early Breast Cancer: Prognostic & Predictive Factors

Adjuvant systemic therapy is administered following primary surgery for early breast cancer to prevent breast cancer recurrence and to improve overall survival. Patient selection is important because not all patients receive benefit from adjuvant therapy and because it is associated with significant toxicities, therefore establishing risk and potential benefit is a priority. A prognostic factor is a factor that is associated with clinical outcome, typically a

time-to-event outcome such as overall survival or recurrence-free survival. (Some individuals adhere to a more strict definition of prognostic marker as applying only to the natural history of patients who received no treatment following local therapy). In contrast, a predictive factor is a factor that is associated with response to a given therapy. In breast cancer management prognostic markers are used to identify patients most at risk of poor outcome and in whom further adjuvant systemic therapy would be beneficial, and predictive markers used to identify which patients will or will not respond to given therapies. A large number of both prognostic and predictive factors have been proposed in breast cancer, yet relatively few of these are in routine clinical use[8].

1.5.1 Identifying "Risk": prognostic markers

Prognostic markers assessed by histopathology include tumour size, lymph node involvement, tumour type, tumour grade and presence of lymphovascular invasion. They are all included in the routine pathological examination using traditional hematoxylin and eosin (H&E) light microscopy[8]. These prognostic markers are indicators of growth, invasion and metastatic potential of the tumour. Tumour burden (anatomical extend of disease) as defined by tumour size and lymph node status has traditionally been the basis for most adjuvant chemotherapy recommendations.

Tumour Size

Tumour size is part of the TNM stage- larger tumours have worse prognosis than smaller tumours [9, 10]. Larger tumours are more frequently associated with nodal involvement, although nodal metastases have been reported in up to 20% of tumours <10mm [11] and in tumours 5mm [12].

Lymph node Involvement

Tumour spread to the axillary nodes is probably the most important factor in predicting disease free and overall survival in early and locally advanced breast cancer[8]. 10 year survival for lymph node negative disease is 75% compared to 25-30% with positive nodes [13]. Nodal involvement may indicate breast cancers that have been present for longer (ie delay in presentation) or may indicate an aggressive tumour subtype. The number of nodes involved is important and staged (different from TNM staging). Prognosis is poorer with increasing stage: Stage1= no nodes involved; Stage 2= less than three positive nodes and Stage 3= four or more lymph nodes involved [11, 14].

Tumour type

Tumour type provides information on tumour differentiation and biological behaviour (ie tendancy to metastases, and expression of markers) but the actual prognostic value on multivariate analysis is small [8, 15]. At least 18 different morphological types of breast cancer have been described [16]. In order to improve reproducibility, stricter classification has been introduced [17].

- When <50% of tumour has no special type characteristics it is No Special Type (NST), Ductal
- When 50-90% of tumour has a specific morphological pattern it is MIXED
- When >90% of tumour has special type characteristics it is pure SPECIAL type

NST (still referred to by many as infiltrating/Invasive Ductal) is the most commonly diagnosed breast cancer and has a tendency to metastasize via lymphatics. This accounts for 70-75% of breast cancers. It has no specific histological characteristics other than invasion through the basement membrane. DCIS is a frequently associated finding on pathologic examination.

20% of breast carcinomas are of special type and the majority of these are lobular carcinomas. Tubular and mucinous carcinomas occur next most frequently and thereafter the remaining special types are seen infrequently. In order to make a diagnosis of a special type of carcinoma >90% of the tumour is required to show the particular pattern. Special types of carcinoma should be distinguished from mixed carcinomas where the special type areas occupy between 50 and 90% of the tumour area with the remaining area being usually of no special type.

Lobular Carcinoma is characterized histologically by the “Indian file” arrangement of small tumor cells. Like ductal carcinoma, infiltrating lobular carcinoma typically metastasizes to axillary lymph nodes first. However, it also has a tendency to be more multifocal. 90% of lobular carcinomas are grade 2. Specific histological features are characteristic of the other special types such as invasive tubular, mucinous, cribiform, medullary and very rarely metaplastic, apocrine, micropapillary and adenoid cystic.

Excellent Group: >80% 10 yr survival • Tubular • Invasive Cribiform • Mucinous • Tubulolobular
Good Group: 60-80% 10 year survival • Tubular mixed • Alveolar lobular • Mixed ductal no special type (NST) and special type
Moderate Group: 50-60% 10 year survival • Medullary • Atypical medullary • Invasive papillary • Classical lobular
Poor Group: <50% 10 year survival • Mixed lobular • Solid lobular • NST (Ductal) • Mixed ductal NST/ lobular

Table 1-1 Prognostic value of tumour type

Prognostic value of tumour type ,adapted from ref [8]

Tumour Grade

The grading of a cancer in the breast depends on the microscopic similarity of breast cancer cells to normal breast tissue, and classifies the cancer as well differentiated (low grade), moderately differentiated (intermediate grade), and poorly differentiated (high grade), reflecting progressively less normal appearing cells that have a worsening prognosis. The Nottingham (also called Elston-Ellis) modification of the Scarff-Bloom-Richardson grading system is recommended [17]. This assesses 3 components of invasive breast cancer (gland/tubule formation, atypical/pleomorphic/nuclear size, mitotic count)

Tubule formation: assesses what proportion of the entire tumour is forming acini with definitive lumen (ie normal duct structure).

- 1 point: tubular formation in more than 75% of the tumour
- 2 points: tubular formation in 10 to 75% of the tumour
- 3 points: tubular formation in less than 10% of the tumour

Nuclear pleomorphism: this assesses the uniformity of the cell nuclei comparing them to normal breast duct epithelial cells nuclei. The areas of greatest pleomorphism should be graded.

- 1 point: nuclei with mimimal variation in size and shape
- 2 points: nuclei with moderate variation in size and shape
- 3 points: nuclei with marked variation in size and shape

Mitotic count: mitotic figures counts are performed in the most mitotically active areas (often the tumour periphery), only unequivocal areas of mitoses are counted (apoptic and anaphase ignored).The score (1-3) depends on the magnification and type of microscope used. Tumour fixation prior to assessment should be performed quickly, as delay may lead to inaccuracy and under scoring of the mitotic count.

Overall grade: the scores for each of these three criteria are added together to give a final overall score and a corresponding grade as follows:

- 3-5 **Grade 1** tumour (**well-differentiated**)
- 6-7 **Grade 2** tumour (**moderately-differentiated**)
- 8-9 **Grade 3** tumor (**poorly-differentiated**)

Histological Grade has been demonstrated in multivariate analysis to have prognostic value similar to lymph node stage [18-20]. A trained pathologist can easily and quickly grade tumour specimens and this is routine practice in all invasive breast cancers [8]. The reproducibility of histological grade procedures has been examined and several studies have shown that with the scoring criteria detailed above, there is 80-87% agreement [21-23]. Grade is associated with the histological type of tumour, whilst invasive ductal carcinomas and invasive cancers of no special type can be grade 1-3, tubular carcinomas are all grade1 and invasive lobular carcinomas are typically grade2, although grades 1 and 3 can occur.

Tumour NPI

Although lymph node stage & grade are well-recognized predictors of outcome, independently they are relatively poor discriminators, for example neither grade or lymph node stage can identify a group of patients with nearly 100% survival. Maximal use of the known prognostic factors can be made when they are combined in a prognostic index identifying groups with a very good and a very poor out-come [8]. The Nottingham Prognostic Index (NPI) includes lymph node stage scored from 1 to 3(as described above), is added to histological grade (1, 2 or 3) and to 0.2 x tumour size (in centimetres). Cut-off points of 2.4, 3.4, 4.4, 5.4 and 6.4 can be used to stratify the patients into groups (excellent, good, moderate I, moderate II, poor and very poor). Based on the NPI score, decisions can be made regarding likelihood of survival and thus the appropriateness of adjuvant therapy.

1.5.2 Identifying 'Benefit': Predictive Markers

Despite the huge amount of resources allocated to translational research endeavours, only three predictive markers are utilised to define therapy of breast cancer patients, oestrogen receptor (ER) and progesterone receptor (PgR), the predictive markers of response to endocrine therapy, and human epidermal growth factor receptor 2 (HER2), the molecular target of trastuzumab (herceptin).

The Hormone Receptors, ER and PgR

Tumour ER expression is a powerful predictor of response to endocrine therapy and its value is undisputed, the role of PgR as a predictive factor is less well defined. As will be described in detail below, the presence of the ER also defines 'ER positive breast cancer' a crude term for a group of heterogenous cancers that are collectively characterised by the presence of the ER+/- PgR. Although endocrine therapy has revolutionized ER+ breast cancer treatment and substantially improved outcomes for patients, the optimal adjuvant therapeutic management remains a significant challenge.

HER2

Approximately 20 percent of breast cancers have amplification and/or overexpression of the gene encoding the cell surface receptor HER2. Over expression is associated with poor prognosis, however interpretation of data is influenced by the survival benefits of trastuzumab therapy in patients with HER2 over expressing tumours, and subsequently in clinical practice its role is that of a predictive factor rather than prognostic. High levels of HER2 expression identify those women who benefit from treatment with agents that target HER2, such as the monoclonal antibody trastuzumab [24]. Multiple randomized trials indicate a significant survival benefit when this drug is applied in the adjuvant setting for early HER2-positive breast cancer.

The most widely used method for measuring HER-2 over expression is immunohistochemistry (IHC) in breast cancer, this is semiquantitative and based of four classes (0/1+/2+/3+), table 1-2.The optimal testing algorithm for assessing HER2 status in breast cancer as well as strategies to assure optimal performance, interpretation, and reporting of individual assays was addressed in a joint guideline from an expert panel of ASCO and the CAP [25]. A positive result is defined as uniform intense membrane staining of >30% of invasive tumour cells, alternatively a positive result is amplified HER2 gene copy number by

FISH (average of more than six gene copies per nucleus for test systems without an internal control probe or an HER2/CEP 17 ratio of more than 2.2 where CEP is a centromeric probe for chromosome 17 on which HER2 resides). The panel defined equivocal categories for HER2 testing that were meant to trigger reflex HER2 testing using an alternative validated assay (IHC if FISH equivocal, FISH for an equivocal IHC). About 24 percent of IHC 2+ tumours have gene amplification when tested by FISH [26, 27].

Score 0	No staining is observed or cell membrane staining is observed in less than 10% of the tumour cells. ("negative").
Score 1+	A faint perceptible membrane staining can be detected in more than 10% of the tumour cells. The cells are only stained in part of their membrane. ("negative").
Score 2+	A weak to moderate complete membrane staining is observed in more than 10% of the tumour cells. ("weakly positive"). Equivocal.
Score 3+	A strong complete membrane staining is observed in more than 30% of the tumour cells. "Positive"

Table 1-2 HER2 immunohistochemistry (IHC) scoring guide

1.6 Adjuvant Medical Therapy for Early Breast Cancer: Treating a Heterogenous Disease

It is no longer tenable to consider breast cancer as a single disease [28]. Microarray based gene expression profiling studies have brought to the fore the concept that breast cancer consists of a collection of different diseases. The class discovery studies carried out by Perou et al and Sorlie et al revealed that ER positive and ER negative cancers are fundamentally distinct diseases at the molecular level [29, 30]. Advances in gene expression microarray analysis have resulted in the recognition at least four molecular instrinsic subtypes of breast cancer which differ in biological behaviour and response to therapy, namely luminal, HER2 enriched, basal like and normal like.

The technique uses microarrays to which cDNA or oligonucleotide probes have been affixed and simultaneously measures the expression of thousands of genes in a breast cancer cell. The gene expression is measured in a semi quantitative manner by determining the level of mRNA that is then compared to the mRNA of the same gene from a reference sample. There are two main types of molecular profiling in common use in the laboratory: unsupervised and supervised analyses. Unsupervised analysis (or clustering) permits examination of gene expression patterns heedless of clinical endpoints and reflects inherent biologic differences. Supervised analyses are those in which the gene sets are designed to differentiate tumours by a defined clinical endpoint. In addition, semi-supervised analysis combines both gene expression data and clinical data, in that some clinical data is used to identify a list of genes that correlate with the clinical variable(s) of interest and then unsupervised clustering techniques are applied to this subset of the genes [31]. The intrinsic subtypes are a semisupervised example, whereas prognostic molecular profiles (prognostic signatures) are examples of supervised analyses.

The list of genes that differentiates the subtypes is called the intrinsic list and is made up of several clusters of genes relating to ER expression (the luminal cluster), HER2 expression, proliferation, and a unique cluster of genes called the basal cluster. The intrinsic subtypes segregate into two groups that correspond to expression of hormone receptor-related genes [29]. The luminal cancers, luminal A and luminal B, have overlap with ER-positive breast cancers. The remaining subtypes characterized by low expression of hormone receptor-related genes (ER-negative).

Luminal subtypes

The name "luminal" derives from similarity in expression between these tumours and the luminal epithelium of the normal breast; they typically express luminal cytokeratins 8 and 18. These are the most common subtypes, make up the majority of ER positive breast cancer, and

are characterized by expression of ER, PR, and other genes associated with ER activation. Luminal A and luminal B have some important molecular and prognostic distinctions.

- Luminal A, approximately 40% of all breast cancers, usually have high expression of ER-related genes, low expression of the HER2 cluster of genes, and low expression of proliferation-related genes [32, 33]. Luminal A has the best prognosis of all breast cancer subtypes [29, 34-37].
- Luminal B, approximately 20% of all breast cancers and have relatively lower (although still present) expression of ER-related genes, variable expression of the HER2 cluster, and higher expression of the proliferation cluster. Luminal B tumours carry a worse prognosis than luminal A [37].

Reliable and reproducible differentiation between luminal A and B has been questioned [38]. The main difference between these sub types is the expression of proliferation-related genes. Unlike ER and HER2 mRNA expression, which displays a bimodal distribution, the expression levels of proliferation related genes form a continuum in luminal cancers [39]. Therefore, no natural cut-off to separate luminal A and B cancers exist.

HER2-enriched

The HER2-enriched subtype (previously the HER2+/ER- subtype) makes up about 10 to 15 percent of breast cancers and is characterized by high expression of the HER2 and proliferation gene clusters, and low expression of the luminal cluster. Tumours are typically negative for ER and PR, and positive for HER2. This subtype comprises only about half of clinically HER2-positive breast cancer (the other half have high expression of both the HER2 and luminal gene clusters and fall in a luminal subtype). In the era before HER2-targeted therapy, this subtype carried a poor prognosis [37]. This adverse natural history has been markedly affected by therapeutic advances in HER2-directed therapy.

Basal-like

The basal-like subtype, so called because of some similarity in expression to that of normal breast basal epithelial cells, makes up about 15 to 20 percent of breast cancers. It is characterized by low expression of the luminal and HER2 gene clusters. For this reason, these tumours are typically ER-, PR-, and HER2-negative on clinical assays, often referred clinically as the "triple negatives". However, while most triple negative tumours are basal like and vice versa, there is about 30% disconcordance between these two classification methods. Basal like tumours have high expression of the proliferation cluster of genes, are virtually always high grade, and evidence widespread genomic instability even early in the disease. They also have high expression of the epidermal growth factor receptor (EGFR, member of the same family of receptors as HER2), as well as a unique cluster of genes called the basal cluster, which includes basal epithelial cytokeratins 5, 14, and 17.

Basal-like breast cancer has a strong association with hereditary cancers arising in women born with a mutation in BRCA1 gene, over 80 percent of these hereditary cancers are basal-like [34, 40-42]. Most basal-like breast cancers are sporadic, however, and the BRCA1 gene and protein appear intact in these tumours. Basal-like breast cancer carries a poor prognosis, and hence is the subject of intense research finding modern chemotherapy agents to target this aggressive subtype.

Claudin-low

The sixth subtype found in non-basal triple-negative breast cancers is the more newly described claudin-low subtype [43].It is uncommon but interesting because of its expression of epithelial-mesenchymal transition genes and characteristics reminiscent of stem cells.

Normal-like

The normal-like subtype was one of the initial subtypes identified by gene expression array and consistently appears in breast cancer clusters. Typified by similar gene expression pattern

as normal breast, it remains unclear as to whether it represents a separate subtype or a technical artefact.

The intrinsic subtypes were developed to identify relevant biology, not as prognostic factors; however, in multiple independent datasets, these subtypes correlate with prognosis. In general, patients with the luminal A subtype have the best prognosis; patients with the other major hormone receptor-positive subtype, luminal B, suffer a significantly worse outcome. Both the basal-like and HER2-enriched subtypes have the worst survival, at least until recently (HER2-targeting therapy has altered the outcome for the HER2-enriched subtype and HER2-positive luminal cancers).

The 12th St Gallen International Breast Cancer Conference (2011) most recent guidelines adopted the intrinsic subtype classification to make recommendations on therapeutic decision making in early breast cancer[28], recognising that each subtypes have different responses to adjuvant medical therapy , table 1-3 summarises the recommendations.

Subtype	Recommended therapy	Notes
Luminal A	Endocrine therapy	Less responsive to chemotherapy, although consider chemo in high risk*
Luminal B/HER2 neg	Endocrine therapy+/- chemotherapy	Inclusion of chemotherapy may depend on level of hormone receptor*
Luminal B/HER2+	Chemotherapy + anti-HER2+ endocrine therapy	
HER2 positive (non-luminal)	Chemotherapy+ antiHER2	
Triple Negative (Basal)	Chemotherapy	

Table 1-3 Systemic treatment recommendations for subtypes in early breast cancer.

*Adapted from Goldhirsch et al, 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 2011 [28]. *There was a lack of complete consensus on the threshold indication for inclusion of chemotherapy in patients with luminal A or luminal B/ HER2 negative disease.*

Although the molecular subtypes have now become part of the lexicon of breast cancer research, oncologists, surgeons and pathologists, importantly the information they provide above and beyond that provided by ER, PgR, HER2 and proliferation remains to be fully established [38]

1.7 ER positive Breast Cancer

One of the most fundamental differences in breast cancer tumour biology is the cancer's dependence on hormonal stimulation. The female sex hormones, oestrogen (E2) and progesterone play a pivotal role in normal development, growth and differentiation of the breast. Central to the hormonal action in both normal and cancer cells is the oestrogen receptor (ER). A breast cancer is considered hormonally responsive if it expresses the ER

accounting for 70-75%. More than half of these cancers also express the progesterone receptor (PgR) [44], the PgR is an oestrogen regulated gene and its synthesis in both normal and cancer cells require a functional ER [45]. The advent of gene expression profiling has highlighted that within ER+ breast cancer is a heterogenous group of diseases, and elucidation of ER signalling and tumour biology is fundamental to tumour response to adjuvant therapy and targeting treatments.

1.7.1 Molecular Biology of ER+ breast cancer

In oncogenesis the cancer cell exploits the "normal" cellular interactions and mechanisms. The exploitation of the normal cellular pathways through mutation, mis-regulation, altered cross-talk, altered protein interaction/ function promotes cell survival, replication, invasion and metastasis, and treatment resistance via escape pathways. The ER and PgR, are members of the nuclear receptor superfamily. Their classic mechanism of action is as a ligand activated transcription factor within the cell nucleus (genomic action) influencing a large number of genes involved in growth and development both in the normal cell and in carcinogenesis. In addition both hormone receptors have non- genomic (extra-nuclear) actions involving interaction/ cross talk with a complex array of growth factor receptor and cell signalling pathways.

1.7.2 The ER structure and cell signalling

Central to oestrogen's action in both normal and cancer cells is the oestrogen receptor (ER). There are at least two receptor subtypes, ERα and ERβ, which are not isoforms of each other but rather distinct proteins encoded by separate genes located on different chromosomes. The human ERα is mapped to chromosome 6 and ERβ to chromosome 14. They have a similar overall domain structure, which they share with other members of the nuclear receptor superfamily and their primary function is as ligand activated transcription factor. The discovery of ERβ is only fairly recent and its role in the pathogenesis of breast cancer, and

endocrine resistance remains fairly elusive. ERα (in contrast to ERβ) has been studied in great detail and it serves as a clinically useful predictive factor for endocrine therapy. In further discussion 'ER' should be read as ERα, unless stated otherwise. The ER protein contains 595 amino acids and has a molecular weight of 66kDa. Structurally it consists of an amino-terminal region that harbours the ligand-independent activation domain (AF-1), a central DNA binding domain (DBD), and a carboxy-terminal hormone binding domain (HBD), which contains the ligand-dependant activation function (AF-2). The ER is predominantly a nuclear protein, although much smaller amounts of ER are found in the cytoplasm and cell membrane.

The classic action of the ER is as a ligand activated transcription factor (TF) that modulates the expression of hundreds of genes, either up or down-regulating, genes important for normal cell function and development and tumour growth and survival. Oestrogen diffuses into the cell and binds the ER. Un-liganded ER is held in an inactive state by chaperone proteins (eg. HSP90). Upon E2 binding the receptor transforms into an active state which undergoes homodimerization and binds oestrogen response elements (EREs) in target genes to activate gene expression. With E2 bound both transactivation domains, AF-1 & AF-2, juxtapose and are able to bind coregulator proteins, co-activators or co-repressors protein complexes, to specific sites on DNA. Co-regulators act to further fine tune the action of the ER as a transcription factor [46]. The ER itself can act as a coregulator for other TFs such as AP-1 (activator protein-1) or SP-1 (specificity protein-1). The nuclear functions of the ER that regulate gene transcription via specific response elements in the promoter of target gens are known as the classical/ genomic ER action. The ER transcriptional activity is governed by binding of ligand, receptor phosphorylation status, presence of co-regulatory proteins and the available promoter sequences on specific genes. The mechanisms by which oestrogen

increases breast tumour growth are multiple- the net effect of the genomic actions is to enhance tumour cell survival and proliferation.

The ER signalling pathway is also regulated by membrane receptor tyrosine kinases (RTK). The ErbB family of receptor tyrosine kinases encompasses 4 closely related transmembrane receptors: erbB-1 (EGFR, epidermal growth factor receptor or HER-1), erbB-2 (HER-2 or *neu*), erbB-3 (HER-3), and erbB-4 (HER-4).These interact either directly with extra-nuclear ER or more commonly indirectly through activation of signalling cascades that phosphorylate the ER or its coregulator proteins to modify specific function [46]. Key cell signalling cascades include the Ras/Raf/MAPK pathway which is implicated in cell proliferation, migration and differentiation and the PI3K/AKT pathway, which is a complex powerful branching signalling network that promotes cell survival and proliferation. PTEN is a negative regulator of the PI3K/AKT pathway and frequently mutated in many human cancers. The Src family of non receptor kinases are key intra cellular messangers involved in cell growth, proliferation, angio-genesis and invasion/metastasis. The activation of ER by growth factor receptor signalling is often referred to as ligand independant receptor activation.

Bidirectional crosstalk exists. A huge research effort has identified multiple interactions between growth factor receptor pathway signalling and ER signalling pathway. Oestrogen can influence the expression of some growth factor ligands, which in turn activates the pathways. However, confusingly, oestrogen signalling can result in downregulation of EGFR and HER2 yet increase expression of other growth factor receptors [46]. Activation of PI3K/AKT and the MAPK pathways, often as a consequence of EGFR and HER2 activation, results in down regulation of the expression of both the ER and PgR. Thus, while these receptor tyrosine kinases can activate the transcriptional function of the ER, they can also reduce oestrogen dependence by down regulating the expression of the ER. Some of the

actions of oestrogen on the target cell occur within minutes, too rapid to be a transcriptional effect. The extra nuclear or membrane ER, activated by oestrogen, associates with the growth factor receptors or the downstream signalling molecules to further activate cell pathways involved in cell proliferation, apoptosis, invasion and angiogenesis, thus the ER, via non-genomic activity can alter the expression of genes normally regulated by growth factors. Signalling from the tumour micro environment can activate stress kinase pathways, such as FAK (downstream of Src), JNK and MAPK pathways that can modulate components of the transcriptional machinery, including the ER. A diagrammatic illustration of the ER signalling pathway and associations with growth factor receptor signalling is shown in fig 1-2.

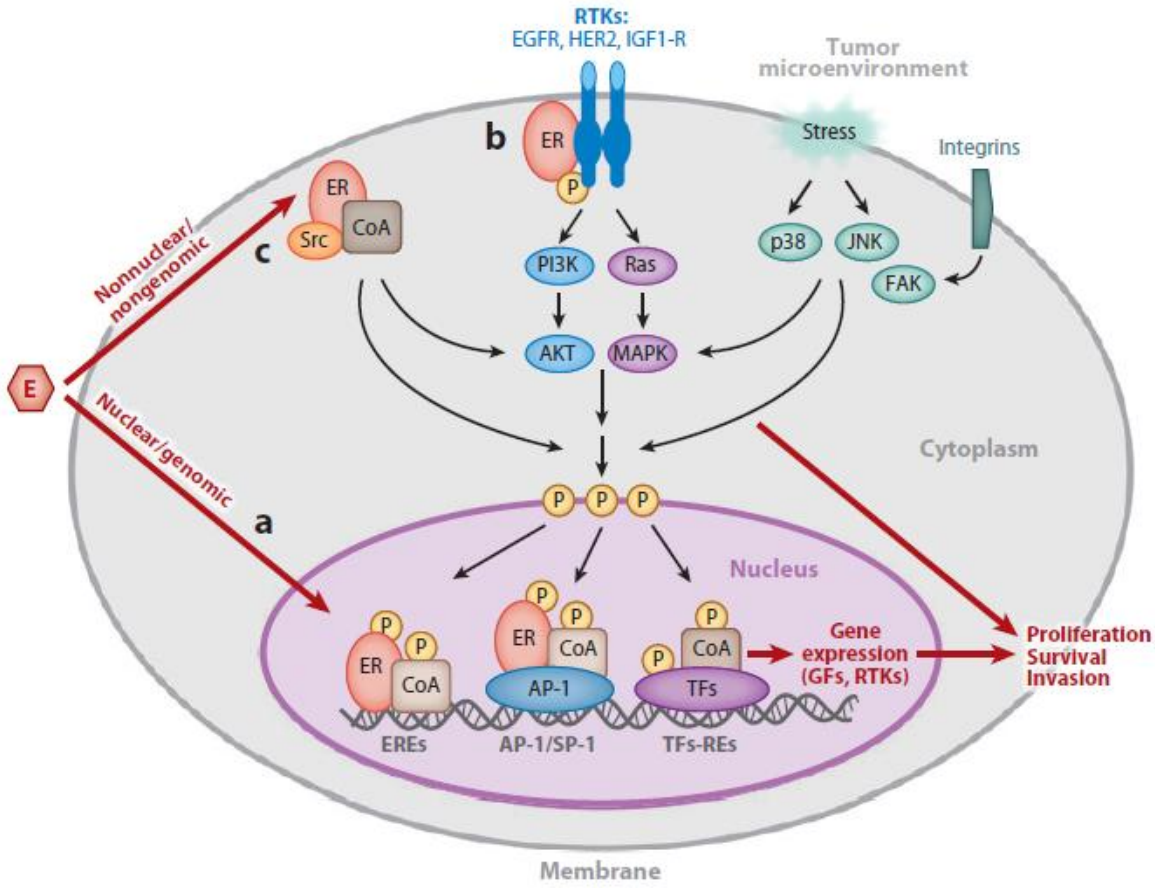


Figure 1-2 Mechanism of ER action in breast cancer

a. Oestrogen (E)-bound ER primarily acts as a TF in the nucleus (genomic action), it binds EREs or other TFs. This recruits other co-activators (CoA) to modulate gene transcription, including genes encoding growth factors and RTKs. b. Extranuclear ER associates in response to oestrogen with RTK. c. Extranuclear ER can also associate with cell signalling molecules, resulting in a similar action to RTK activation, which results activation of multiple downstream pathways including the PI3K/AKT and Ras-Raf-MAPK pathway, which phosphorylate various TFs, including coregulators which are components of the ER pathway to enhance gene expression.d. Signalling from the tumour microenvironment trigger downstream kinase pathways that in turn can modulate ER transcription. Overall both the genomic and non-genomic ER activities work in concert to provide tumour cells with survival, proliferation and invasion stimuli. Adapted from Osborne & Schiff. Mechanisms of Endocrine Resistance. Annu Rev Med, 2011. 62: p. 233-47 [46].

1.7.3 Progesterone Receptor

Like the ER, the progesterone receptor (PgR) is a member of the steroid hormone receptor superfamily. It shares a common overall domain structure, containing an N-terminal domain, a DNA binding domain, a ligand binding domain, multiple activation function sites and a C terminal domain [47]. The PgR exists as 2 isoforms, PR-A and PR-B (the former being a truncated form of the latter), encoded by a single gene. The PgR functions as a transcription factor and regulates a number of genes involved in normal mammary development and breast cancer. PgR is critical for lobuloalveolar development in the normal mammary gland [48]. Recent interest into the different ratio of isoform expression (PR-A and PR-B) in cancer development reports over expression of PR-A may be associated with tamoxifen resistance [49]. Additionally, over abundance of PR-B as a result of a functional promoter polymorphism has been associated with increased risk of breast cancer[50]. The molecular mechanisms of PgR expression (and the isoforms) and its effects on cell biology remain far from elucidated, particularly in breast cancer and is an active area of research. In common with clinical practice and the literature, for the purposes of clarification PgR refers to both PR-A and PR-B unless specifically clarified.

PgR is an oestrogen regulated gene, and its synthesis in normal and breast cancer cells requires oestrogen and the ER[45].The working hypothesis is that tumour PgR expression represents an intact oestrogen- ER response pathway[51]. The expression of PgR in breast cancer cells in the absence of ER expression is less than 1% of all cancers, and expert opinion suggests that this molecular subtype does not exist and ER status in such cases should be retested [52]. Approximately 75% of primary breast cancers express the ER, and more than half of these cancers also express PgR [44].

The aetiology of ER+/PgR- tumours remains unclear. Nearly 30 years ago, it was recognized that transcription of the PgR gene was regulated by oestrogen in breast and reproductive

tissues and that ER^+ breast tumours that lacked PgR expression were less responsive to endocrine therapy than those that express PgR. At that time, Horwitz and McGuire [53] hypothesized that PgR loss was due to loss of ER activity, due to either low circulating oestrogen in some older women or a nonfunctioning ER pathway[54, 55]. This hypothesis, however, does not fully explain why some ER^+/PgR^- tumours respond to endocrine therapy, albeit at a lower frequency, than tumours that are ER^+/PgR^+.

Some studies have shown that the ER and PgR status change over the natural history of the disease or during treatment [56, 57] and thus a ER+/PgR- tumour may simply have evolved during tumour progression in a subclinical cancer. During tamoxifen therapy, levels of both PgR and ER decrease but PgR levels decrease more dramatically than ER levels, with up to half of the tumours completely losing PgR expression as they develop tamoxifen resistance[57]. In patients with such tumours, the loss of PgR translates into a more aggressive disease and worse overall survival, suggesting that other alterations in the molecular machinery driving tumour growth accompany the loss of PgR receptor expression[58].

There is increasing evidence that complex cell signalling and cross talk between growth factor signalling pathways and the ER (both genomic and non-genomic) contribute to PgR downregulation [59]. Growth factors can also independently cause PgR down regulation. HER 2 over expression causes PgR to be 500 fold lower, whilst ER expression is only lowered by half [45]. The expression of PgR is lower in T47D cell line (in which PgR expression is independent of ER) that over express HER2 [45]. Short term treatment (a few hours) with insulin-like growth factor (IGF-1), EGF and heregulin all sharply lower PgR levels and progesterone induced PgR activity in cell lines[45]. Growth factors also cause activation of the PI3K-Akt-mTOR pathway and can down regulate PgR [45, 60]. Loss of PTEN, a negative regulator of the PI3K-Akt pathway, causes upregulation of this survival

pathway and is correlated with loss of PgR in clinical breast cancer specimens [61] and it has also been reported that loss of heterozygosity in the chromosome harbouring the PTEN gene occurs in 30-40% of breast cancers and is associated with higher histological grade and specific loss of PgR but not ER[62]. Another potential mechanism of loss of PgR expression is methylation at the PgR gene promoter, thus silencing PgR expression [45].

The various theories to explain PgR downregulation and decreased expression of PgR in ER positive breast cancer are summarised in table 1-4.

Molecular mechanisms to explain loss of PgR in ER+ Breast Cancer
1. Nonfunctional ER 2. Low circulating levels of oestradiol 3. Hypermethylation of the PgR gene promoter 4. Loss of heterozygosity at the PgR gene locus 5. Growth factor (GF) regulation 6. SERM or Growth Factor- induced non genomic ER activity 7. Altered ER coregulator activity (or levels)

Table 1-4 Molecular mechanisms to explain loss of Progesterone Receptor (PgR) in ER+ Breast Cancer

Arpino et al assessed the clinical and biological features of 31,415 patients with ER+/PgR- breast cancer and compared it to 13,404 ER+/PgR- cases[63]. Clinically, ER+/PgR- tumours was significantly more frequent in older patients. They were also larger in size and were more frequently associated with 4 or more positive lymph nodes. The median level of ER expression was found approximately half that of the ER+/PgR+ cohort. In addition they had higher S-phase fraction resulting in a higher proliferation rate and were more likely to be aneuploid. Importantly, ER+/PgR- tumours had higher levels of HER-1 (25% of cases versus 8% in ER+/PgR-) and HER-2 expression (21% cases vs. 14%).

The differences in biology and outcome of ER+/PgR- tumours suggests that this tumour type may represent a breast cancer phenotype of its own, and this hypothesis is supported with the

advent of molecular profiling[64]. ER+/PgR- defined by gene signatures are associated with the luminal B subtype. Creighton et al [64] suggested ER+/PgR- tumours represent a subtype that is distinct at the molecular level from ER+/PgR+ and ER-/PgR-, these tumours were mixture of three different subtypes: ER+/PgR- that associates with ER+/PgR+ tumours by gene signature; ER+/PgR- tumours associating with ER-/PgR- tumour by gene signature and ER+/PgR- tumours not aligning with either ER+/PgR+ or ER-/PgR- gene signatures, the 'true' ER+/PgR-. In addition ER+/PgR- cancers have their own epidemiological risk factors- combination of receptor expression differs with age, preganancy, post menopausal hormone use and BMI after menopause[45].

1.7.4 Endocrine Therapy Strategies

Endocrine therapy refers to therapeutic strategies to prevent breast cancer cells from receiving stimulation from oestrogen. The two most common strategies in use for Early Breast Cancer include either blocking oestrogen-ER binding or lowering the levels of endogenous oestrogen production (oestrogen deprivation).

Tamoxifen

Tamoxifen is a Selective Oestrogen Receptor Modulator (SERM). It binds to the ER and prevents oestrogen binding. Depending on the target cell, tissue or species type Tamxoifen-ER binding can exert either agonist (oestrogenic) and antagonist effects.

Tamoxifen binds to the ligand-binding domain, the ER is then released from heat shock protein (HSP)-90 thus inducing receptor dimerisation and binding to ER-response elements on target genes [65]. In the presence of oestrogen, mRNA transcription is promoted though AF2, Tamoxifen inhibits AF2 function in breast cancer cells[66]. In addition, the conformation of the receptor is different when bound by Tamoxifen, and the Tamoxifen-ER associates with a different set of co-regulatory molecules and co-repressors [67]. In the

breast, tamoxifen acts as an antagonist, at least on genes important for cell survival and proliferation. In bone and endometrium, Tamoxifen exerts agonist effects. The agonist/antagonist profile of Tamoxifen is thought to be related to the particular milieu of co-activators and corepressors within a cell [68].

Oestrogen Deprivation

During reproductive years the predominant source of endogenous oestrogen (oestradiol, E2) is from the ovaries. Ovarian suppression or ovarian ablation methods prevent oestrogen synthesis. Several methods exist, including surgical oophrectomy, radiation induced ablation or medically with the use of LNRH analogues such as goserelin. In post menopausal woman, the main oestrogen is oestrone, E1. The liver, adrenal glands, breast and adipose tissue produce androstenedione which is converted by the enzyme aromatase to E1. This synthesis can be blocked using drugs that target aromatase, the aromatase inhibitors (AIs).The ER is not active in the absence of ligand, and as a result there is profound reduction in ER-mediated transcriptional activation and suppression of oestrogen induced tumour growth.

1.7.5 Endocrine Resistance – molecular insights

Tamoxifen, until recently has been the gold standard endocrine therapy, subsequently much of the research into therapy resistance has focused on tamoxifen. Aromatase Inhibitors (AIs) have only been in routine clinic use for less than a decade and long term recurrence data is limited. One third of woman treated with tamoxifen for 5 years will have recurrent disease within 15 years [69]. Two types of resistance are recognised, *de novo* (instrinsic) resistance or acquired resistance. A plethora of mechanisms have been proposed for both types, and this is an active area of research. The primary mechanism of de novo resistance is absence of the ER. In acquired resistance, not all tumours lose ER expression yet become oestrogen independent, the hypothesis here is that the cell finds an escape pathway [46].

EGFR and HER2 expression and their downstream cell survival pathways including PI3K/AKT and MAPK pathways are heavily implicated as potential escape mechanisms. These pathways may assume the driving role in tumour progression by 'providing' an alternative survival pathway, or they may downregulate ER expression. Preclinical and clinical data suggest that some tumours can alternate between ER and HER2 as being the dominant survival pathway, and therapy targeted at one may cause reactivation of the other.

Co-regulatory proteins that bind to tamoxifen-ER transcription complex are implicated in resistance, similar to how these factors influence the agonist/antagonist effect of tamoxifen in tissue type the balance within the breast cancer cell can become oestrogenic/ stimulatory. As a result of growth factor signalling pathways the availability of coregulatory proteins can be altered by modifications such as phosphorylation, methylation, ubiquitination, altering the ER transcriptional apparatus [46]. The advent of gene expression profiling is hopeful for providing further mechanistic insights into endocrine resistance [70]. An important issue however, is how much does a tumour's gene signature (when analysed in terms of treated patients outcome), correspond to therapeutic response or simple biological aggressiveness or indolence.

1.8 Endocrine therapy for Early Breast Cancer in Clinical Practice

1.8.1 Aromatase Inhibitors (AIs) vs. Tamxifen

The last decade has witnessed a significant change in endocrine therapy strategy for post menopausal woman. Tamoxifen has now been largely replaced by the aromatase inhibitors (AI). Concerns regarding tamoxifen resistance and its side effect profile (namely venous thrombo-embolism and endometrial cancer), combined with encouraging results of AI compared to tamoxifen in patients with metastatic cancer and in the neoadjuvant setting led to a number of multinational trials of adjuvant AI in post menopausal early breast cancer. The third generation AIs include exemestane, letrozole and anastrozole. These landmark trials

were undertaken to establish the efficacy and safety profile of AIs. The employed strategies included head to head comparisons of upfront AI vs upfront tamoxifen, or switching strategy, in which AI then tamoxifen is given sequentially or vice versa. The timing of the switch has also been studied- early (after2-3 years) or late (after 5 years). A combined analysis of data from the ATAC (Anastrozole, Tamoxifen Alone or in Combination) and Breast International Group (BIG) 1-98 (testing letrozole) found that head to head comparison of upfront AI versus tamoxifen there was a lower incidence of all breast cancer related events, which was small in magnitude but statistically significant with AIs, however there was not a significant reduction in breast cancer mortality [71]. A combined analysis was also performed of data from switching trials and there was a significantly lower incidence of all breast cancer related events, with a larger magnitude of benefit and a small but statistically lower breast cancer mortality rate[71]. These trials have demonstrated upfront AI treatment or switching to an AI, compared to adjuvant therapy with tamoxifen alone, reduces breast cancer recurrence and the incidence of contralateral breast cancer, and probably improves overall survival. There is currently no 'gold standard' strategy, although most would employ upfront AI for all high risk post menopausal breast cancer ER+, particularly node positive disease. In the West of Scotland, the Breast Managed Clinical Network current recommendation for use of AIs (selection of which AI is per current license and health board policy) in post menopausal women is as follows:

- 5years AI if ***any*** of following- Grade 3 disease, node+, weakly ER+ (defined as Allred ER scores 3-5), HER2+, Tumour size>5cm or neoadjuvant therapy to downstage
- 5 years Tamoxifen if ***all*** of following- Grade 1, node negative and tumour size<20mm
- Extended Adjuvant (5 years Tamoxifen+ 3 years AI) or Switch (2.5 years Tamoxifen + 2.5 years AI) in Grade 1 or 2 node negative.

1.8.2 Endocrine therapy side effect profiles

AIs have a different mechanism of action to Tamoxifen, and subsequently the side effect profile is different. AIs do not have oestrogen receptor agonist function and therefore not associated with increased risk of thrombo-embolism or uterine cancer. However, as they lack Tamoxifen's protective oestrogenic effects on the bones and actually cause bone loss by lowering circulating oestrogen levels, AI induced bone loss and osteoporosis (including increased risk of fractures) is a concern. In the ATAC trial, anastrozole was associated with fewer ischemic cerebrovascular events, endometrial cancers, venous thromboembolism, hot flashes and vaginal bleeding compared with tamoxifen. However, fractures and musculoskeletal disorders, such as joint pain or stiffness, were more frequent with anastrozole. Nausea, fatigue, mood disturbances, cataracts, and ischemic cardiovascular disease were similar for anastrozole and tamoxifen. A meta-analysis of seven trials comparing an AI versus tamoxifen, either as initial therapy or as sequential therapy [72], longer duration of AI therapy or use of an AI alone was associated with significantly greater likelihood of developing bone fractures & significantly greater odds of developing cardiovascular disease as compared to tamoxifen alone or tamoxifen plus short duration of an AI. The meta- analysis confirmed a decreased risk of venous thrombo-embolism and endometrial cancer.

AIs are not recommended in pre menopausal patients as indirectly they can result in ovarian stimulation. In premenopausal women, tamoxifen alone, or in combination with ovarian suppression/ablation are effective endocrine strategies for the adjuvant treatment of ER+ breast cancer in premenopausal women. The 11th St Gallen Expert Consensus [52] recommend the combination of OA/OS with tamoxifen following chemotherapy in premenopausal, hormone receptor positive patients who are at risk of not having a

chemotherpy induced ovarian failure and in premenopausal hormone receptor positive patients with high risk/ node positive disease.

1.8.3 Endocrine Therapy Benefit

Tumour ER expression is one of the best examples of a predictive factor in cancer management and its value undisputed, as is the value of adjuvant endocrine therapy in hormone responsive early breast cancer. The most recent meta-analysis update from the EBCTCG, report for women with ER-positive breast cancer five years of tamoxifen reduced the 15-year probability of recurrence by 39 percent and reduced breast cancer mortality by 30 percent compared with no adjuvant therapy[73] The EBCTCG reported 5years of adjuvant tamoxifen was just as effective for younger as compared to older women, in those with node-positive and node-negative disease, and in patients receiving versus not receiving chemotherapy. Equally importantly the relapse curves do not converge, this means that 5 years Tamoxifen can prevent (rather than delay the inevitable) and potentially cure many patients. The message is clear, endocrine therapy saves lives. Encouraging results from the recent landmark trials of AIs in ER+ breast cancer, suggest that even more woman will benefit from this strategy, as already exemplified by lower rates of recurrence and possibly improved overall survival. Combined with the relatively well tolerated safety profile of all endocrine therapy agents, it is of absolute importance that patients be offered this treatment if the tumour is endocrine responsive. Importantly, what meta-analysis [73] has reconfirmed, and supported by a wealth of other studies, is that tumours that do not express the ER i.e. ER negative tumours, derive no benefit from endocrine therapy.

1.9 Receptor Testing controversies

1.9.1 Immunohistochemistry testing variation

Semi quantitative immunohistochemistry (IHC)IHC is the near universal choice of tumour hormone receptor (ER & PgR)testing. Despite its extensive use there are still issues around

IHC testing methodology, interpretation and quantification[74] resulting in intra and inter laboratory variability . Key areas of testing variation include pre-analytical factors such as time to fixation, analytical factors such as lack of utilisation of validated assay, post analytical factors including reporting of results and lack of quality assurance programs have contributed to inconsistency in assay results with the net result being that it is estimated that up to 20% of results may be false negative [74]. One of the areas of greatest variation was threshold values for defining positive and negative ER breast cancer [75-80]. Until 2010 [81], no established cut-off value was in widespread acceptance (this is despite IHC being the near universal method of ER assay for over twenty years!) The recommended cut-off to distinguish positive tumours from negative is ≥1% ER positive tumour cells and endocrine therapy should be considered in all patients whose breast tumours show at least 1% ER+ cells [81].

1.9.2 Should PgR be routinely tested?

The value of PgR as a predictive factor for endocrine therapy has recently been subject of controversy [82-85]. Historically, PgR testing was undertaken to ensure that ER negative cancer patients (who may benefit) were not denied endocrine therapy. Consensus opinion is that this subtype (ER-/PgR+) does not actually exist, and subsequently the value of measuring tumour PgR has been questioned. A number of studies suggest that the predictive value of PgR is not as importantly clinically as ER [86-88] and an earlier Oxford EBCTCG overview of all trials of tamoxifen therapy in EBC found PgR status did not predict endocrine benefit [89]. Although recent concerns regarding assay variability and quality control may limit the value of this data. In 2009 the National Institute of Clinical Excellence (NICE) no longer recommends PgR measurement in routine pathological assessment of early breast cancer samples. In addition Adjuvant! Online (www.adjuvantonline.com) does not evaluate PgR expression as a part of its routine assessment of relapse and mortality risk in early breast

cancer. In contrast other studies have shown that PgR status provides additional predictive value [90] independent of ER values [91, 92] , especially in premenopausal woman [93, 94]. A large retrospective analysis of two large data sets of early breast cancer patients treated with endocrine therapy (n = 15,871) measured ER and PR levels in two standardized quality-controlled clinical laboratories demonstrated the predictive value of PgR [95]. In this study, patients with ER+/PR+ tumours benefited much more from adjuvant tamoxifen therapy than patients with ER+/PR- tumours. Multivariate analyses showed that both ER and PR were independent predictors of overall survival, with the reduction in relative risk of death being significantly greater in ER+/PR+ compared with ER+/PR- tumours. Importantly, PR still had predictive value even when ER was considered as a continuous variable, indicating that the predictive information is independent of quantitative ER levels and that PR adds predictive information to ER. The predictive value of PR in the adjuvant tamoxifen setting has also been shown in two smaller studies [96, 97]. Although the precise role of PgR in patient management has not been firmly established, current international guidelines recommend PgR testing. Predictive validity for PgR has been demonstrated with as few as 1% of stained nuclei in retrospective studies, therefore they recommend the cut-off for defining PgR positivity as ≥1% of tumour cells [81].

1.10 Endocrine Responsiveness: Does the level of ER expression influence endocrine Response?

The most important purpose of evaluating the ER and PgR tumour status for individual patients is to predict whether a clinically important benefit from a particular therapy is likely [98]. This would ideally involve a comprehensive assessment of the functionality of ER and PgR, including an evaluation of the activated downstream proteins [74]. However, IHC assays of ER and PgR are limited to determining whether these receptors are present in tumour cells and providing some information on the levels of ER and PgR in the tumour. The

importance of quantifying hormone receptor expression level by IHC remains an open question.

As a consequence of the experience with LBA (a functional assay), there is an expectation that IHC assays should result in a broad range of values among ER positive patients, similar to the range observed with LBA and that there is a direct proportional benefit with level of receptor and response to endocrine therapy. However, this assumes there is a direct, linear relationship between the amount of ER protein present in the tumour cells and the amount of ER antigen detected by IHC. IHC is a semi-quantitative technique and preanalytical, analytical and post-analytical factors can influence the result and result in test variation. Two recent studies that together included the analysis of 7000 breast cancers found that the distribution of ER values using contemporary IHC methodology was essentially bimodal, with more than 90% of tumours being either completely ER negative or unequivocally and strongly ER positive [99, 100]. Fisher et al [101] compared various methods of scoring ER and PgR, involving percentage ranges and intensity, both summated and as a product, and concluded that the "any-or-none" method was just as good at prediction and simpler. Certainly a highly sensitive IHC assay combined with a dichotomous reporting system appears to have advantages, limiting potential variability incurred by receptor quantification and minimize the likelihood of false negative results in tumours with low levels of ER.

There is, however, a growing body of evidence suggesting that the level of the hormone receptor expression measured by IHC predicts response to adjuvant therapy. Evidence for a linear relationship between level of ER expression as determined by IHC and response to both tamoxifen and letrozole was reported in a neoadjuvant study [102]. A number of other studies have shown that the proportional benefits of endocrine therapy vary with the relative quantitative expression of ER [74, 91, 103, 104]. Higher amounts of hormone receptor levels as determined by IHC has also been associated with improved patient outcomes [86, 91, 94,

104-108] including both the adjuvant treatment and advanced disease. Overall survival, [104, 107, 108], disease free survival [108], recurrence free survival [94, 107], 5 years survival [106], time to treatment failure [86, 104], response to endocrine therapy [91, 104] and time to recurrence [105] were all positively associated with ER levels. These studies suggest that patients with higher ER IHC levels will have a higher probability of positive outcomes. The 2007 version of the St Gallen guidelines[109] included a description of 3 categories of endocrine responsiveness: "highly endocrine responsive" (tumours express high levels of both steroid receptors in the majority of cells), "incompletely endocrine responsive" (some expression of steroid hormone receptors but at lower levels or lacking either ER or PgR) and "endocrine nonresponsive" (tumours have no detectable expression of steroid hormone receptors). For the purposes of *selecting* endocrine therapy in patients this categorisation is not relevant, it is the status (positive or negative using ≥1% positive tumour cells) that is of primary importance. However, this categorisation is clinically useful in the context of guiding decision making regarding the requirement for additional adjuvant chemotherapy.

1.11 Chemotherapy in ER+ Early Breast Cancer

1.11.1 Benefit of chemotherapy

The most recent EBCTCG overview of poly-chemotherapy included 100,000 woman in over 100 trials [110]. They updated the older 25year trials that used older regimens of CMF vs. AC and demonstrated that survival benefits were equivalent for both AC and CMF. Both reduced mortality rates by 20-25% compared to no chemotherapy and underpin the benefit adjuvant chemotherapy confers to woman with EBC. The overview also compared these older regimens to newer modern regimens that use taxanes added to anthracyclines or anthracycline regimens that use higher cumulative doses than traditional AC. The meta-analysis showed that modern regimens reduce breast cancer mortality by one sixth compared to older. When all the trials analysed together it was calculated that modern regimens reduce

breast cancer mortality by one third compared to no chemotherapy and this applies to ALL women, irrespective of age, nodal status, size of tumour and ER status.

1.11.2 Selecting ER+ patients for Chemotherapy

The improvements in outcome represent population wide benefits, and one limitation of the EBCTCG overviews is that they do not allow for the molecular heterogeneity of breast cancer. Individual risk-benefit assessments are challenging and about 60% of all early breast cancer patients receive adjuvant chemotherapy, of which only a small proportion 2-15% [69] will ultimately derive benefit, while all remain at risk of toxic side effects. The threshold for adjuvant chemotherapy is very difficult to define. Patients receiving anti HER2 therapy conventionally also receive chemotherapy either preceding or concurrent with the anti-HER2 therapy. Chemotherapy remains the mainstay of adjuvant treatment for patients with triple negative disease who are at sufficient risk of relapse to justify its utilisation. Identifying which patients with ER+/HER2 negative early breast cancer may benefit or safely avoid adjuvant chemotherapy is one of the most challenging areas in early breast cancer management. These patients include a spectrum from those at low risk from who there is little evidence supporting the addition of chemotherapy and to those at high risk, where chemotherapy appears to be clearly justified. In clinical practice adjuvant chemotherapy treatment decisions in this difficult group are commonly aided by algorithms such as Adjuvant! Online, an online web tool which is based on clinic-pathological prognostic and predictive factors and combined to provide risk-benefit estimates and treatment specific benefits. The 11th St Gallen Expert Conference issued guidance in thresholds for selection in ER+/HER2 negative patients. Table1-3 details the characteristics which favour the use of chemotherapy, or justify the use of endocrine therapy alone. This is based on histopathological assessment, and the likely endocrine response of a tumour as measured using IHC assay of ER and PgR expression level.

	Relative indications for chemoendocrine therapy	Factors **not** useful for decision	Relative indications for endocrine therapy alone
Clinicopathological features			
• ER and PgR	Lower ER and PgR level		Higher ER and PgR level
• Histological grade	Grade 3	Grade 2	Grade 1
• Proliferation[a]	High	Intermediate	Low
• Nodes	Node positive (four or more involved nodes)	1-3 positive nodes	Node negative
• PVI	Extensive PVI		Absence of PVI
• pT size	>5cm	2.1-5cm	≤2cm
• Patient preference	Use all available treatments		Advoid chemo related side effects
Multigene assays			
• Gene Signature	High score	Intermediate score	Low score

Table1-3 Chemoendocrine therapy in patients with ER+/ HER2-negative disease.

ER , oestrogen receptor; PgR, progesterone receptor; pT, pathological tumour size; PVI, peritumoral vascular invasion. [a]see text for discussion on proliferation markers. Adapted from ref[52]

1.11.3 ER+ patients with intermediate prognostic indices

The difficulty arises in the intermediate group. This is the group of patients in which clinicians are not confident that endocrine therapy alone will be sufficient to prevent disease recurrence and prevent poor patient outcome. The role of proliferation markers such as Ki-67 labelling index is advocated in decision making [52]. However its routine use is limited by assay variability and reproducibility. In addition variability in defining established cut-offs, as it is measured as a continuum, exists and limits its routine use.

1.11.4 Gene Prognostic Signatures

The development of microarray-based prognostic gene signatures was heralded as a major breakthrough for the management of breast cancer patients [111-118]. It was thought then that these signatures would provide a more objective assessment of the risk of relapse of breast cancer patients and would be more reproducible than the methods currently used. The first prognostic gene signatures (the 70-gene signature, Mammaprint [118], and the 76-gene signature [115]) were developed to be applied to all breast cancer patients, and did not take into account the molecular heterogeneity of the disease. Their performance in the training and validation datasets demonstrated objectively that the prognostic information provided by these signatures is independent of the information provided by tumour size, presence of lymph node metastasis and histological grade [111, 112, 116]. This has contributed to the recognition that in early breast cancer, tumour biology is as important as tumour burden in terms of outcome. However, importantly the molecular prognostic profiles can augment, but do not replace, traditional prognostic factors. Several groups have now developed their own prognostic signatures. In many cases, these are measuring common pathways; virtually all of the existing profiles distinguish hormone receptor-positive from negative, HER2-driven from not, and highly proliferative from more indolent tumours. Meta-analyses performed by independent groups revealed that different gene signatures identify similar groups of patients with poor outcome; that the assignment of cases with poor outcome is based on the expression of proliferation-related genes [39].

Currently these first generation signatures only have discriminatory power in ER+ disease and proliferation is perhaps the strongest determinant of outcome in ER+ disease [32, 39, 119, 120]. The 21-gene recurrence score (RS, Oncotype Dx®) is among the best-validated prognostic assays and is relatively unique in that it can be used in fixed tissue. It is recommended by the American Society of Clinical Oncology (ASCO) for use in women with

node-negative, ER+ breast cancer [98]. The RS was developed using a different approach from the supervised analyses of gene expression arrays used by the other molecular prognostic profiles. In this approach, the investigators started with the 250 most promising candidate genes selected from the literature. They then used a reverse transcription polymerase chain reaction (RT-PCR)-based method for generating quantitative expression levels of these genes in fixed tissue from 447 patients collected from three largely hormone receptor-positive, node-negative datasets. The result is the RS, which is actually a mathematical formula that includes 16 genes (plus five reference genes) weighted to optimize prediction of distant relapse despite tamoxifen therapy. The RS was validated in an independent dataset derived from 668 samples collected in the tamoxifen-treated arm of National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14, a prospective randomized clinical trial examining the benefit of adjuvant tamoxifen in hormone receptor-positive, node-negative breast cancer[121]. Study participants were largely postmenopausal (71 percent), stage I (62 percent), and with a good prognosis (85 percent free of distant metastasis at 10 years). Although this population had a generally good prognosis, reflecting the low stage and treatment with tamoxifen, the RS was able to distinguish prognostic groups: of those with low RS (<18), 93 percent were free of distant disease compared with only 70 percent of those with high RS (>31). Similar findings have been reported with aromatase inhibitors in postmenopausal women. As an example, when the RS was assessed for tumour samples from the TransATAC study, it was predictive of distant recurrence in patients treated with the aromatase inhibitor, anastrozole [122]. The question that remains germane is whether molecular profiling offers more than the information provided by traditional clinicopathological biomarkers. It is not clear how much of the prognostic value of the RS might be obtained by better pathologic grading and quantitative hormone receptor scoring as opposed to the biological properties being assayed by RT-PCR. This was in part

addressed by Dunkler and colleagues [123], who re-analysed the data from the cohort employed to validate the 70-gene signature and demonstrated that the contribution of this signature to the prognostication of breast cancer patients above and beyond that offered by the clinicopathological parameters was minimal. Furthermore, a recent comparison of the prognostic information provided by OncotypeDx™ or four immunohistochemical markers (that is, ER, PR, HER2 and Ki67 - a proliferation marker) semi-quantitatively assessed in the material from the ATAC (Arimidex, Tamoxifen, Alone or in Combination) prospective trial demonstrated that these four markers would at least be equivalent to OncotypeDx™[124].

1.12 Chemotherapy response in ER+ Breast Cancer

Beyond the question of whether additional adjuvant chemotherapy is indicated as endocrine therapy alone is unlikely to be entirely protective, is the question of whether ER expressing tumours and the level of expression influences response to chemotherapy. The predicted benefit from chemotherapy in the adjuvant setting has been assessed in several studies of patients with ER+ tumours undergoing chemotherapy plus tamoxifen compared with those undergoing chemotherapy alone for the treatment of ER negative breast cancer [69, 125]. These analyses suggest that the benefits of chemotherapy are significantly greater in patients with ER negative tumours. A number of neoadjuvant studies have also reported improved pathological complete response (PCR) in ER negative breast cancer compared to ER+. Other studies that have considered the molecular instrinsic subtype of ER+ tumours (eg. luminal A and B) and incorporated tumour markers such as HER2 and/or Ki-67 have demonstrated increased chemosensitivity in these subtypes of ER+ tumours [126]. Furthermore, several studies have shown that the tumour level of ER may help select the subsets of patients with ER+ disease who are likely to benefit from the addition of chemotherapy to endocrine therapy[125, 127, 128]. The Oncotype DX RS assay may not only predict the likelihood of tumour recurrence, but also could predict the magnitude of chemotherapy benefit [128].The

usefulness for prediction of chemo response is relatively clear at the extremes of the RS, but there is uncertainty with intermediate RS, ie. the point at which the addition of chemotherapy is beneficial is unclear. Node-negative patients with a low RS are least likely to benefit from chemotherapy whereas high RS patients are expected to achieve benefit. The ongoing TAILORx trial will provide high-level evidence for the role of the RS in identifying those who may and those who may not benefit from chemotherapy. In this trial, women with intermediate RS (between 11 and 25) are randomly assigned to endocrine therapy versus the same plus chemotherapy. Neither the RS, nor any other genomic profile at this time, provides insight into choice of chemotherapy regimen. The prognostic ability of the RS in node-positive breast cancer has also been examined [129], investigators found that the RS was prognostic, and predicted benefit from CAF chemotherapy added to tamoxifen. The combined prognostic and predictive ability of RS, albeit limited, is the reason that the RS is incorporated into adjuvant decision-making for node-negative disease, as suggested by the American Society of Clinical Oncology (ASCO), the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines for breast cancer and the St Gallen International Expert Consensus[52].

Molecular profiling has confirmed the concept of instrinsic chemosensitivity. The common theme from studies is that tumours with features of high tumour proliferation, such as absent or low ER, HER2 overexpression, high grade and high risk as assessed by multigene assay have increased chemo-responsiveness compared to ‘biological low risk’. High ER and PR expression, low grade, low Ki67 immunostaining and lobular type histology have been shown to be associated with impaired response chemotherapy and lack of meaningful benefit [130].

Perhaps the greatest challenge for clinicians is the treatment of patients with tumour biology that indicates low risk (such as luminal A subtype, or low recurrence score), and who are unlikely to obtain any meaningful benefit from chemotherapy, yet remain at considerable risk

of distant recurrence with endocrine therapy alone because of large tumour size and/ or extensive nodal involvement. The so called 'predictive markers of relative chemotherapy insensitivity' including ER expression, lobular histology, low Grade, low proliferation, luminal A subtype and low recurrence scores are not necessarily markers of resistance and do not exclude the possibility of a smaller absolute benefit from adjuvant chemotherapy. The perception of 'meaningful benefit' is subjective and each individual has different expectations and attitudes regarding treatment. For some patients a potential absolute benefit of 1% is sufficient to justify the potential toxicity of adjuvant chemotherapy[130]. Currently, adjuvant therapy decision making involves an integration of available clinical tools to assess risk-benefit, and open conversation with each individual patient.

1.13 Novel Strategies in ER+ breast cancer

Endocrine resistance is a significant problem and one third of woman treated with tamoxifen will have recurrent disease within 15 years [69]. There is increasing awareness of the concept of instrinsic chemo-sensitivity and consensus view is that ER+ breast tumours, especially the biologically less aggressive luminal A type, derive limited benefit from adjuvant chemotherapy, balancing this against the significant impact on quality of life and rare but potentially life threatening side effects new treatment strategies need to be considered.

Recent progress in our understanding of the molecular biology of oestrogen receptor signalling and adaptive cross talk with growth factor receptor and cell signalling pathways have resulted in strategies being developed that combine endocrine therapy with inhibitors of growth factor receptors or downstream signalling pathways to prevent or treat endocrine escape pathways that may become operative in impaired endocrine responders. The nonreceptor tyrosine kinase, c-Src, has been implicated in the progression of human breast cancer [131] and evidence suggests it is a key mediator in cell signalling pathways.

Surprisingly translational studies examining its expression in the subtypes of breast are limited. Src expression in ER+ and its potential targetable is discussed in more detail in chapter 6.

The sodium iodide symporter, NIS, is a transmembrane glycoprotein which has been exploited for the safe delivery of radio-iodide in the treatment of thyroid cancers for many years. NIS is expressed in many breast cancers. In vitro evidence suggests the ER may play a key role in NIS regulation, the potential to exploit the expression of NIS in ER+ Breast cancer is discussed in detail in chapter 5.

1.14 Thesis Aims

Over 1 million women a year are diagnosed with Breast Cancer. The majority, approximately 70% express the oestrogen receptor (ER). ER positive breast cancer has historically been perceived as a 'good cancer', although many woman with ER+ breast cancer still succumb to their disease and globally breast cancer is the leading cause of female cancer deaths. The advent of gene expression profiling and the definition of the molecular instrinsic subtypes has defined at least two subtypes of ER positive breast cancers (luminal A and luminal B) that differ markedly in terms of biological behaviour, response to adjuvant therapies and most importantly patient outcome. In the clinic identifying which ER+ have expected poor outcome with endocrine therapy alone remains a priority, as further adjuvant therapy will be indicated. Mounting evidence suggests that ER+ cancer is less responsive, if at all, to chemotherapy. New therapies targeted at ER+ breast cancer are sought.

The focus of this research is ER+ breast cancer and targeting patient therapy in this heterogeneous group. This work attempts to translate our understanding of the biology of the ER and cell signalling interactions to aid the correct identification of patients for both current therapy and more novel therapeutic approaches.

This thesis addresses a number of controversial issues in ER+ early breast cancer patients' management, specific aims were:

1. Examine Endocrine Response- "how much benefit do we expect this patient to derive from endocrine therapy?"
2. Identification of high risk ER+ breast cancer and aid adjuvant therapy decision making by developing a pragmatic equivalent of gene prognostic profiles utilising currently routinely measured tumour markers
3. Novel strategies
    - Examine the relationship between NIS expression and the ER and assess NIS function, expression level and location in ER+ breast cancer
    - Perform a pilot study examining Src expression in ER+ breast cancer

2 Pilot Study- ER expression level and response to Endocrine therapy

2.1 Introduction

Tumour oestrogen receptor (ER) expression is a powerful predictor of breast cancer response to endocrine therapy. Virtually all determinations of tumour ER status of breast cancers are performed today using immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded tissue (FFPE). ER determination using IHC has been in widespread use 20 years, yet surprisingly until recently, no consensus cut-off value defining ER positive from negative existed.

Results from the Early Breast Cancer Trialists Collaborative Group (EBCTCG) overview show that tamoxifen substantially reduces risk for breast cancer recurrence and death across all age groups in patients with ER-positive early stage breast cancer, whereas patients with ER negative disease do not show benefit from tamoxifen [69, 73]. In addition, the importance of quantifying hormone receptor expression by IHC remains an open question. There is a growing body of evidence suggesting that the level of the hormone receptor expression measured by IHC predicts response to both endocrine therapy (and may display an inverse relationship with response to chemotherapy), and this is influencing treatment strategies in clinical practice [52].

This pilot study was undertaken in 2007 to examine whether the benefit from endocrine therapy follows a linear relationship with tumour expression of ER as measured by IHC and examine whether a threshold value exists that will define response (ER positivity).

2.2 Materials and methods

2.2.1 Data Collection & Patient database Creation

Ethical approval was granted by the Research Ethics Committee of the North Glasgow University Hospitals NHS Trust for the collection of patient data. Retrospective data on all operable invasive breast cancer cases diagnosed between October 1995 and September 1998 (3years) in Greater Glasgow NHS hospitals (Glasgow Royal Infirmary, Victoria Infirmary, Stobhill Hospital and Southern General) was collected in March 2006 in a secure password protected database.

All patients were female, and identified by their Glasgow unique identifier and pathology number. Patient details were date of birth and age at presentation. For every patient, details on the date of first sample, date of definitive surgery and date of most recent review was collected to calculate time to outcome. Date of deaths and cause of deaths, were confirmed with the registrar general or patient case records. Status of patients at most recent review was recorded (alive and well- no recurrence; alive with recurrence; Dead- breast cancer specific death or other; and recurrence now disease free). For cases with recurrence the site, local or distant was documented and in non breast cancer specific deaths the cause of death documented. Accuracy of follow up data was maintained by the gatekeeper of the database, Aileen Kesson, personnel in NHS Greater Glasgow.

Details of treatment including type of surgery, radiotherapy, chemotherapy and 5 years tamoxifen therapy or participation in the ATAC trial were documented. Pathological variables such as tumour type, grade, size, number of nodes involved and ER status at time of definitive surgery was collected from the pathology reports. Tumours were all evaluated by IHC and scored for ER by a trained pathologist using the percentage staining method, a direct count of positively staining tumour cell nuclei to give a value of 0-100%. Methods of IHC assay were those that were utilised in the pathology department during 1995-1998.

2.2.2 Statistical analysis

Statistical analysis was performed using SPSS version 15. It was then repeated with the aid from medical statistician, Caroline Bray, Dept of Public Health, Glasgow University using mini tab software. Univariate analysis and multivariate survival analysis with calculation of hazard ratios (HR) were performed using Cox's proportional-hazards model.

2.3 Results

2.3.1 Patient and tumour Characteristics

Between October 1995-September 1998 in Greater Glasgow hospitals, 1711 woman were diagnosed with operable breast cancer. Patient and tumour characteristics for the entire cohort are shown in table 2-1.

	Cohort (n=1711)
Age	
≤50	447 (26%)
>50	1264(74%)
Nodal Status	
0	989 (58%)
1-3+	441 (26%)
>3	231 (13.5%)
unknown	50 (3%)
Tumour Size	
<20mm	1088 (64%)
20-50mm	577 (34%)
>50	42 (2%)
Tumour Grade	
1	393 (23%)
2	807 (47%)
3	507 (30%)
ER %	
Unknown	51 (3%)
0	392(23%)
1-100%	1268 (74%)
Local Therapy	
WLE+Axilla	682 (40%)
No Radiotherapy	76 (11%)
Radiotherapy	605(89%)
Mastectomy + Axilla	996 (58%)
No Radiotherapy	786(79%)
Radiotherapy	207(21%)
unknown	3 (<1%)
WLE or Mastectomy only	33 (2%)
Systemic Therapy	
EndocrineTherapy	
None	254(15%)
Tamoxifen	1316 (77%)
ATAC trial	128 (7%)
unknown	13 (<1%)
Chemotherapy	
Yes	546 (32%)
No	1162 (68%)
Unknown	3 (<1%)
Survival- Mean (range), years	5.4 (0-8.8) yrs
Deaths (any)	430 (25%)
Breast Cancer Related Deaths	244 (14%)
Recurrence-Mean (range), years	5.3 (0-8.8)yrs
Recurrence	310 (19%)
Local	44(15%)
Distant	228(75%)
Site not documented	38 (10%)

Table 2-1 Patient and tumour characteristics in Pilot Cohort (n=1711)

2.3.2 Distribution of ER percentile scores

Figure 2-1 shows a histogram of the distribution of ER scores in all endocrine treated patients (n=1444), 1316 (91%) tamoxifen treated and 128 (9%) ATAC participants.

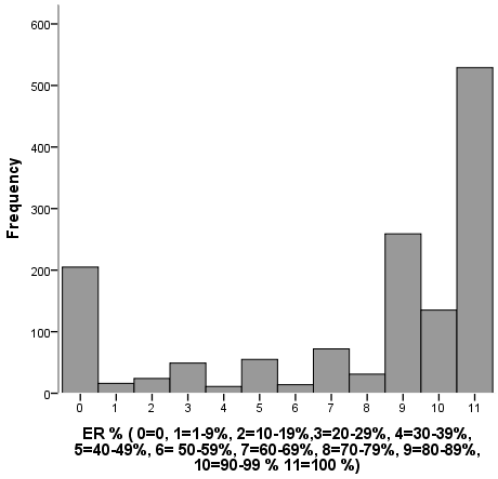


Figure 2-1 Histogram of distribution of ER percentile scores in Pilot cohort

Histogram demonstrating distribution of ER percentile scores in all endocrine treated patients (n=1444), the pilot cohort.

2.3.3 Level of ER expression and Outcome in all endocrine treated patients

1444 patients were treated with endocrine therapy, the ER % score was known for 1400 patients. Using the histogram groups of ER expression, low (0-9%), intermediate (10-79%), high (80-100%) were analysed in terms of outcome.

i). Low (ER 0-9%), Intermediate (ER10-79%), High (ER80-100%)

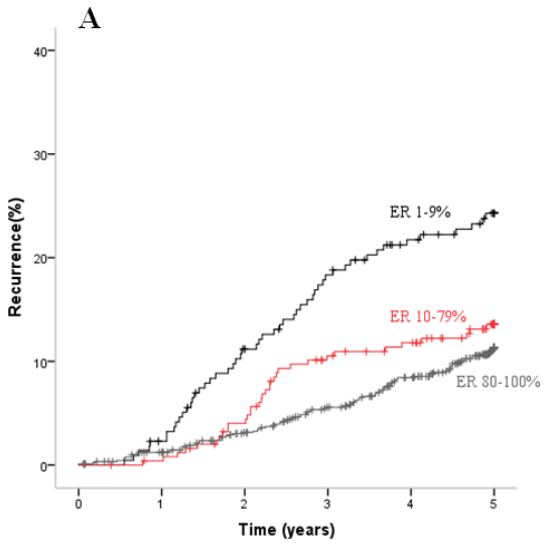
Recurrence was time to first recurrence, either local or distant, or death. Events were censored at five years as this is the average time of tamoxifen therapy and considered early recurrence. Patients categorised as being in the low group (n=221) had 51 events and a mean disease free survival (DFS) of 4.3 years (range 4.2-4.5years). Patients in the intermediate

group (n=251) had 33 events and a mean DFS of 4.65 years (range 4.5-4.7 years) and patients in the high group (n=914) had 96 early events and a mean DFS of 4.76 years (range 4.7-4.8 years). A significant association between ER% expression between these groups was observed, figure 2-2A p= 0.1×10^{-5}. Patients in the low group had significantly poorer outcome compared with patients in the intermediate group (p=0.003, HR 0.52) and patients in the high group ($p=1.5 \times 10^{-7}$, HR 0.41). Whereas the difference between intermediate and high was not significant (p=0.26), although both the time to event and HR indicate that patients categorised as high have improved early DFS compared to the intermediate group.

When, analysing later recurrence events (range of follow up 0-8 years) it was observed that patients in the low group had 63 events and mean DFS time 6.7 years (range 6.3-7.1 years), patients in the intermediate group had 40 events and mean DFS 7.4 years (range 7.4-8 years) and patients in the high group had 124 events, mean DFS 7.7 years (range 7.5-7.9 years). The significant difference is between low and high ($p=2 \times 10^{-8}$, HR 0.43) and low and intermediate (p=0.001, HR 0.51). Similar to early events, the difference between intermediate and high is not significant (p=0.36) and in fact examining the Kaplan Meier curve (figure 2-2 B) demonstrates the outcome curves for intermediate and high crossover.

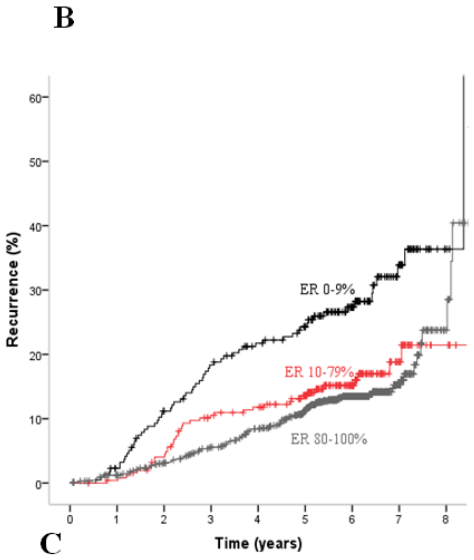
In terms of breast cancer specific survival patients categorised in the low group had 51 events, mean survival time 7 years (range 6.6-7.3 years), patients in the intermediate group had 28 events, mean survival time 8.1 years (range 7.8-8.3 years) and patients in the high group had 97 events, mean survival time 7.9 years (range 7.7-8.8 years). The survival advantage of increasing ER% expression is significant between low and high groups ($p=7 \times 10^{-7}$, HR 0.43) and between low and intermediate (p=0.001, HR 0.51). There is no significant difference between intermediate and high, p=0.7 (HR 1.1). It is noteworthy from figure 2-2 that after 6 years there is cross-over between intermediate and high outcome curves

and it appears that intermediate expressers may have better forecast outcomes than high ER expressers.



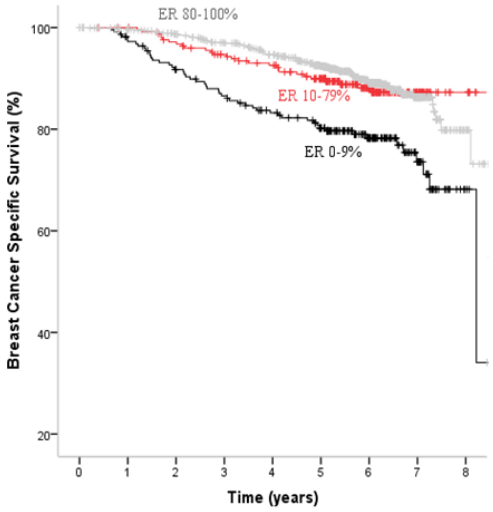
Overall **p=0.1 x10^{-5}**

	ER (10-79%)	ER (80-100%)
ER (0-9%)	**p=0.003**	**p=1.5x10^{-7}**
ER (10-79%)		**p=0.26**



Overall **p=1.5 x10^{-7}**

	ER (10-79%)	ER (80-100%)
ER (0-9%)	**p=0.001**	**p=2x10^{-8}**
ER (10-79%)		**p=0.36**



Overall **p=2.5 x10^{-6}**

	ER (10-79%)	ER (80-100%)
ER (0-9%)	**p=0.001**	**p=7x10^{-7}**
ER (10-79%)		**p=0.7**

Figure 2-2 Low (0-9%), intermediate (10-79%) and high (80-100%) ER expression and patient outcome

Patient outcome by ER% level(low, intermediate and high) A) 5 year recurrence; B Late recurrence; C)Breast cancer specific survival

ii. Low (ER 0-9%), Intermediate (ER 10-79%), High (ER80-99%) and Very High (ER 100%)

A large number of tumours had complete, 100% ER expression (n=529), Table 2-2. We hypothesised that the cross over and lack of significance between intermediate and high reported may be as a result of non complete ER expressers influencing the benefit of complete 100% ER expression, assuming that 'more is better'. Therefore to further investigate whether level of expression influenced outcome the groups were reclassified and ER 100% was analysed separately as 'very high'/complete expressers and ER 80-99% as 'high' expressers.

ER % Group	No of patients (n)	% of Endocrine treated Cohort
0-9% (low)	221	15
10-79% (intermediate)	256	18
80-99% (high)	394	27
100% (very high)	529	37
unknown	44	3

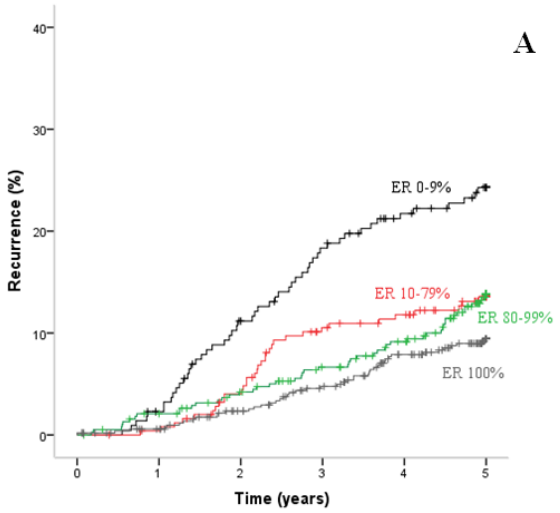
Table 2-2 Low, intermediate, high and very high %ER expression groups

Examining early recurrence as the outcome suggests a linear response between ER% expression level and outcome in this cohort of endocrine treated patients and supports our hypothesis that 100% ER expression is associated with best outcome. The DFS times for low and intermediate groups are as above. In patients with high (80-99%) ER expression there were 50 early events, mean DFS time 4.7 years (range 4.6-4.8). In patients categorised as having complete (100%) ER expression there were 46 events and mean DFS time was 4.8 years (range 4.7-4.86 years). A significant association between ER% expression between these groups was observed (figure 2-3A, $p=1x10^{-6}$). The difference was most significant

between low and very high ($p=4 \times 10^{-8}$, HR 0.34). Between very high and high a difference was suggested (p=0.052, HR 1.5) and between very high and intermediate (p=0.069, HR 1.5). No significant difference was observed between intermediate and high (p=0.95). Both intermediate and high were also significantly associated with improved outcome compared to low (p=0.003, HR 0.52 and p=0.001, HR 0.51 respectively).

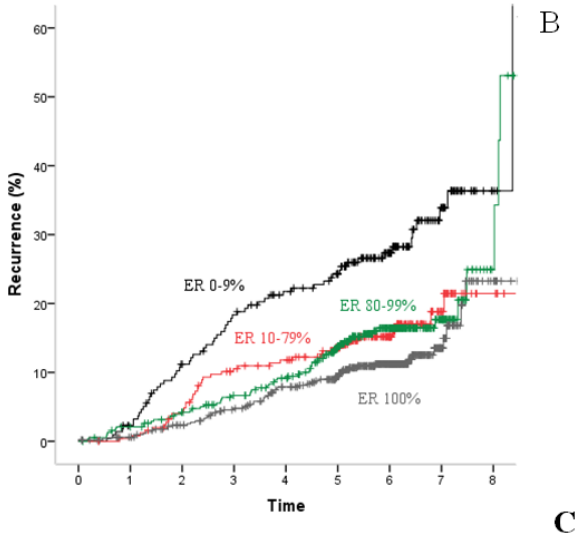
Interestingly, examining later recurrence events the distinction between levels is less clear. For patients categorised as very high there were 61 events and mean DFS time was 7.7 years (range 7.5-7.9 years), this is the same as the intermediate group. Patients with high ER expression (80-99%) had 63 events and have shorter mean DFS than the intermediate group (high group mean DFS 7.5 years (range 7.3-7.9 years). There is no significant difference between very high and high (p=0.073 or very high and intermediate (p=0.1), although compared to the low group, intermediate (p=0.001, HR 0.5), high ($p=1.4 \times 10^{-4}$, HR 0.5) and very high ($p=9 \times 10^{-9}$, HR 0.3) are all associated with improved outcome.

Examining the groups with breast cancer specific survival does not support that level beyond being low is important. In the very high group there were 42 events, and mean survival time was 8 years (range 7.8-8.2 years), in the high group there were 55 events and mean survival time was 7.8 years. As detailed in the previous section the intermediate group has the highest mean survival time (8.1 years, range 7.8-8.3 years). The most significant finding is the poor outcome associated between the low group and other categories: low compared to intermediate (p=0.001, HR 0.47), low compared to high (p=0.004, HR 0.6) and low compared to very high ($p=2.5 \times 10^{-8}$, HR 0.3). In addition, and interestingly there is a significant difference between very high and high (p=0.007, HR 1.7), but not the intermediate group, in fact the Kaplan Meier curve (figure 2-3C) suggest the intermediate group may have long term outcome comparable to patients in the very high group.



A Overall **$p=1x10^{-6}$**

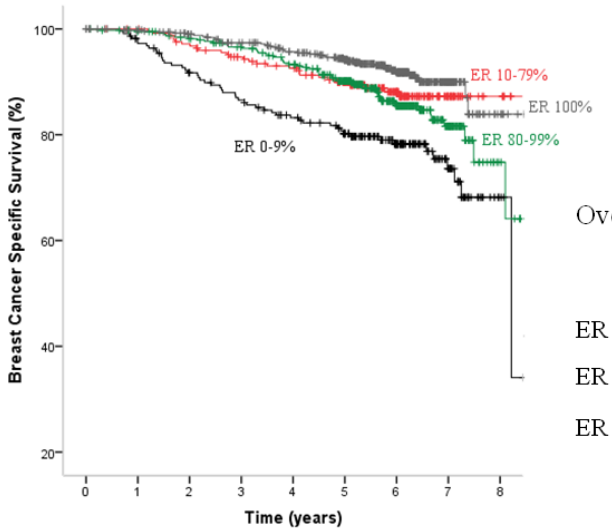
	ER (10-79%)	ER (80-99%)	ER (100%)
ER (0-9%)	**p=0.003**	**p=0.001**	**$p=4x10^{-8}$**
ER (10-79%)		**p=0.95**	**p=0.07**
ER (80-99%)			**p=0.052**



B Overall **$p=2\ x10^{-7}$**

	ER (10-79%)	ER (80-99%)	ER (100%)
ER (0-9%)	**p=0.001**	**$p=1x10^{-4}$**	**$p=9\ x10^{-9}$**
ER (10-79%)		**p=0.9**	**p=0.1**
ER (80-99%)			**p=0.07**

C



Overall **$p=6x10^{-7}$**

	ER (10-79%)	ER (80-99%)	ER (100%)
ER (0-9%)	**p=0.001**	**p=0.004**	**$p=2\ x10^{-8}$**
ER (10-79%)		**p=0.4**	**p=0.15**
ER (80-99%)			**p=0.007**

Figure 2-3 Low (0-9%), intermediate (10-79%), high (80-99%) and Very High/ Complete (100%) ER expression and patient outcome

Patient outcome by ER% level (low, intermediate, high and very high) A) 5 year recurrence; B) Late recurrence; C)Breast cancer specific survival

2.3.4 Multivariate analysis

In multivariate analysis when combined with lymph node status, grade and tumour size categorisation of ER expression level into low (0-9%), intermediate (10-79%, high (80-99%) and very high (100%) remained independently significant for early (p=0.03) and late recurrence (p=0.02) but not breast cancer specific survival (p=0.08) in this cohort of endocrine treated early breast cancer patients. Table 2-4 details the adjusted HR for each ER% category when analysed in combination with tumour grade, lymph node stage and tumour size. The significance is between low and very high, although the adjusted HR's do indicate that there is increased risk with intermediate and high ER% values (not significant).

	Early Recurrence (p=0.03)		**Late Recurrence** (p=0.02)		**Breast Cancer Specific Survival** (p=0.08)	
ER Group	Adjusted RHR (CI)	Signif	Adjusted RHR (CI)	Signif	Adjusted RHR (CI)	Signif
0-9%	1.8 (1.2-2.7)	p=0.004	1.7 (1.2-2.6)	p=0.004	1.7 (1.1-1.7)	p=0.01
10-79%	1.3 (0.8-2)	-	1.2 (0.7-1.7)	-	1.2 (0.7-1.9)	-
80-99%	1.1 (0.7-1.7)	-	1.1 (0.7-1.5)	-	1.2 (0.8-1.9)	-
100%	1		1		1	-

Table 2-3 Adjusted Relative Hazard Ratios (RHR) for ER expression level

2.4 Discussion

This pilot study was undertaken to examine whether the level of ER expression as determined by IHC has a linear relationship to endocrine therapy response. The pilot results do not demonstrate a direct linear relationship, importantly though, they do not exclude it. Our early recurrence data (when events were censored at 5 years) are suggestive that as hypothesised, tumours with a greater % of ER positive cells (especially complete 100%) are associated with improved response however with increasing time (coinciding with endocrine therapy withdrawal) the distinction between tumours expressing greater than 10% ER becomes less clear.

A significant finding in this pilot study is the poor outcome associated with being categorised as low ER. Almost all the low ER group (n=205, >99%) were actually ER negative (recorded as 0% of cells staining positively) patients who had received endocrine therapy, therefore it is little surprise that this group have poor outcome and ideally should have been excluded. However at the time of undertaking this pilot study there was no consensus on what defined ER negative breast cancer, a national survey of practising lead breast surgeons in all UK breast cancer units reported that the absolute cut-off point for positivity varied widely from 5-80% [77] and a lack of hormone receptor status definition was recognised in the recent NCCN task force report as contributing to hormone receptor testing variation [74]. Interestingly, as a result of including the ER negatives in the low category, we observed a trend- a 'widening gap', the statistical significance increased in most cases when low ER was compared to intermediate, high and very high ER groups, although patient numbers in groups may have influenced this.

Hormone receptor testing by IHC has been the gold standard technique for determining the ER 'content' in breast cancer specimens for over 20 years and subsequently influencing the decision making on patients' adjuvant hormonal therapy. IHC replaced radiolabelled ligand

binding assays (LBA) in the 1990s as the primary ER assay. LBA is a quantitative technique and the magnitude of benefit from endocrine therapy was related to the quantity of ER protein [74]. As a result of the LBA experience there is an expectation that IHC determination of the hormone receptors will follow a linear distribution, supported by earlier studies [86, 103, 132]. IHC as a technique is semi-quantitative. A number of methodological factors have been demonstrated to influence results and result in testing variation, most recently addressed in the NCCN task force and ASCO/CAP guidelines [74, 81], important factors contributing to testing variation include: pre-analytical (such as tissue fixation variations), analytical (antigen retrieval techniques, utilisation of un-validated antibodies) and post analytical (observer error, reporting criteria including lack of consensus on hormonal status of the tumour and scoring methods). We hypothesised that this pilot study data, collected prior to centralisation of services and reporting the % of staining cells rather than the validated Allred scoring system [103, 133] or Histoscore method, both which incorporate intensity of staining *and* percentage of cells staining, may be have an influence on the analysis.

There is little dispute that the ER is an excellent predictive factor [69, 73]. Its predominant role currently appears to be as a negative predictor, i.e. lack of the ER predicts lack of response or benefit with endocrine therapy and our pilot data does suggest this. Over expression of HER2 and increased expression of other cell signalling pathways implicated in carcinogenesis and endocrine resistance are recognised to influence endocrine response[46]. In addition, this analysis does not consider PgR. As discussed in detail in the introduction, the predictive role of PgR in endocrine response remains poorly defined and controversial. However, there is strong evidence that the co-expression of both hormone receptors (ER and PgR) is associated with improved endocrine response [95] and PgR loss associated with biological aggressiveness and impaired endocrine response [45, 63, 95]. Importantly, in this

pilot study PgR and HER2 expression were unknown. We hypothesised that the co-expression or lack of expression of these factors would influence and add valuable insight into the biology of the hormone receptors and endocrine response within this cohort, perhaps explaining why tumours with intermediate ER expression had improved DFS times compared to high expressers. Based on the current literature and the pilot results we strongly suspected tumour PgR expression in ER positive breast cancer would provide some insight into addressing the question of whether level of hormone receptor expression and patient outcome with endocrine therapy were related.

The 2010 published hormone receptor testing guidelines based on meta-analysis, systemic review and expert panel opinion [81] strongly advice that all patients with tumours expressing $\geq$1% cells staining positive for ER as determined by IHC, should be defined as ER positive and endocrine therapy should be considered. The question of whether ER level influences response remains an open one, especially topical is the influence the ER may have on chemotherapy response. The conclusion of our hypothesis generating pilot study when it was undertaken was in keeping with the new guidelines, whilst we suspected that ER expression level influences (at least in part) the response to adjuvant hormonal therapy based on this pilot data patients with very low levels appear to derive benefit, and subsequently in clinical practice we recommend adjuvant endocrine therapy to all patients without a contra-indication if their tumour expresses *any* ER as determined by IHC.

3 ER, PgR expression and the Combined Endocrine Receptor and Endocrine Response

3.1 Introduction

The most important purpose of evaluating the ER/ PgR tumour status for individual patients is to predict whether a clinically important benefit from a particular therapy is likely [98, 134]. This would ideally involve a comprehensive assessment of the functionality of the receptors, including an evaluation of the activated downstream proteins of these receptors[74] . IHC assays of ER and PgR are limited to determining whether ER and PgR are present in tumour cells and provide some information on the levels of ER and PgR in the breast cancer cells.

The predictive power of ER is undisputed, and currently its major role is as a negative predictor, the absence of ER predicts lack of benefit from endocrine therapy. In addition the level of tumour ER expression may influence response to endocrine therapy and may inversely be associated with enhanced response to chemotherapy. The precise predictive value of PgR is controversial. PgR is an oestrogen regulated gene, and its synthesis is dependent on a functioning ER. There is good evidence that in breast cancer cells, enhanced growth factor signalling can down regulate PgR expression and ER+/PgR- breast cancer are associated with increased biological aggressiveness and impaired response to endocrine therapy[45].

The 2007 version of the St Gallen guidelines [109] included a description of 3 categories of endocrine responsiveness: “highly endocrine responsive” (tumours express high levels of both steroid receptors in the majority of cells), “incompletely endocrine responsive” (some expression of steroid hormones but at lower levels or lacking either ER or PgR), and “endocrine non responsive disease” (tumours have no detectable expression of steroid

hormone receptor)[109]. The 2009 guidelines suggests consideration of these categories of endocrine responsiveness in the context of guiding decisions regarding use of chemo-endocrine therapy in patients with ER+ HER2 negative early breast cancer [52].

The hypothesis for this study was simple- combining the receptor score for ER and PgR will be more informative of ER function and oestrogen signalling within the tumour, compared to either steroid receptor independently, producing values to (semi)-quantitate likely endocrine response and hence categorise tumours by endocrine response.

There were 3 aims for this study. Firstly, to re-analyse archival FFPE breast cancer specimens from early breast cancer patients within the database, using central IHC testing (thus reducing test variation) to determine Allred scores for ER, PgR and also examine other important biomarkers such as HER2 and Ki67. Secondly, examine tumour ER expression, PgR expression and a novel combined ER/PgR score (the Combined Endocrine Receptor, CER) and perform retrospective analysis of patient outcome. Lastly, it was anticipated that the combined endocrine receptor, would be used as a surrogate marker of oestrogen receptor signalling in a prognostic score system utilising traditional pathological markers to identify ER+ breast cancer patients at risk of poor outcome and potential candidates for adjuvant chemotherapy.

3.2 Material and Methods

3.2.1 Patient Database

Patients were diagnosed with operable invasive breast cancer between October 1995 and September 1998 in Greater Glasgow NHS hospitals. Tumour samples were analysed for 557 patients, randomly selected from the 1711 patients (33%) within the Greater Glasgow Database described in chapter 2. Survival status (alive, dead, breast cancer related death) was re-confirmed and updated in March 2010, for early recurrence events were censored at 5

years from patient diagnosis and late recurrence follow up details confirmed in March 2006. The Reseach Ethics Committee of North Glasgow University Hospital approved the collection of patient data and use of human tissue in this study.

3.2.2 Tissue microarray (TMA) construction

Formalin fixed paraffin embedded (FFPE) tissue, taken at the time of surgical resection was used for tissue microarray construction as described previously [135, 136].

3.2.3 Immunohistochemistry (IHC)

IHC for ER, PgR and HER2 was conducted as described previously [135, 136] applying protocols established in the CPA accredited Diagnostic Pathology laboratory, Glasgow Royal Infirmary with appropriate positive and negative controls. In addition, immunohistochemistry for Ki67 was performed by Dr Zara Mohammed as described in [137].

3.2.4 IHC scoring

IHC scoring was performed in collaboration with Dr Zara Mohammed's work comparing and validating automated image analysis assessment with observer assessment for steroid hormone receptors, HER2 expression and Ki67 labelling index in breast cancer [[137].
In this study ER and PgR were quantified using the Allred Scoring System [103, 133] an internationally recognised and validated scoring system [81] which incorporates both percentage and intensity of cells staining. The Allred score is simply calculated, first the percentage is scored 0-6 as detailed in table 3-1 and then the intensity score is added, resulting in an Allred score 0 or 2-8.

Percentage description	% Score	Intensity	Score
no cells staining	**0**	no cells staining	**0**
<l% of cells	**1**	Weak	**1**
1-10% (1/10)	**2**	Intermediate	**2**
10-33% (1/3)	**3**	Strong	**3**
34-66% (2/3)	**4**		
67-100%	**5**		
Allred Score= Percentage Score+ Intensity Score			

Table 3-1 Calculation of the Allred Score

Each tumour was scored in triplicate and the mean Allred Score calculated and used for analysis. Allred Scores were performed by two independent observers that where blinded to patient outcome and other observers score (Observer 2, scoring one core for every 76 patients, the intraclass correlation coefficient (ICCC) for ER was 0.96 and 0.97 for PgR [135].

HER2 membrane staining was scored according to the NICE-approved DAKO HercepTest scoring system: 0, no membrane staining; 1+, faint, partial membrane staining; 2+, weak, complete membrane staining in more than 10% of invasive cancer cells; 3+, intense, complete membrane staining in more than 10% of invasive cancer cells [136]. Ki67 scores were recorded as the percentage of positively staining nuclei[137].

3.2.5 Statistical Methods

Correlations were calculated using both Spearman's Correlation and Pearson's Correlation methods. Univariate outcome analysis was performed using Kaplan Meier method and calculation of hazard ratios (HR) for both univariate and multivariate analysis performed using Cox's proportional-hazards model, a stepwise backward procedure was used to derive a final model of variables that had a significant independent relationship with patient outcome. Any patient with uncertain follow up was excluded from analysis. All statistical analysis was performed using SPSS software version 19 (SPSS Inc., Chicago IL, USA).

3.3 Results I

3.3.1 Patient and tumour Characteristics

557 early invasive breast cancer patient tumour samples were centrally tested and scored for ER, PgR, HER2 and Ki67. Outcome data (survival and recurrence) was confirmed for 517 (93%) patients. Patient and tumour characteristics are detailed in table 3-2 and patient treatment and follow up details are detailed in table 3-3.

Patient & Tumour Characteristics	Cohort (n=517)
Age	
≤50	151 (29%)
>50	366 (71%)
Nodal Status	
0	295 (57%)
1-3+	132 (26%)
>3	85 (16%)
unknown	7 (1%)
Tumour Size	
<20mm	308(59%)
20-50mm	191 (37%)
>50	18 (3.5%)
Tumour Grade	
1	103 (20%)
2	222 (43%)
3	191 (37%)
Tumour Type	
Invasive Ductal	439 (85%)
Invasive Lobular	38 (7%)
Invasive Other *	40 (8%)
ER %	
0	168 (32%)
1-50%	47 (9%)
51-100%	303 (59%)
ER-Allred Score	
<3	186 (36%)
≥3	331 (64%)
PgR-Allred Score	
<3	286 (55%)
≥3	231 (45%)
HER2 (IHC 2+)	
Unknown	26 (5%)
Negative	419 (81%)
Positive	73 (14%)

Table 3-2 Patient and tumour characteristics for all patients (n=517)

*Clinico-pathological details for patient cohort (n=517) in which tumour hormone receptors (ER and PgR) and HER2 were centrally retested. * Invasive Other including Medullary, Mixed, Mucoid, Tubular and other special type)*

Treatment and Outcome Details	Cohort (n=517)
Local Therapy	
WLE+Axilla	189 (37%)
No Radiotherapy	12 (6%)
Radiotherapy	177 (94%)
Mastectomy + Axilla	322 (62%)
No Radiotherapy	269 (84%)
Radiotherapy	31 (16%)
unknown	2(<1%)
WLE or Mastectomy only	7 (1%)
Systemic Therapy	
EndocrineTherapy	
None	138 (27%)
Tamoxifen	369 (71%)
ATAC trial	3 (<1%)
unknown	8 (1.5%)
Chemotherapy	
Yes	218 (42%)
No	289 (58%)
unknown	1 (<1%)
Survival- Mean (range), months	125 (2-180)
Deaths (any)	207 (40%)
Breast Cancer Related Deaths	105 (20%)

Table 3-3 Treatment and outcome details for all patients

3.3.2 Tumour ER and PgR expression

Hormone Receptor Status

An Allred ≥3 was used as cut-off to define hormone receptor positive cases. Within the cohort (n=517) 64% were ER+, Allred ER score <3 (n= 186, 36%) and Allred ER ≥3 (n=331, 64%). Less than half of the cohort were PgR +, Allred PgR <3 (n= 286, 55%) and Allred PgR ≥3 (n=231, 45%).

Tumour expression for ER and PgR had a significant 2 tailed correlation (Pearson correlation coefficient 0.631, Spearman's 0.625). Almost all ER negative tumours were also PgR negative (n=174, 94%). 6% of ER negative (n= 12) were PgR +, this is unusually high as it is estimated that only 1% of ER negative tumours are PgR+ (and some experts question

whether this phenotype exists at all). The majority of ER+ tumours were also PgR+, ER+/PgR+ (n=219, 66%) and ER+/PgR- (n=112, 34%).

Hormone Receptor Level

In hormone receptor + breast cancer, the amount of protein as detected by semi-quantitative IHC is considered helpful in categorising likelihood of endocrine response. Allred scores 3-5 were categorised as 'low' and scores ≥6 'high'. Over 80% of all ER+ tumours had 'high' ER (Allred ER high, n=273, 82% vs Allred ER low, n=58, 18%).

The level of PgR expression within tumours with high ER was variable. 70% of high ER were PgR+ (n=190). The majority of ER high also expressed high PgR (n=151, 55%) and 15% had low PgR (n=39). 30% were PgR negative (n=83).

In tumours with low ER (n=58), half of the tumours were PgR negative (Allred PgR <3, n=29). In low ER/PgR+, 16 tumours had high PgR (28%) and n=13 had low (22%).

The distribution of PgR expression within each Allred ER Score (0, 2-8) is demonstrated in figure 3-1 and table 3-4.

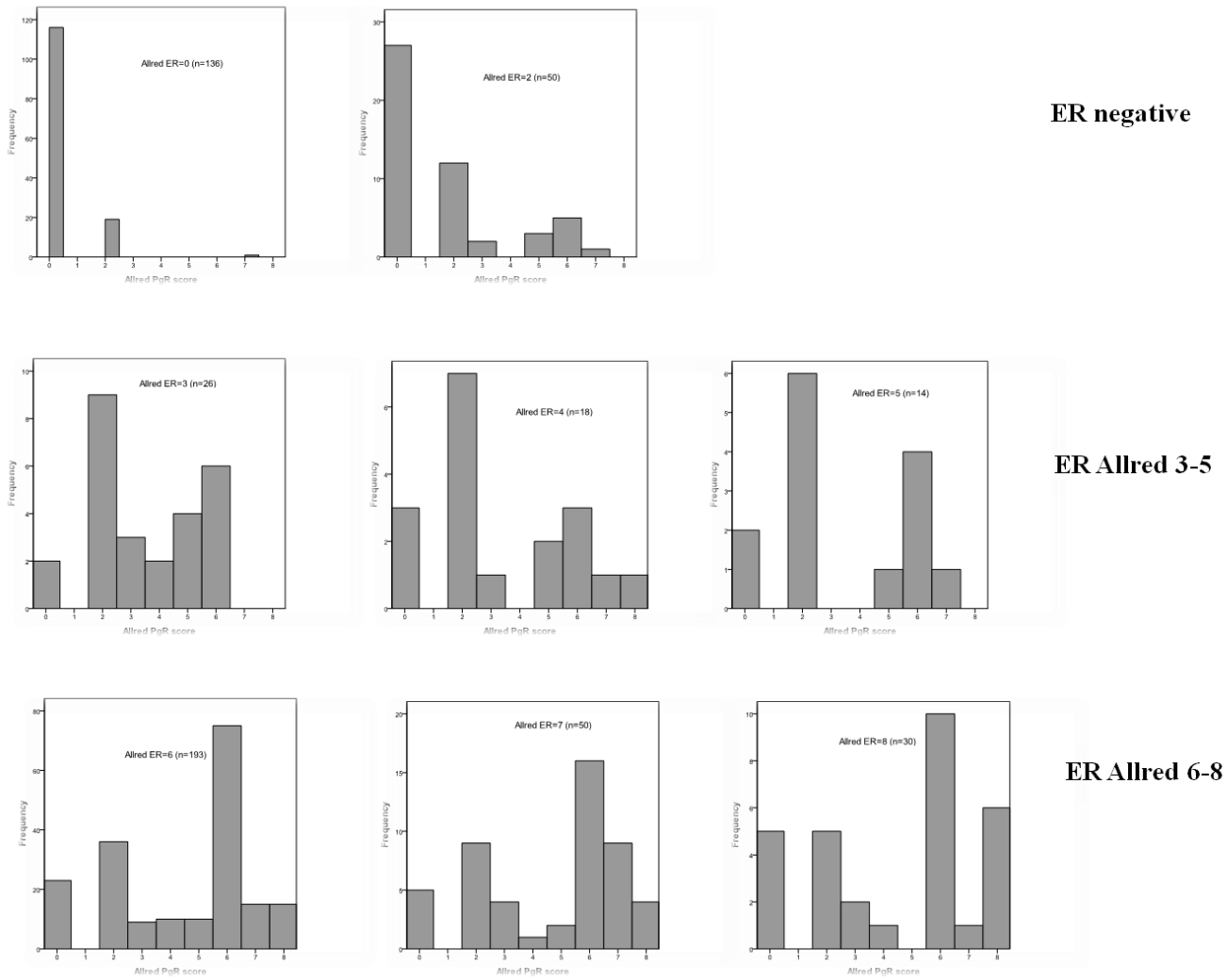


Figure 3-1 Histograms of distribution of PgR Allred Score within each Allred ER score

Histograms demonstrating the wide range of Allred PgR scores for each Allred ER score, 0 and 2-8. ER negative is defined as Allred ER<3. Table 4-3 details the number and Allred PgR score within each Allred ER score.

		Distribution of PgR Allred Score							
		0	**2**	**3**	**4**	**5**	**6**	**7**	**8**
ER Allred score	**0 (n=136)**	**116**	19					1	
	2 (n=50)	27	12	2		3	5	1	
	3 (n=26)	2	9	3	2	4	6		
	4 (n=18)	3	7	1		2	3	1	1
	5 (n=14)	2	6			1	4	1	
	6 (n=193)	23	36	9	10	10	75	15	15
	7 (n=50)	5	9	4	1	2	16	9	4
	8 (n=30)	5	5	2	1		10	1	6

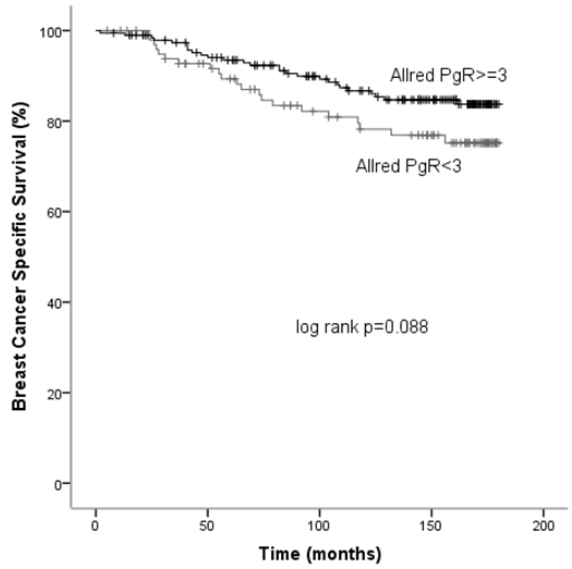
Table 3-4 Numbers of patients & Allred PgR scores within each Allred ER score 0-8

Details the exact numbers of tumours with Allred PgR scores 0-8 for each Allred ER score, corresponds to histograms figure 4-1.

3.3.3 PgR status influences outcome in ER+/Tamoxifen treated patients.

Analysis of PgR expression in all ER positive patients treated with tamoxifen (n=292) suggested PgR expression was associated with improved breast cancer specific survival (figure 3-2A, p=0.088, HR 0.6 (CI 0.3-1.1). In patients with high levels of ER expression (Allred≥6) treated with tamoxifen PgR negative tumours (n=76) had significantly shorter breast cancer specific survival (148 months, range 136-161 months) than tumours expressing PgR (n=167, mean survival time 163 months, range 156-170 months), p=0.025, HR 0.5 (CI 0.3-0.9) , figure 3-2B.

A



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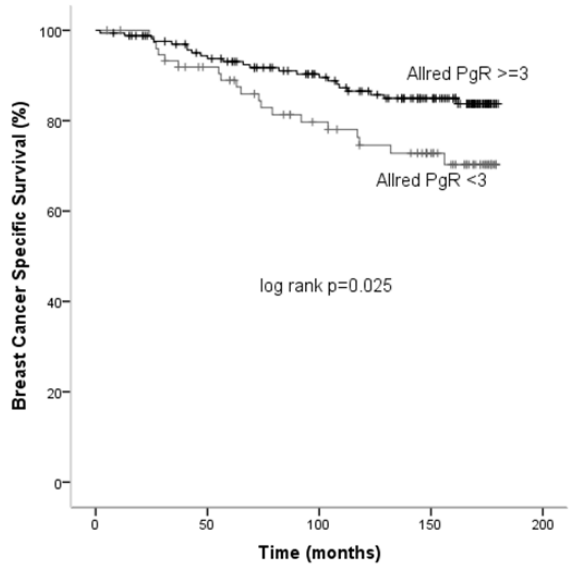


Figure 3-2 Influence of PgR in ER+ breast cancer patient outcome

Kaplan Meier survival curves. A) Influence of PgR status in all ER+ endocrine treated patients (n=292), p=0.088, HR 0.6 (using negative as reference category). B) PgR positive tumours associated with lower risk in Allred ER≥6 (n=243) p=0.025, HR 0.5

3.4 Results II The Combined Endocrine Receptor

3.4.1 Calculation of the Combined Endocrine Receptor (CER) Score and Cut-off definition

The Combined Endocrine Receptor (CER) score was calculated from the summation of Allred ER and Allred PgR and dividing by 2, resulting in a score range 0-8:

$$(CER = \{Allred\ ER + Allred\ PgR\} \div 2)$$

The predictive value of the ER is undisputed and the recommended cut-off value for defining hormone receptor positive tumours is an Allred score ≥3 (1-10% of weakly staining cells). To account for using two variables and division of the sum by 2, tumours with a CER ≥ 1.5 were considered positive; CER+, n=355, 69% and CER-, n=162, 31%. Using this cut-off ensured tumours with any (≥1% of tumour cells) of *either* ER or PgR were considered positive. Compared to using Allred ER alone, 24 tumours were therefore reclassified as positive, 5% of the cohort.

3.4.2 CER and definitions of Endocrine Response

In keeping with the St Gallen classification of endocrine response [52], which is based on the philosophy of defining categories according to their implications for treatment selection, the CER scores were categorised as follows:

- High CER (CER 2) - defined as combined score ≥6
- Low (impaired) CER (CER 1)-defined as combined score 1.5-5.5
- True negatives (CER 0)- defined as combined score <1.5

Compared to using ER alone, applying the combined score markedly altered the distribution of tumours into low and high endocrine response categories. Table 3-5 details the number of patients and the Allred 0-8 score for ER and PgR in low and high CER. No PgR negative

tumours are in the high CER, all tumours expressed *both* the ER and PgR. In addition, high ER tumours (Allred ER scores 6-8) are now fairly evenly divided between the low and high endocrine response categories.

	Impaired Endocrine Receptor (CER 1) CER score 1.5-5.5 (n=196)		**High Endocrine Response (CER 2), CER scores ≥6 (n=156)**	
Allred Score	**ER (no. patients)**	**PgR (no. Patients)**	**ER (no. patients)**	**PgR (no. Patients)**
0	1	40		
2	21	84		
3	26	21		2
4	16	13	2	2
5	13	20	5	12
6	88	16	101	91
7	19	2	30	25
8	12		18	24

Table 3-5 Distribution of Allred ER and PgR scores within Impaired and High Combined Endocrine Receptor Categories.

Table 3-6, summarises how the cohort is divided using ER, PgR, CER for status and level. With the CER score, all patients with either ER or PgR expression are considered positive, resulting in a small increase in positive tumours. Additionally it substantially increases the number of cases within the low/impaired group compared to ER (or PgR) independently, figure 3-3.

Cohort (n=517) number of patients (%)			
STATUS	**ER**	**PgR**	**CER**
negative	186 (36%)	285 (55%)	162 (31%)
positive	331 (64%)	231 (45%)	355 (69%)
LEVEL	**ER**	**PgR**	**CER**
negative	186 (36%)	286 (55%)	162 (31%)
Low/ impaired	58 (11%)	57 (11%)	199 (39%)
High	273 (53%)	174 (34%)	156 (30%)

Table 3-6 Comparison of distribution of ER, PgR and CER scores by status and level

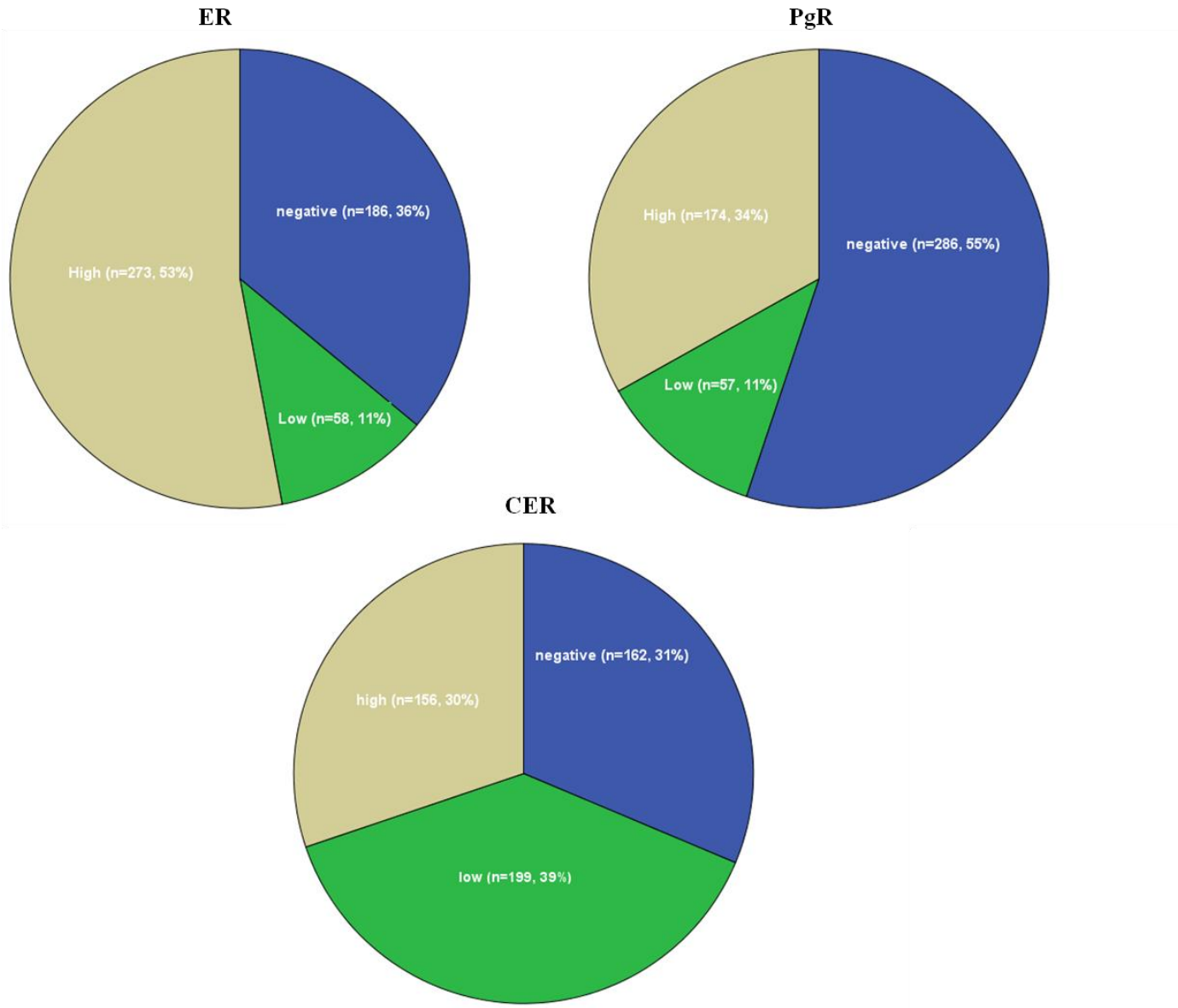


Figure 3-3 Comparison of distribution of receptor levels in ER, PgR and CER

CER results in less tumours being classed as negative and more tumours being re-classed as low (impaired endocrine response), compared to either ER or PgR alone. ER and PgR negative defined as Allred <3, low Allred scores 3-5 and high≥6. CER (a combined Allred ER &PgR/2) definitions of levels: ≤1.5 negative; 1.5-5.5 low and ≥6 high.

3.4.3 ER, PgR and CER Status and Survival

For the entire cohort, independently, both ER and PgR status of tumours was associated with improved breast cancer specific survival. ER negative patients had significantly shorter survival, with a mean survival time of 137 months (range 127-148) compared to ER positive, mean survival time 160 months (range 154-165), log rank p=0.001, HR 0.75 (CI 0.6-0.9), figure 3-4A.

PgR negative patients had a longer mean survival compared to ER negative, PgR negative mean survival time was 143 months (range 135-150). PgR positive patients mean survival time was 164 months (range 158-169), p=0.0004, HR 0.6 (CI 0.5-0.8), figure 3-4B.

The CER status was associated with a greater survival difference than either ER or PgR status. CER negative (n=157) had the shortest mean survival time (134 months, range 125-145), suggesting that this group are the 'true' hormone receptor negatives. CER positive (n=322) had a mean survival time of 160 months (range 154-164), p=0.0001, HR 0.58 (CI 0.4-0.7), figure 3-4C.

In multivariate analysis, when combined with grade, size, lymph node status and the ER and PgR status the CER was independently significant, CER HR 0.6 (95% CI 0.5-0.8, p=0.001). However PgR or ER alone were not deemed independently significant when combined with grade, size and lymph node status.

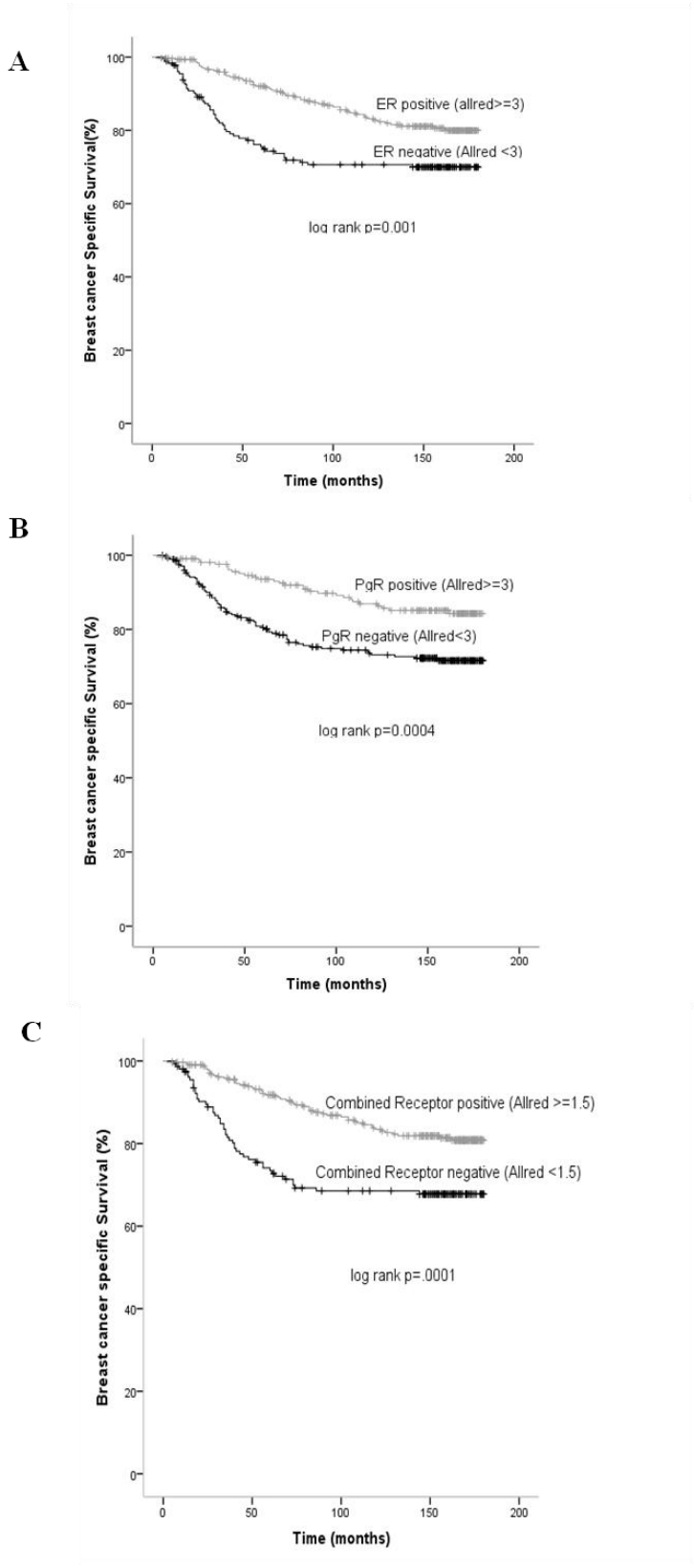


Figure 3-4 Kaplan Meier Survival Curves for ER, PgR and CER by status

(Allred score of ≥3 to define ER and PgR positive, and a CER≥1.5 to define CER positive cases).

3.4.4 ER, PgR and CER Level and Survival

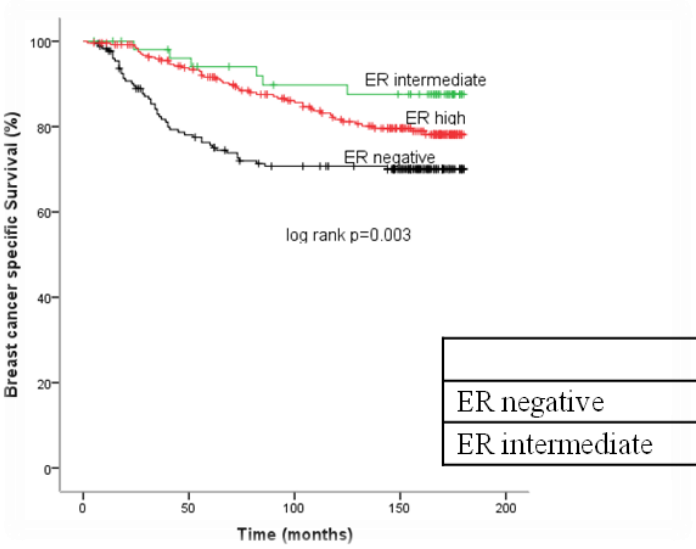
Expression levels of ER and PgR; low (Allred 3-5) and high (Allred≥6) were analysed in the entire cohort. Interestingly, ER high expressers had shorter mean survival time (157 months, range 151-163) than ER low expressers (mean survival time 166 months, range 155-176). In addition, in the Kaplan Meier survival curve (fig 3-5) the separation of ER low and high is not convincing, supporting that status (+/neg) is more informative than level of expression. As hypothesised, both were significantly better than ER negative (mean survival time 137 months), log rank p=0.003. Applying the negative groups as the indicator risk group-ER low HR 0.334 (CI 0.1-0.8) and ER high HR 0.6 (CI 0.4-0.8).

For PgR, the survival time improved with higher PgR expression and was associated with improved breast cancer specific survival. Over half of these patients were PgR negative, and only a small number were included in the low/ impaired group (n=57, 11%). The survival time for PgR negative was as before, low PgR has a mean survival time of 154 months (range 141-167) compared to patients with high PgR (mean survival time 166 months, range 160-172),log rank p=0.001, PgR low HR 0.6 (CI 0.3-1.2) and PgR high HR 0.4 (CI 0.2-0.6).

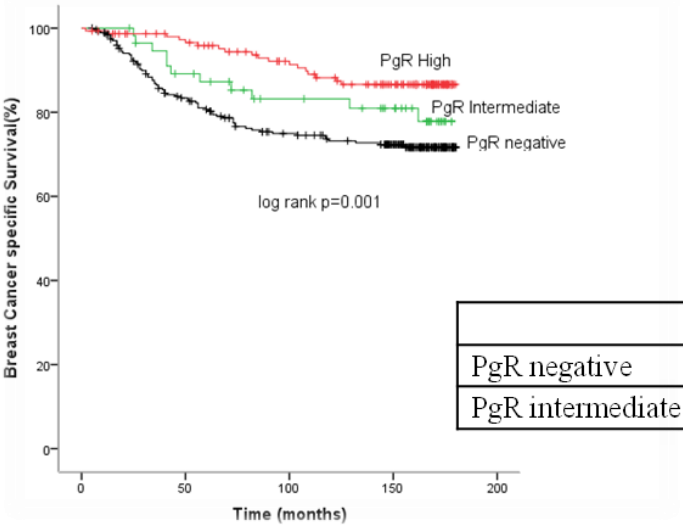
Applying the CER, and categories (CER<1.5= neg; CER 1.5-5.5 = low/impaired & CER ≥6= high) resulted in a fairly even distribution of number of patients in each category (neg n=162; low n=199 and high n=156) compared to ER or PgR alone. CER level was significantly associated with breast cancer specific survival. Negative CER mean survival time was 134 months (range 13-145), low CER 155 months (range 148-163) and high CER 163 months (range 157-171), log rank p=0.0003. CER low HR 0.5 (CI 0.3-0.8) and CER high HR 0.3 (CI 0.2-0.6), figure 3-5.

In multivariate analysis when combined with Allred ER, PgR, grade, lymph node status and tumour size the level of CER was independently significant (p=0.003), low CER HR 0.62 (CI

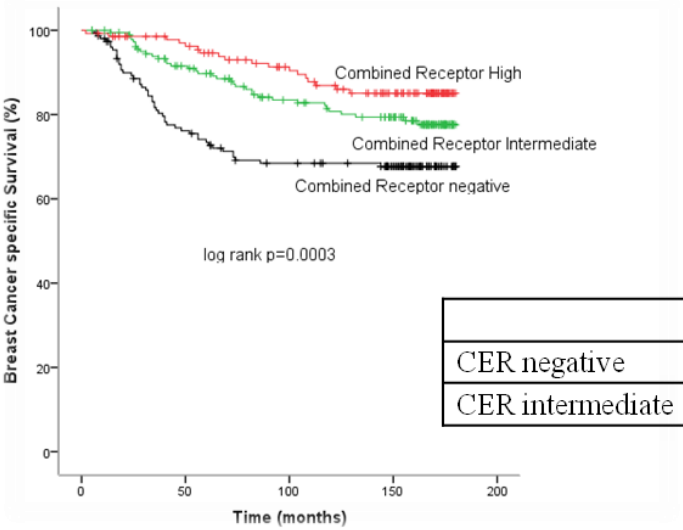
0.4-0.97, p=0.035) and high CER HR 0.4 (0.23-0.7, p=0.001). The levels of ER was not significant in multivariate analysis and whilst overall PgR was independently associated with disease specific survival (p=0.021), however when categorised into levels of PgR this association was lost, low PgR HR 0.9 (CI 0.47-1.7, p=0.78) and high PgR HR 0.47, (CI 0.273-0.8, p=0.006).



	ER intermediate	ER high
ER negative	p=0.01	p=0.005
ER intermediate		p=0.19



	PgR intermediate	PgR high
PgR negative	p=0.16	$p=9x10^{-5}$
PgR intermediate		p=0.14



	CER intermediate	CER high
CER negative	p=0.004	$p=7x10^{-5}$
CER intermediate		p=0.1

Figure 3-5 Kaplan Meier Survival Curves for ER, PgR and CER by level

3.5 Results III Hormone Receptor Levels in Endocrine Treated Cohort

3.5.1 Endocrine Cohort Characteristics

379 patients received endocrine therapy (>99% were treated with Tamoxifen and <1% (n=3) were enrolled in the ATAC trial). 80% (n=302) were ER+ and 54% patients were PgR+ (n=207). A surprising number of ER negative (20%) patients received endocrine therapy, a subtype of breast cancer characterised for its lack of response to hormonal agents. Within this cohort most patients were diagnosed prior to 1998 and this was the year that EBCTCG meta-analysis confirmed lack of benefit in ER negative disease, prior to this a small number of patients were occasionally given the "benefit of doubt" if other adjuvant therapy was contra-indicated or because of patient choice.

At 5 years 15% (n=49) of the endocrine treated patients had recurrence, the majority of these (78%) were documented distant recurrence, the site was not documented in 8 cases (16%). Local recurrence accounted for the minority of cases (n=3, 6%). For long term recurrence (10 years follow up), 71% were recurrence free (n=270). At 15 years, only 53% of the cohort were alive (n=200). Documented breast cancer related deaths accounted for 68 cases (18% endocrine treated cohort), non breast cancer related deaths or unknown cause accounting for the remaining. Breast cancer specific survival was the chosen survival outcome, given the high rate of deaths from other causes.

The distribution of ER, PgR and CER, both in terms of status and level, for the endocrine treated patients is detailed in table 3-7.

	Endocrine treated Cohort (n=379) number of patients (%)		
STATUS	**ER**	**PgR**	**CER**
negative	77 (20%)	172 (45%)	65 (17%)
positive	302 (80%)	207 (55%)	314 (83%)
LEVEL	**ER**	**PgR**	**CER**
negative	77 (20%)	172 (45%)	65 (17%)
Low/ impaired	51 (13%)	53 (15%)	172 (45%)
High	251 (67%)	154 (40%)	142 (38%)

Table 3-7 Hormone receptor status and level in endocrine treated cohort

3.5.2 Early Recurrence

Events were censored at 5 years, as this is the average period patients were receiving endocrine therapy for. Analysing ER independently, for early recurrence in endocrine treated patients the significant difference was between ER negative and ER positive tumours. ER negative tumours had 20 events, mean disease free survival time was 4.24 years (51 months, range 47-54) compared to ER high (25 events), mean DFS time 4.77 years (57 months, range 55-58). Although there were more events within the ER high, as table 3-7 details, this represented the majority of the cohort. There was no difference between low and high ER, low ER mean DFS (4.78 years, 57 months), p=0.94. Cox regression analysis confirmed that at 5 years, ER level does not influence risk beyond the ER status. Applying ER negative as the indicator category, at 5 years in tamoxifen treated patients ER low HR 0.3 (CI 0.1-0.8) and ER high HR 0.3 (CI 0.2-0.6). It is not the level of ER but rather the status of the tumour cell that influences early response to tamoxifen.

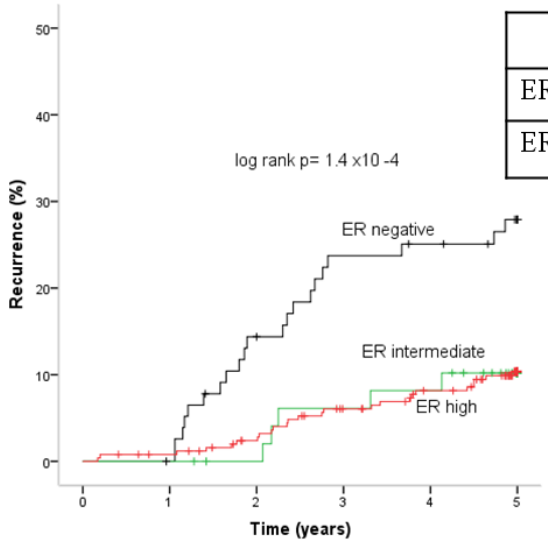
Analysing PgR independently, for early recurrence in tamoxifen treated patients nearly all events occurred within the PgR negative and low PgR group (35 and 9 events respectively, compared to 6 in PgR high). DFS times followed a more linear distribution. PgR negative

mean DFS time was 4.48years (54 months, range 52-56), PgR low DFS 4.64 years (56 months, range 53-59) and PgR high DFS 4.88 years (59months, range 56-60), log rank $p=0.7 \times 10^{-4}$. For PgR the significant difference is between high and low (p=0.004, fig 3-6) suggesting the amount of PgR as detected by IHC is important for optimal endocrine response. It is noteworthy that for both PgR negative and PgR high the DFS time is longer than ER negative and ER high, this likely reflects that within the PgR negative group some patients are ER positive and thus deriving some benefit from endocrine therapy. The longer DFS in PgR high supports that maximal endocrine benefit is associated with tumours (ER positive) expressing high PgR. Cox regression analysis supports that the significant difference in outcome was associated with high levels of PgR expression, low PgR HR 0.7 (CI 0.35-1.5) and high PgR HR 0.2 (0.07-0.4).

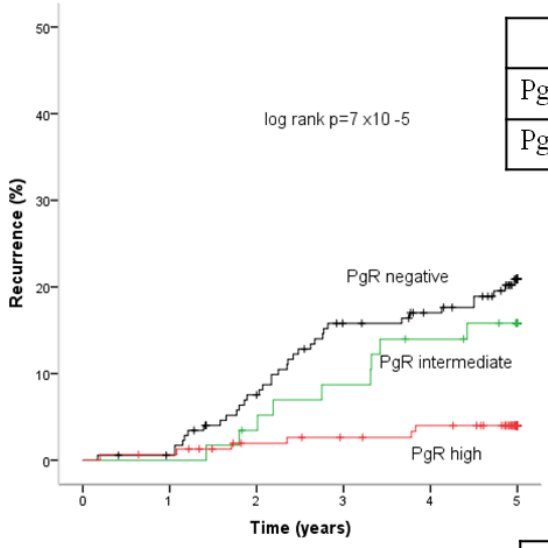
CER was more predictive than either ER or PgR independently for determining early recurrence (log rank $p=1 \times 10^{-5}$) in endocrine treated patients. CER negative patients (19 events) had the lowest mean DFS time 4.23 yrs (51 months, range 47-55). CER low (24 events) mean DFS time was 4.66 years (55 months, range 54-58) and CER high (only 7 events) mean DFS time 4.87 years, (58 months range 56-60). Cox regression analysis was highly significant between the CER categories, $p=8.7 \times 10^{-5}$. Low CER HR 0.45 (CI 0.2-0.8) and High CER HR 0.15 (CI 0.06-0.4).

At 5 years CER was independently significant in multivariate analysis, p=0.001 when combined with nodal status, tumour size, grade, ER and PgR. The Kaplan-Meier curves fig 4.5 demonstrate how using the combined endocrine score and categories results in marked, statistically significant divergence of the categories, p=0.008 between negative and low, and p=0.009 between low and high. For early recurrence applying the combined endocrine receptor score results in a statistically significant linear response between level of tumour

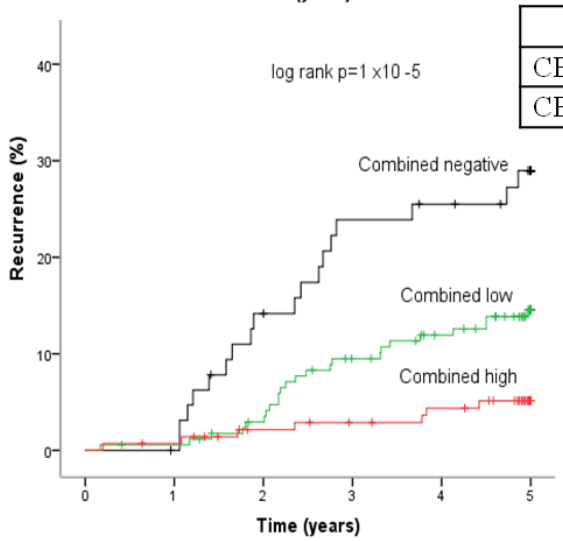
hormone receptors and outcome in a tamoxifen treated cohort and remains significant in multivariate analysis.



	ER intermediate	ER high
ER negative	p=0.026	p=4 x10^{-4}
ER intermediate		p=0.94



	PgR intermediate	PgR high
PgR negative	p=0.5	p=4x10^{-5}
PgR intermediate		p=0.004



	CER intermediate	CER high
CER negative	p=0.008	p=8x10^{-6}
CER intermediate		p=0.009

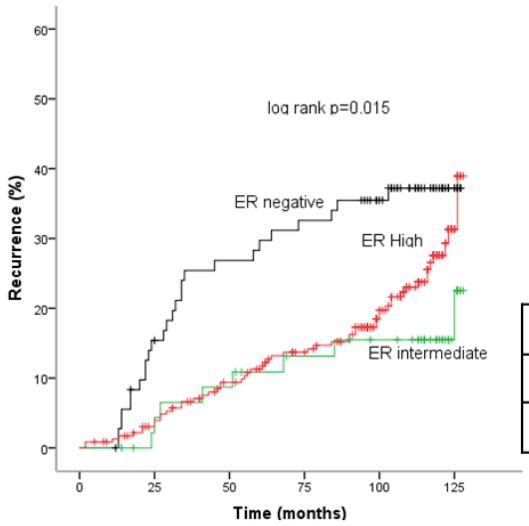
Figure 3-6 Early Recurrence Curves for ER, PgR and CER level in a endocrine treated cohort

3.5.3 Late Recurrence in Endocrine treated patients

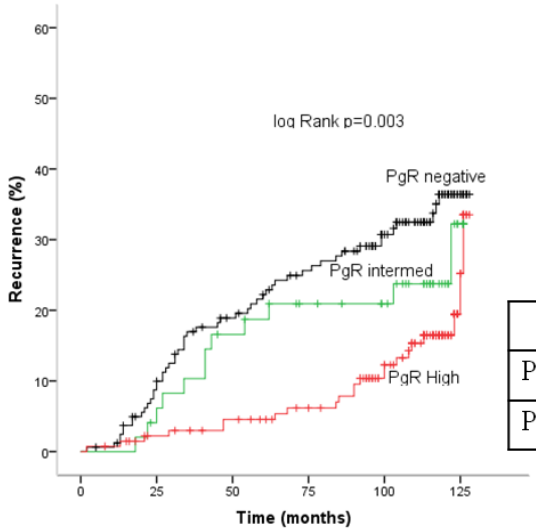
With longer follow up the advantage of having hormone receptor expression lessens in patients treated with endocrine therapy. At 10 years follow up, the Kaplan Meier curves for ER, PgR and CER are shown in fig3-7. For ER alone, the majority of events within ER negative occur within the first five years, and at 10 years ER high expressers have crossed with ER negative, although there is still significant difference in mean DFS time. For ER negative, the mean DFS time is 94 months (range 84-105) compared to 115 months (106-124) for ER low and 111months for ER high (range 107-116), log rank p=0.015. At 10 years the ER low HR 0.4 (CI 0.2-0.8) and ER high HR 0.6 (CI 0.4-1).

Analysing PgR independently, in keeping with early recurrence analysis, PgR negative cases have a longer DFS compared to ER negative, 101 months (range 95-108). The benefit of expressing high PgR lessens as seen by the narrowing of the curves, although at 10 years there is still significant advantage compared to PgR negative, mean DFS for PgR high 118 months (range 115-122), log rank p=0.003. It is noteworthy that the difference between PgR low and high is no longer significant, and actually the recurrence curves converge, although mean DFS time in PgR low is less (106 months, range 96-117). At 10 years the PgR low HR 0.65 (CI 0.4-1.2) and PgR high HR 0.4 (CI 0.3-7), p=0.003.

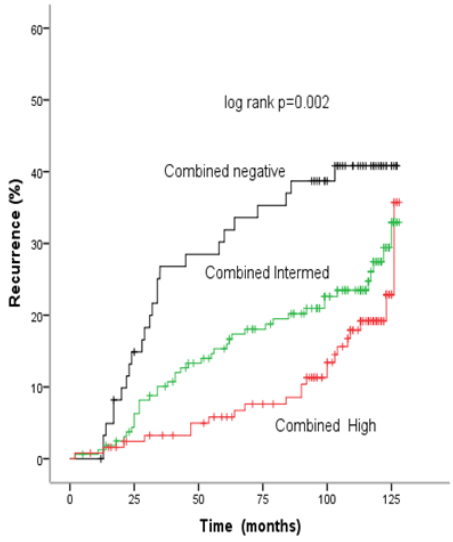
Similar to the results for early recurrence, applying a combined endocrine receptor score appears to more clearly distinguish the tumour differences in hormone receptor expression categories (most pronounced for low expressers, which when analysed for ER and PgR independently follow a less distinct course) and DFS. Mean DFS for CER negative is 92 months(range 80-103), in the low/ impaired group mean DFS is 107 months (range 103-114) and high mean DFS 117 months (113-122), log rank p=0.002. At 125 months the curves for CER low and high converge, but remain clearly distinct from CER negative. At 10 years low CER HR 0.6 (CI 0.3-0.9) and high CER HR 0.4 (CI 0.2-0.7).



	ER intermediate	ER high
ER negative	p=0.02	p=0.03
ER intermediate		p=0.16



	PgR intermediate	PgR high
PgR negative	p=0.2	p=0.001
PgR intermediate		p=0.2

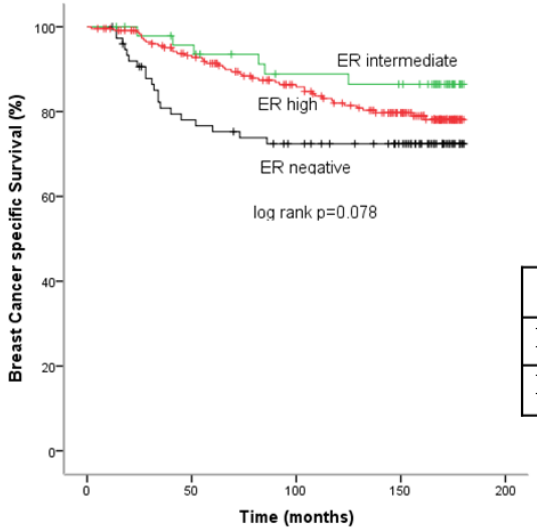


	CER intermediate	CER high
CER negative	p=0.024	p=0.001
CER intermediate		p=0.1

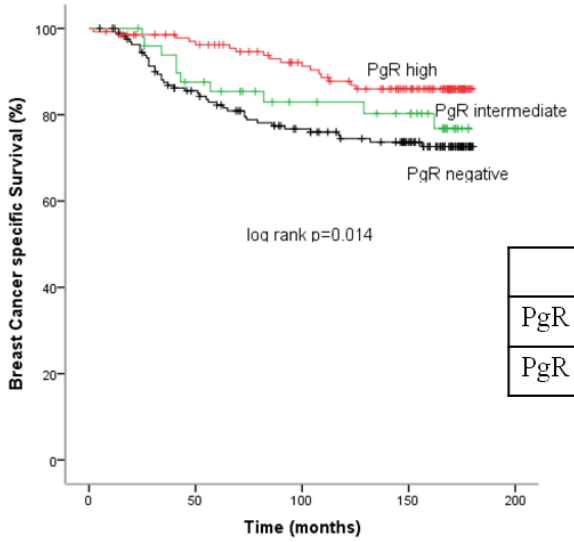
Figure 3-7 Late Recurrence Curves for ER, PgR and CER by level in an endocrine treated cohort

3.5.4 Breast Cancer Specific Survival in endocrine treated patients

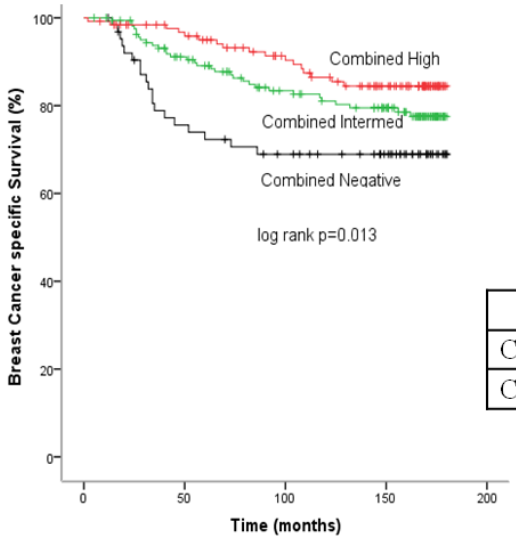
With 15 years follow up, the CER categorisation was statistically stronger in predicting outcome than either ER or PgR independently in the cohort of endocrine treated patients. ER categorised into negative, low and high was not significantly associated long term survival (log rank p=0.058), low ER HR 0.4 (CI 0.16-0.9) and high ER HR 0.6 (0.35-1), although the HR suggest improved outcome these are barely significant (table 3-9). For PgR, the significant difference appears to be between PgR negative and PgR high. There were 41 events in the PgR negative, mean survival time 146 months (range 137-155), 10 events in low (intermediate) group, mean survival time 153 months (138-167) and for PgR high 17 events, mean survival time 166 months (range 160-172), log rank p=0.014, low PgR HR 0.67 (CI 0.3-1.4) and high PgR HR 0.4 (CI 0.25-0.8). For CER negative, there were 19 events with a mean survival time of 135 months (range 118-152). In CER low group, 32 events mean survival time 155 months (range 147-163) and for CER high 17 events with a mean survival time of 164 months (range 157-171), log rank p=0.013, low CER HR 0.6 (0.3-0.99) and high CER HR 0.3 (HR 0.2-0.7).



	ER intermediate	ER high
ER negative	p=0.047	p=0.054
ER intermediate		p=0.3



	PgR intermediate	PgR high
PgR negative	p=0.26	p=0.003
PgR intermediate		p=0.25



	CER intermediate	CER high
CER negative	p=0.004	p=0.002
CER intermediate		p=0.1

Figure 3-8 Breast Cancer Specific Survival Curves for ER, PgR and CER by level in a tamoxifen treated cohort

Table 3-8 summarises the Hazard Ratios (HR) and 95% Confidence Intervals (CI) calculated for ER, PgR and CER levels at 5 years, 10 years and 15 years. High CER has the lowest HR that is statistically significant, compared to ER or PgR. In addition the CI for CER categories are narrow. In Cox regression analysis there is significant difference between the CER low and high categories. In terms of risk, within this cohort increasing ER level is not associated with improved outcome. In fact with time tumours with high ER expression have significant higher risk than tumours with low level of ER expression, although the presence of ER is associated with better outcome than ER negative. In contrast the overall level of PgR is significantly associated with improved outcome at all time points, most pronounced in the first 5 years.

In multivariate analysis in the endocrine treated cohort the CER was independently significant at 5 years (HR 0.45, CI 0.3-0.7, $p=3x10^{-4}$), 10 years (HR 0.65 CI 0.48-0.88, p=0.005) and 15 years (HR 0.69, CI 0.49-0.98, p=0.036) when combined with grade, lymph node status and tumour size.

	5 years- outcome recurrence			**10 years- outcome recurrence**			**15 years- outcome Breast Cancer Specific Survival**		
	HR	**CI**	**p**	**HR**	**CI**	**p**	**HR**	**CI**	**p**
CER	$8.7x10^{-5}$			0.003			0.009		
Low	0.45	0.2-0.8	0.01	0.6	0.3-0.9	0.027	0.6	0.3-0.99	0.046
high	0.15	0.1-0.4	$2.5x10^{-5}$	0.4	0.2-0.7	0.001	0.3	0.2-0.7	0.002
ER	$3.5x10^{-4}$			0.022			0.065		
Low	0.3	0.1-0.8	0.02	0.4	0.2-0.8	0.014	0.4	0.16-0.9	0.045
high	0.3	0.2-0.6	$1.3x10^{-4}$	0.6	0.4-1	0.03	0.6	0.35-1	0.057
PgR	$4.7x10^{-4}$			0.003			0.013		
low	0.7	0.35-1.5	0.4	0.64	0.3-1.2	0.17	0.67	0.3-1.4	0.26
high	0.2	0.07-0.4	$9x10^{-5}$	0.43	0.3-0.7	0.001	0.4	0.2-0.8	0.004

Table 3-8 Summary of Hazard Ratio's for CER, ER & PgR in an endocrine treated cohort

3.6 Results III- Expression of biological markers in Low and High CER

Molecular Profiling studies have identified that ER positive breast cancer is characterised by luminal A and luminal B subtypes. Luminal A, approximately 40% of all breast cancers, usually have high expression of ER-related genes, low expression of the HER2 cluster of genes, and low expression of proliferation-related genes [32, 33]. Luminal A has the best prognosis of all breast cancer subtypes [29, 34-37]. Luminal B, approximately 20% of all breast cancers, have relatively lower (although still present) expression of ER-related genes, variable expression of the HER2 cluster, and higher expression of the proliferation cluster. Luminal B tumours carry a worse prognosis than luminal A [37]. In all tumours Ki67 and HER2 were analysed to determine if low/impaired CER was associated with the expression of these two markers.

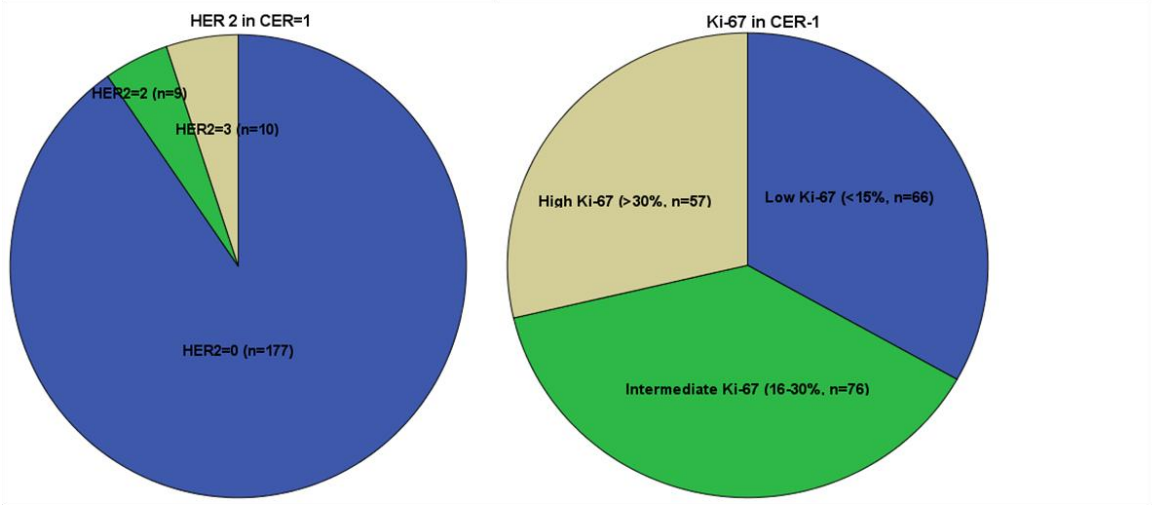
3.6.1 Ki67

Ki67 was classified as low (≤15%), intermediate (16-30%) and high (>30%) [52, 138]. Both low CER and high CER had similar proportion of tumours with low Ki67. Low CER was associated with more tumours having high Ki67 (29% versus 13% of high CER expressing high Ki67) suggesting that low CER may be associated with luminal B subtype. Interestingly, however, CER negative tumours did not have high proportion of tumours with high proliferation scores as would be expected. Ki67 was analysed independently in terms of outcome and was not associated with recurrence or survival.

3.6.2 HER 2

No significant difference was noted in HER2 expression between low and high CER, both groups have similar distribution of HER2 IHC scores. In negative CER, at least 25% of the cohort were HER2 positive (as defined by an IHC score of 3, and a further 10% had IHC scores of 2, which are termed indeterminate and would require further analysis). In all groups HER2 over expression was associated with poor patient outcome. Figure 3-9 illustrates the frequencies of Ki67 and HER2 IHC scores between low CER and high CER for the entire cohort.

Impaired Endocrine Response



High Endocrine Response

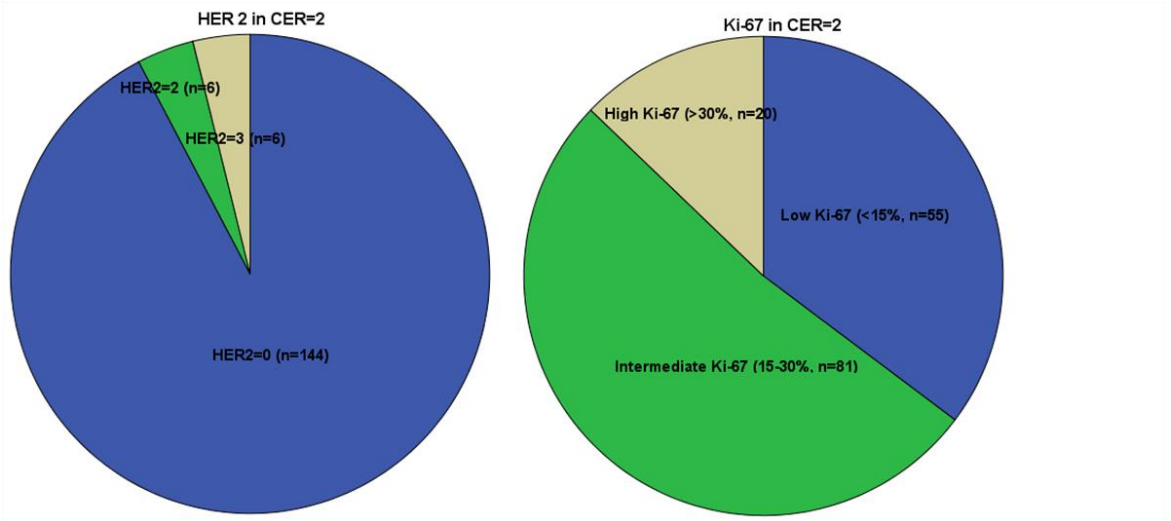


Figure 3-9 HER2 expression as measured by IHC and Ki-67 expression in low and high CER

Similar distributions of HER2, although high Ki67proliferation score (>30% positively staining nuclei) were more common in impaired CER than high CER, in CER negative Ki67 scores were low (see table 3-10).

3.6.3 Tumour size, lymph node involvement and Grade

Examining other recognised prognostic factors such as tumour size, lymph node involvement and grade, demonstrated that the low CER and high CER had almost exactly the same distribution of these prognostic factors. Negative CER was associated with Grade 3, more patients with >3 nodes involved and more patients with tumours 20-50mm, reflecting the biological aggressiveness of hormone negative early breast cancer. The distribution of prognostic factors, HER2 and Ki67 for the entire cohort divided into CER category is detailed in table 3-9.

	Combined Endocrine Receptor Category (entire cohort n=517)		
	Negative (n=162)	**Impaired (n=199)**	**High (n=156)**
Nodes			
Negative	55%	59%	58%
0-3 nodes+	24%	26%	28%
>3 nodes+	20%	14%	13%
Grade			
1	2%	27%	26%
2	20%	54%	54%
3	78%	19%	19%
Tumour Size			
<20mm	47%	65%	65%
20-50mm	49%	32%	32%
>50mm	4%	3%	3%
HER2			
0	70%	89%	92%
2	5%	4.5%	4%
3	25%	5%	4%
unknown		1.5%	
Ki67			
<15% Low	63%	33%	33%
16-30% Med	23%	38%	52%
>30% High	15%	29%	13%

Table 3-9 Distribution of Prognostic factors in CER categories

3.7 Discussion

The combined endocrine receptor (CER) is a novel and easily reproducible marker. In this explorative, retrospective study of patients with early breast cancer, using the current cohort the CER appears to offer a better method of discrimination for both recurrence (early and late) and breast cancer specific survival than either ER or PgR independently in an endocrine treated cohort. In addition the CER is statistically significant in terms of level of response and independently significant in multivariate analysis when combined with grade, lymph node status and tumour size.

This data confirms that ER status is the most important factor in determining benefit from tamoxifen, and demonstrates no evidence to support level of ER expression influences response to endocrine therapy beyond its presence in the tumour cell or when its level is combined with the level of PgR expression. In concordance with other studies, this study demonstrates that tumour cells that are ER+ and have absent PgR still benefit from endocrine therapy and PgR status is a poor discriminator of potential benefit. The role of PgR in this study is in addition to ER, maximal endocrine response is in ER+/PgR+. The concept of *both* ER and PgR expression influencing response to endocrine therapy is not new. It has been demonstrated previously that patients with ER+/PgR- tumours have poorer response and outcome with endocrine treatment and this has been re-confirmed in recent adjuvant endocrine therapy trials comparing AIs and tamoxifen, in which biomarkers have been centrally tested and compared with outcome [87, 105, 139]. Yet, despite decades of measuring the PgR its role as a predictor factor remains poorly defined.

This data demonstrates that PgR expression level appears to follow a linear relationship with endocrine response and highlights the importance of tumour PgR expression level in hormone responsive breast cancer. In patients with high levels of ER expression who normally would be classified as ‘high endocrine response’, the absence of PgR was significantly associated

with poor outcome. In addition, at 15 years follow up, PgR expression was significantly associated with breast cancer specific survival, and ER expression was not. It could be argued that PgR is simply an excellent prognostic factor, and thus influencing results. Yet at the cellular level we know that PgR is a marker of a functional oestrogen response pathway. The absence of PgR in tumours with any ER expression likely represents an *impaired* oestrogen signalling pathway, one that can function albeit to a lesser degree, perhaps due to enhanced growth factor signalling or other biological processes discussed in the introduction.

This exploratory study adheres to established research principles for potential markers [140]. It uses a simple hypothesis based on biological theory- that ER and PgR together represent a fully functioning intact oestrogen response pathway, and reduced expression of either represent that this is impaired. Expression level of both receptors were centrally tested using validated IHC method to avoid testing variation, and expression level assumed to be important on the basis of a wealth of literature supporting this. Clearly defined cut-offs were applied and as ER and PgR are routinely assessed by IHC universally, this study will be easily reproducible. In 2005 guidelines were published on reporting recommendations for tumour marker prognostic studies (REMARK). [141] This emphasised the requirement for clear study design, a pre planned hypothesis, detailed patient /specimen characteristics; detailed and reproducible study assay methods and detailed statistical analysis. The recommendations are for prognostic markers and it is recognised that reporting of potential predictive markers is more complex and less frequent although the recommendations are still applicable. Certainly, the simplicity of this study and the fact it is retrospective in cohort of early breast cancer patients diagnosed almost two decades ago leaves it vulnerable to ridicule or critism, however it has been conducted in adherence to guidelines. Although simple in hypothesis and calculation, the results are highly significant and encouraging for aiding the identification of likely tumour response to endocrine therapy and ‘functionality’ of the ER.

Applying the combined endocrine receptor score resulted in a marked redistribution of patients in terms of endocrine response. Firstly, more patients would be considered hormone 'responders', applying a CER score ≥1.5 would result in a small increase of patients being recommended endocrine therapy and potentially gaining in survival advantage. Endocrine therapy, although very rarely associated with life-threatening side effects is in general a very well tolerated form of therapy, and consensus opinion is that a low threshold should be employed to ensure that any patient that may derive benefit will be recommended adjuvant endocrine treatment. Importantly, this data demonstrates that high ER Allred scores were not always associated with optimal outcome. More patients would be labelled as 'impaired' endocrine responders, and subsequently this may result in more patients being considered for both adjuvant chemo-endocrine therapy. Importantly, a high combined endocrine score was associated with low risk, most pronounced within the first five years and this remained significant in univariate and multivariate analysis with time. High CER is potentially a marker that will reassure both clinicians and patients alike that endocrine therapy alone is a safe option. In this cohort , at 15 years in terms of breast cancer specific survival high CER HR 0.3 (CI 0.2-0.7, p=0.002), comparable with Grade 1 tumours (a well established marker of excellent outcome) 15 year HR 0.27 (0.12-0.6, p=0.001) (data not shown in results). More 'good markers' will be helpful in treatment decisions for the challenging (and heterogenous) group of ER+ HER2 negative breast cancer patients. Current evidence from landmark studies demonstrate that AIs in post menopausal woman are associated with decreased recurrence and likely overall survival, its therefore anticipated that in current practice should the CER be applied that HR will be even lower. The cut-off values between endocrine response categories are fairly arbitrary, and were selected based on literature review and definition of high and low receptor expression in adjuvant studies. These values would benefit from further refinement.

It is increasingly recognised that ER+ breast cancer is heterogenous. Applying the CER, ER+ cancer was divided into 2 cohorts with significant differences in patient outcome. As a secondary analysis it was hypothesised that this may represent luminal A and luminal B subtypes, however analysis of HER2 expression and Ki67 within the CER categories did not support this. In addition, in terms of tumour grade, size, and lymph node involvement both low and high CER were indistinguishable. Together these results suggest that CER is an excellent predictor of tumour endocrine response and it is not aggressive biological behaviour of the tumour cell that is influencing the outcome differences between low and high CER in endocrine treated patients. The combined endocrine receptor score may be a novel method of utilising IHC markers currently routinely measured to provide some insight into the function of oestrogen signalling in the tumour cell.

4 Clinical Outcome Score

4.1 Introduction

ER positive breast cancer is heterogeneous. The optimal management of these patients remains a significant challenge. The threshold for using adjuvant chemotherapy in addition to adjuvant endocrine therapy remains one of the most controversial issues in the treatment of women with breast cancer. Traditionally the decision to administer adjuvant chemotherapy was based on tumour burden or anatomical extent of disease. The advent of gene expression profiling, in particular gene prognostic signatures has emphasised the importance of tumour biology for patient outcome. Studies comparing various gene prognostic profiles, indicate commonality in sampling groups of genes representing activation of the oestrogen receptor signalling pathway, EGFR and HER2 signalling pathways and markers of proliferation [39, 111].

The aim was to produce a scoring system, using biological markers available from standard pathological reports, that reproduce biological processes important in breast cancer progression, that is simple to calculate and easy to reproduce. The reason we require such a system is to aide identification of patients with ER+ early breast cancer at risk of poorer outcome, and who may potentially benefit from adjuvant chemotherapy.

4.2 Results I- Calculating the Clinical Outcome Score

The Clinical Outcome Score (COS) was calculated using conventional pathological markers, and employed the use of our novel Combined Endocrine Receptor Score described in the previous chapter. Grade (1-3) was used as a surrogate marker of proliferation, HER2 IHC score (0-3) for HER2 expression and CER as a surrogate marker of the oestrogen signalling pathway. CER was categorised as in chapter 3, and detailed in table 4-1. Age was included as a risk, with 1 point being awarded for over 50.

Combined Endocrine Receptor (CER) Score ($CER = \{Allred\ ER + Allred\ PgR\} \div 2$)	**Combined Endocrine Receptor Category & code for COS Calculation**
≤1.5	*Negative* : **0**
1.5-5.5	*Low:* **1**
≥6	*High:* **2**

Table 4-1 Combined Endocrine Receptor (CER) category & code for COS calculation

The formula to calculate the clinical outcome score was

$$COS = (Grade + HER2 + \{3 - CER\ code\} + 1\ for\ age > 50)$$

The minimum score was 2 (grade 1 tumour, HER2 IHC 0, high CER, code $2^{(3-2=1)}$ and age under 50). The maximum score was 10 (Grade 3, HER 2 IHC score 3, negative CER, code 0 $^{(3-0=3)}$ and age over 50).

4.3 Results II - Clinical Outcome Score in Entire Cohort

4.3.1 Patient and tumour Characteristics- Entire Cohort

The patient cohort was the same as detailed in chapter 3, although slightly less in number (n=511) as details were missing from 6 patients tumours preventing COS calculation. COS scores and 15 year breast cancer specific survival data was available for 495 samples (96%), 10 year recurrence data for 485 (94%) and 5 year recurrence data for 511 (100%). Patient and tumour characteristics and clinical outcome are demonstrated in table 4-2 and 4-3.

	Cohort (n=511)
Age	
≤50	146 (29%)
>50	365 (71%)
Nodal Status	
0	290 (57%)
1-3+	133 (26%)
>3	82 (16%)
unknown	6 (1%)
Tumour Size	
<20mm	305 (60%)
20-50mm	189 (37%)
>50	16 (3%)
unknown	1 (<1%)
Tumour Grade	
1	95 (19%)
2	222 (43%)
3	194 (38%)
Local Therapy	
WLE+Axilla	184 (36%)
No Radiotherapy	7 (4%)
Radiotherapy	177 (96%)
Mastectomy + Axilla	321 (63%)
No Radiotherapy	267 (83%)
Radiotherapy	52 (16%)
unknown	2(<1%)
WLE or Mastectomy only	6 (1%)
Systemic Therapy	
EndoTherapy	
None	128 (25%)
Tamoxifen	373 (73%)
ATAC trial	3 (<1%)
unknown	7 (1%)
Chemotherapy	
Yes	211 (41%)
No	298 (58%)
Unknown	2 (<1%)
ER-Allred Score	
<3	183 (36%)
≥3	328 (64%)
PgR-Allred Score	
<3	282 (55%)
≥3	329 (45%)
HER2 (IHC)	
0	432 (84.5%)
2	23 (4.5%)
3	56 (11%)
Combined Endocrine Receptor (CER)	
Negative	
Low	159 (31%)
High	196 (38%)
	156 (31%)
Combined Outcome Score	
2	10 (2%)
3	58 (11%)
4	137 (27%)
5	103 (20%)
6	72 (14%)
7	66 (13%)
8	18 (3%)
9	24 (5%)
10	23 (4.5%)
COS category	
Low (0)	205 (40%)
High (1)	306 (60%)

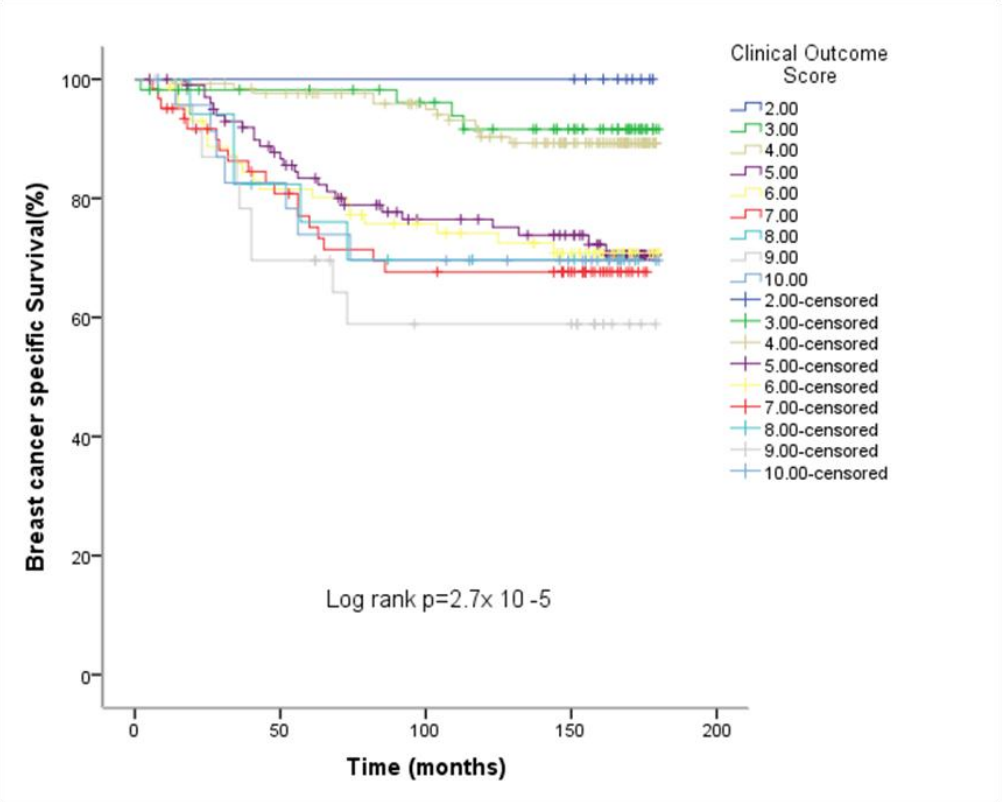
Table 4-2 Patient and tumour characteristics for entire cohort (n=511)

Follow Up Details for Clinical Outcome Score 'Entire' Cohort n=511	
Breast Cancer Specific Survival	n=497
Range	2-180 months
Mean survival	123 months
Median survival	152 months
Deaths (any)	197 (40%)
Breast Cancer related Deaths	101 (20%)
Early Recurrence (events censored at 5 years)	n=511
Range	0-5 years (0-60 months)
Mean DFS	4.37 years (52 months)
Median DFS	5 years
Recurrence type	
No	430 (84%)
Local	8 (1.5%)
Distant	60 (12%)
Site not documented	13 (2.5%)
Late Recurrence (10 years follow up)	n=485
Range	2-128 months
Mean DFS	89 months
Median DFS	104 months
Recurrence type	
No	356 (73%)
Local	19 (4%)
Distant	79 (16%)
Site not documented	31 (6%)

Table 4-3 Follow up details for entire cohort

4.3.2 Distribution of COS scores in the entire cohort and outcome

The distribution of scores and long term breast cancer specific survival in all patients is shown in figure 4-1. There was an almost linear distribution within the cohort, the higher the score the worse the outcome, $p= 2.7x10^{-5}$



Clinical Outcome Score	No. Patients n=511	Significance
2	10 (2%)	
3	58 (11%)	p=0.38
4	137 (27%)	p=0.71
5	103 (20%)	**$p=3.6x10^{-4}$**
6	72 (14%)	p=0.74
7	66 (12%)	p=0.55
8	18 (3%)	p=0.78
9	24 (5%)	p=0.5
10	23 (4.5%)	p=0.343
Overall		**$p=2.7x10^{-5}$**

Figure 4-1 Clinical Outcome Score (2-10) and Survival for entire cohort

There is a clear division in the cohort, COS 2-4 have improved outcome compared to scores 5-10. Although score 2 is associated with nearly 100% survival, this included only 9 patients. In addition, score 9 had the poorest outcome.

Low Risk was therefore defined as scores 2-4, and high risk scores 5-10. At 15 years for breast cancer specific survival for the entire cohort, 195 patients scored low and there were 16 breast cancer related deaths in this group, the mean survival time for low COS was 171 months (range 166-175 months). For high COS (n=300), there were 85 breast cancer related deaths, the mean survival time was 140 months (range 133-147), $p=2.5x10^{-8}$. Fig 4-2 displays the breast cancers specific survival for the cohort divided into low and high COS.

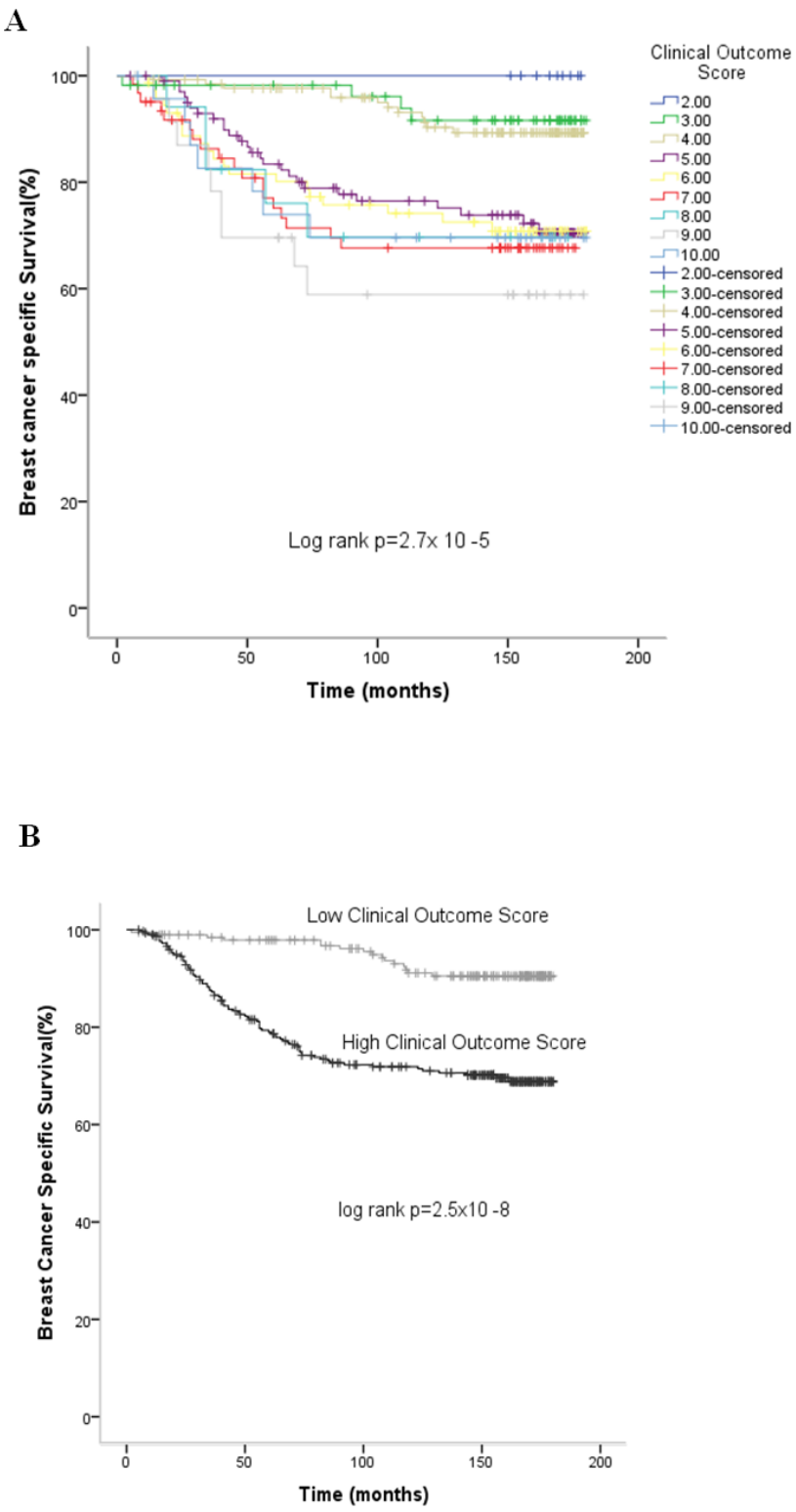
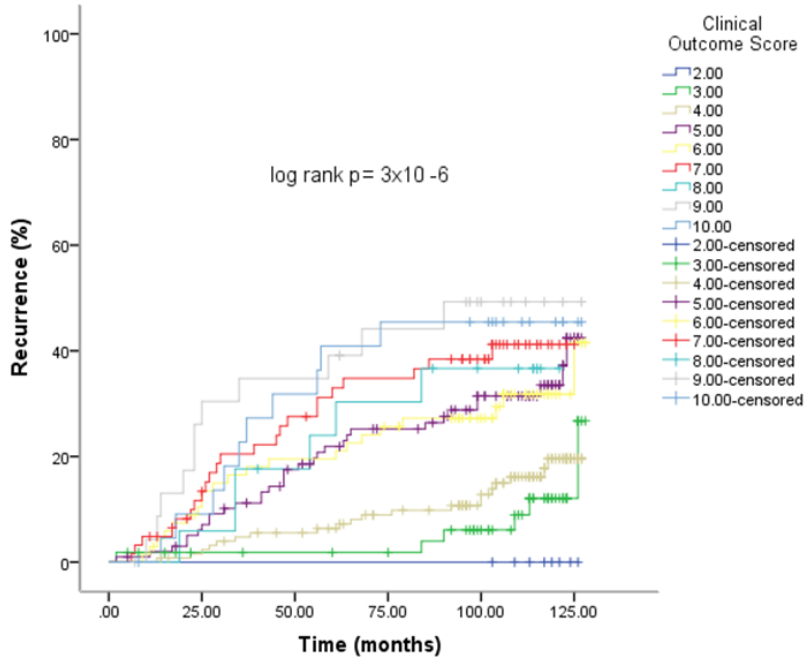


Figure 4-2 Low and High COS and Breast Cancer Specific Survival (entire cohort)

Kaplan Meier Survival Curves for Clinical Outcome Score (COS) and Survival. A) COS scores 2-10, as fig 4-1. B) Low Cos, scores 2-4 (n=195) and High COS, scores 5-10 (n=300).

10 year recurrence data (n=485) in the entire cohort followed a similar linear distribution, $p=3 \times 10^{-6}$. There was a similar division between low (2-4) and high (5-10) scores that was highly significant. In the low risk group (n=191) there was 26 events, and mean DFS time was 119 months (range 115-122). In high risk group (n=294), there were 103 events and mean DFS time was 97 months (range 92-102), $p=2 \times 10^{-8}$. Fig 4-3.

A



B

Score	Significance
2	
3	p=0.3
4	p=0.4
5	**p=0.001**
6	p=0.92
7	p=0.20
8	p=0.63
9	p=0.35
10	p=0.56
Overall	**$p=3x10^{-6}$**

C

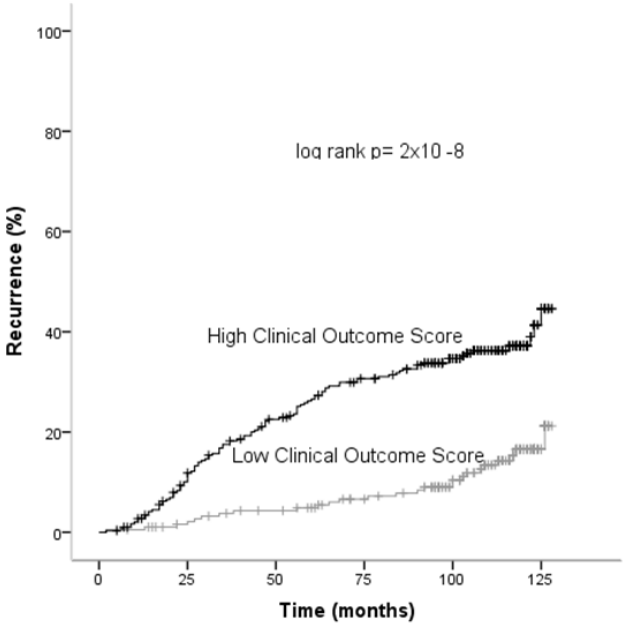


Figure 4-3 Low and High COS and 10 year Recurrence (entire cohort)

Kaplan Meier Survival curves for Breast Cancer Recurrence. A) COS scores 2-10. B) Significance between scores 2-10 sequentially and justification for selecting cut-offs between low/high. C). Low COS, scores 2-4 (n=191) and high COS, scores 5-10 (n=294)

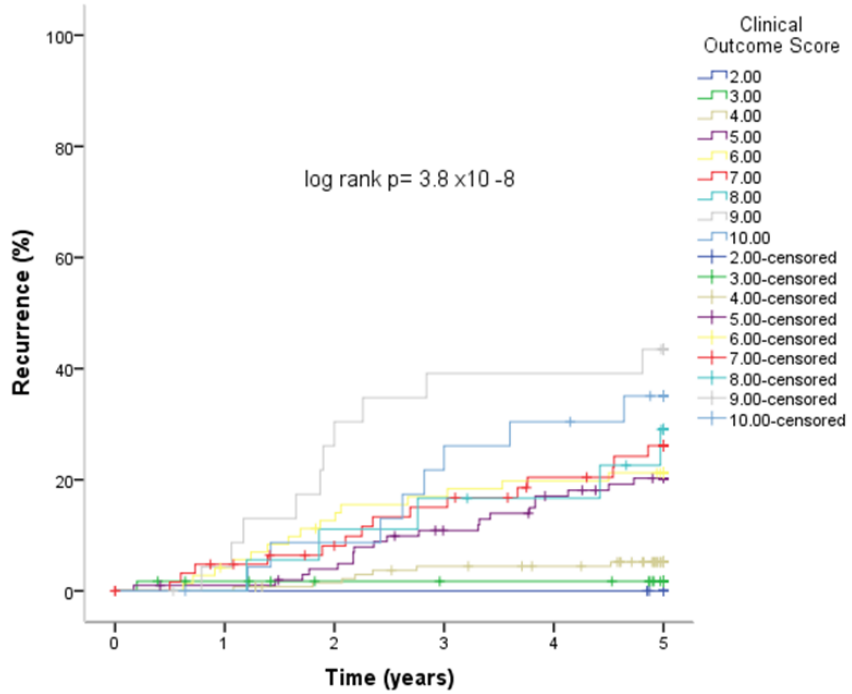
4.3.3 COS predicts early recurrence

COS appears to be an excellent predictor for risk of early recurrence. Events were censored at 5 years, recurrence and COS was known for 511 patients. At 5 years, scores follow a linear distribution, $p=3.8x10^{-8}$ fig 4-4. One notable difference is that very high scores (9 and 10) appear very high risk. Scores 9 and 10 accounted for 48 patients, nearly 10% of the entire cohort. 37 patients were over age 50, no tumours were grade 1 (88%, n=42 were grade 3), almost all (n=44) were CER negative with only 4 (8%) low CER. 96% (n=46) had HER2 expression (n=40 IHC HER2 3+). This cohort will not have been treated with anti HER2 therapy, as patients were diagnosed prior to its routine use in adjuvant therapy for early breast cancer. Interestingly nodal involvement or tumour size did not appear to heavily influence this 'very high risk' group. 21 (44%) were lymph node negative, 11 (23%) had 1-3 nodes+ and 15 (31%) had >3 nodes involved. Nearly half (n=22, 45%) of this very high risk group had tumours <20mm, and 22 (50%) had tumours 20-55mm, and only 4 patients had tumours >50mm.

Applying the defined classification of low (2-4) and high (5-10), there were 8 recurrences in the low group (n=205). 1 recurrence was local, 6 distant and site not known for 1. The DFS time was 4.88 years (59 months, range 57.5-59months). For high (n=306), there were 73 recurrence events (7 local, 54 distant and 12 site not documented). High DFS was 4.38 years (52.5 months, range 50-54), the DFS difference was highly statistically significant, log rank $p=1.6x10^{-9}$. Fig 4-4

COS as a predictor for early recurrence was more significant than grade ($p=1.8x10^{-5}$), lymph node status ($p=1.4x10^{-4}$) and tumour size ($p=5x10^{-5}$).

A



B

Score	Significance
2	
3	p=0.68
4	p=0.31
5	**p=4.6x10^{-4}**
6	p=0.72
7	p=0.53
8	p=0.97
9	p=0.27
10	p=0..305
Overall	**p=3.8 x10^{-8}**

C

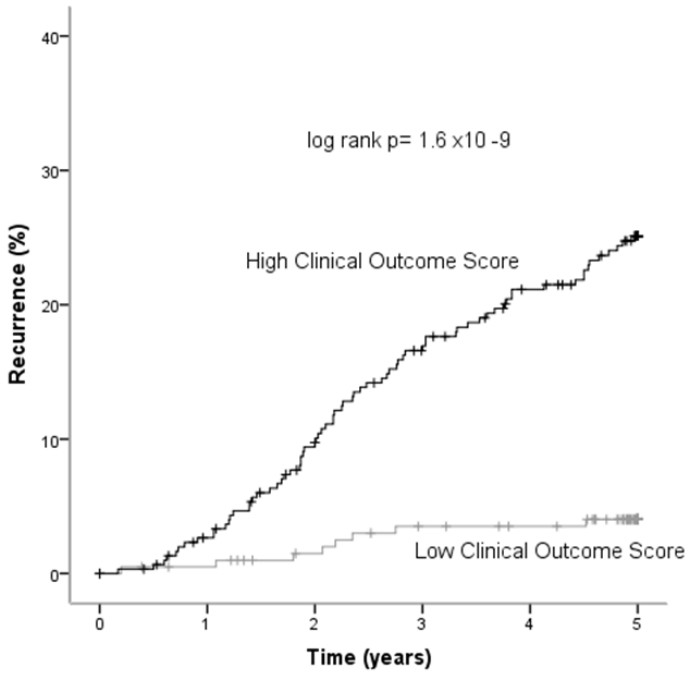


Figure 4-4 Low and High COS and Early Recurrence (entire cohort)

Kaplan Meier survival for Early (5 year) Recurrence. COS appears to be an excellent predictor for early recurrence. There were 81 early events, including 8 (10%) local recurrence, 60 (74%) distant recurrence and 13 (16%) early recurrence events in which site was not documented. A). COS scores 2-10 B) Significance between scores sequentially and justification for selecting cut-offs between low/high. C) Low COS, scores 2-4 (n=205) and high COS, scores 5-10 (n=306).

4.3.4 COS predicts outcome in Grade2, lymph node negative or light and small tumours

Recognised prognostic factors such as tumour size, lymph node status and grade are routinely used to determine risk of recurrence, or poor outcome and influence adjuvant therapeutic recommendations. Adjuvant chemotherapy decision making is more difficult for tumours with intermediate risk, for example, tumour size 20-50mm or grade 2, and lymph node light (1-3+). In addition, growing awareness of the importance of tumour biology rather than tumour burden has resulted in clinicians considering adjuvant chemotherapy in lymph node negative disease and small tumours (<20mm) in certain circumstances.

High COS was significantly associated with shorter DFS and poorer long term survival in the entire cohort when analysed in all grade 2 early breast cancer patients, all tumours 20-50mm and patients with 1-3 nodes positive. In addition COS was significantly associated with shorter DFS and poorer breast cancer specific survival in lymph node negative disease and tumours < 20mm.

In all grade 2 patients (n=215), 101 tumours had low COS, with only 8 events and a mean breast cancer specific survival time of 171months (range 166-177). High COS (n=114) had 30 events and significantly shorter mean breast cancer specific survival, 144 months (range 132-155), log rank $p=2x10^{-4}$ (HR 3.96).

187 patients had a tumour size of 20-50mm, in low COS (n=58) there were 7 events and mean breast cancer specific survival time of 164 months (range 154-175). In high COS (n=129) there were 40 events, and mean survival time was 138 months (range 120-149), log rank p=0.006 (HR 2.93). For tumours < 20mm (n=294), in low COS (n=133) there were 8 events, and mean breast cancer specific survival time was 173 months (range 169-177), in

high COS (n=161) there were 35 events and mean survival time was 149 months (range 140-158), log rank p= $0.7x10^{-4}$ (HR 4.2)

129 patients had lymph node light disease (1-3+ nodes), in node light disease with low COS (n=53) there were 5 events and mean breast cancer specific survival was 170 months (range 161-179). High COS (n=76) there were 26 events, mean survival 132 months (range 117-147), log rank p=0.001 (HR 4.4). More patients had node negative disease (n=281), n=111 had low COS with only 5 events, mean breast cancer specific survival time was 174 months (range 170-178) compared to high COS (n=170), 29 events, mean survival time 157 months (range 149-165), log rank p=0.001 (HR 4.32). The Kaplan Meier survival curves are shown in figure 4-5.

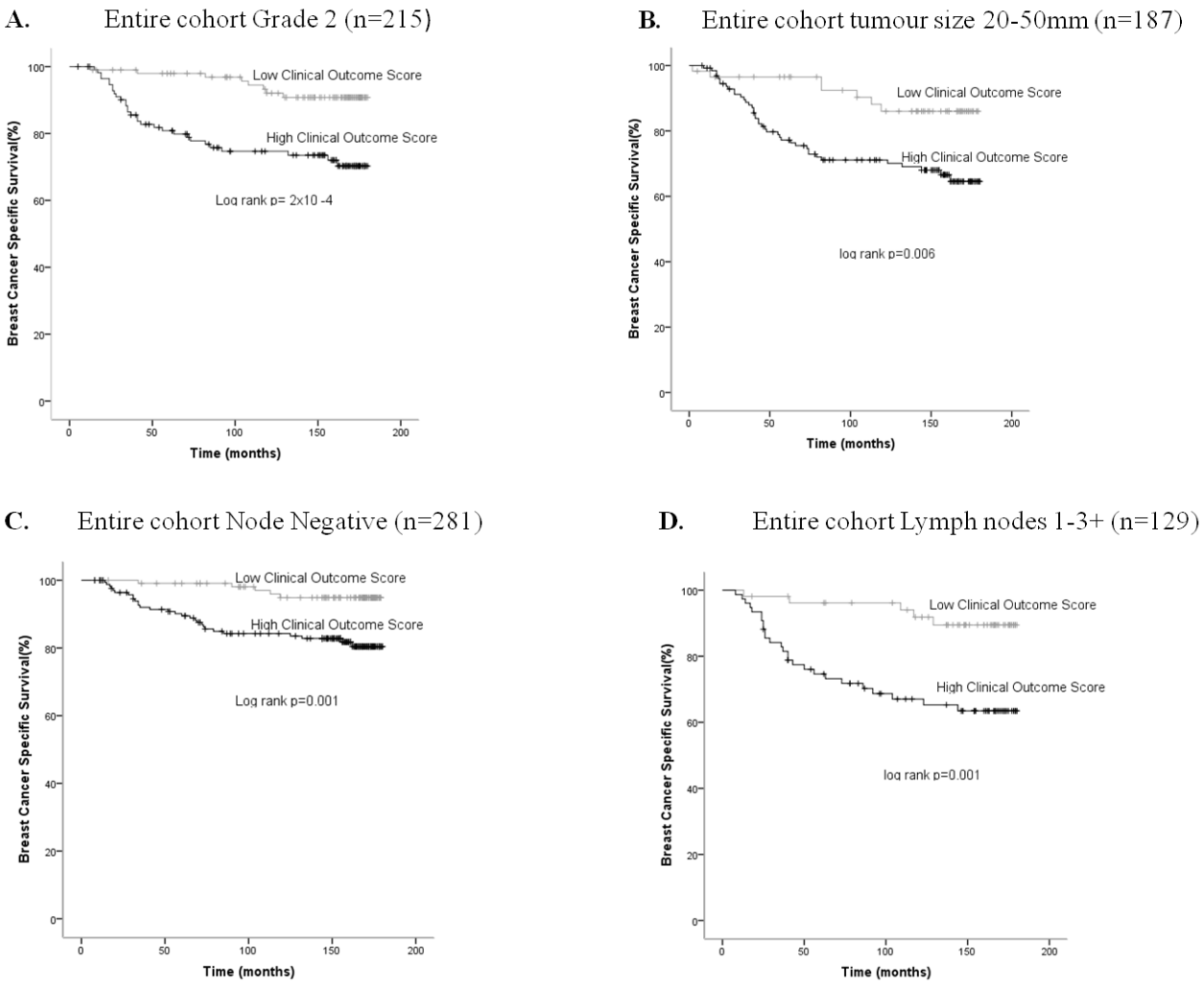


Figure 4-5 Entire Cohort sub group analysis for COS and Survival

Identifying increased risk using COS in Grade 2 (A), Size 20-50mm (B) and lymph node stage (C and D) with breast cancer specific survival as outcome.

For early recurrence, at 5 years high COS was invariably associated with significant shorter DFS for in all the factor subgroups. The Kaplan Meier curves are shown in figure 5-6, the patient numbers in each group are slightly higher as accurate 5 year recurrence was known for more patients. Table 4-4, details the numbers in each COS category per subgroup analysis, the number of events and mean DFS.

Prognostic index sub-group	COS	No of events	Mean DFS in months (range)	significance	Hazard Ratio
Grade 2 (n=222)	Low (n=107) High(n=115)	5 25	58.5 (57-60) 53 (51-56)	$p=2x10^{-4}$	HR 5.1
Tumour Size 20-50mm (n=189)	Low(n=59) High(n=130)	4 30	58 (55-60) 52 (50-55)	p=0.007	HR 3.8
Tumour Size <20mm (n=305)	Low(n=141) High(n=164)	4 34	59 (58-60) 54 (52-56)	$p=2x10^{-6}$	HR 8.1
Lymph nodes: 1-3+ (n=133)	Low(n=54) High(n=79)	2 20	58.5 (56-60) 51 (47-55)	p=0.001	HR 7.7
Lymph node negative (n=290)	Low(n=119) High(n=171)	3 25	59(59-60) 55(53.5-57)	p=0.001	HR 6.3

Table 4-4 Entire cohort sub group analysis for COS and early recurrence

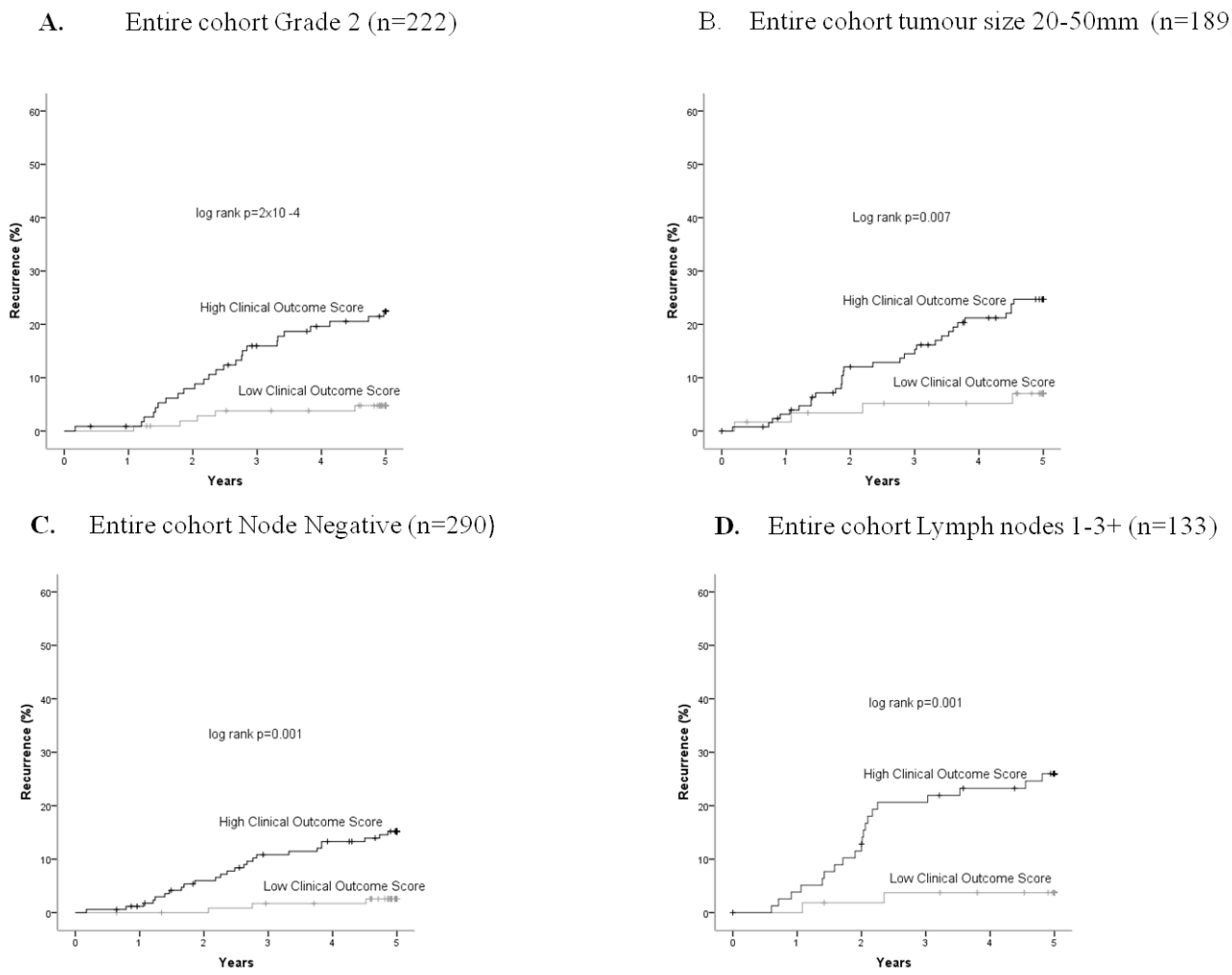


Figure 4-6 Entire Cohort sub group analysis for COS and Early Recurrence

Identifying increased risk using COS in Grade 2 (A), Size 20-50mm (B) and lymph node stage (C and D) with early recurrence the outcome.

4.3.5 Risk analysis in the Entire Cohort- COS, Grade 2, Tumour Size <50mm and Lymph node status

Applying Cox Regression model, risk estimates based on the clinical outcome score were calculated in each subgroup (all grade 2 patients, all tumours <50mm, lymph node 1-3+ and lymph node negative) for the entire cohort and breast cancer specific survival as outcome. Grade 2 patients with high COS HR 4 (CI 1.8-8.6, $p=1.2x10^{-4}$), tumours less than 50mm with high COS HR 3.8 (CI 2.19-6.6, $p=7x10^{-8}$), lymph nodes 1-3 positive and high COS HR 4.4

(CI 2.16-8.3, $p=1.7x10^{-6}$) and in lymph node negative patients high COS HR 4.3(CI 1.6-11, $p=4x10^{-4}$).

In multivariate analysis, combined with Grade, lymph node stage, tumour size, Allred ER, PgR and CER, COS was independently associated with disease specific survival. At 15 years in terms of breast cancer specific survival high COS HR 3.75 (CI 2.2-6.5, $p=1.6x10^{-8}$). Multivariate analysis confirmed COS is an excellent predictor of early recurrence, high COS HR 6.5(CI 3.15-13.6, $p=5\ x10^{-8}$).

4.4 Results III. Clinical outcome score in ER+/ Endocrine treated cohort

4.4.1 Patient and tumour characteristics

300 patients were ER+ (Allred≥3) and treated with endocrine therapy (298 tamoxifen and 2 enrolled in the ATAC trial). Compared to the entire cohort, the ER+ endocrine treated patients were similar in terms of nodal involvement and tumour size. There were less grade 3 tumours and more grade 2 tumours, in addition over 90% of the cohort had HER2 IHC score 0. Within this cohort there was an even distribution of tumours into low CER and high CER. ER+ endocrine treated patients had improved early DFS (mean time 55 months) and longer breast cancer specific survival (mean 128 months), there were less breast cancer related deaths. Compared to the entire cohort, where 60% had high COS, in this cohort only 40% score high. Patient and tumour characteristics are shown in tables 4-5 and 4-6.

	Cohort (n=300)
Age	
≤50	58 (19%)
>50	242 (81%)
Nodal Status	
0	168 (56%)
1-3+	87 (29%)
>3	40 (13%)
unknown	5 (2%)
Tumour Size	
<20mm	188 (63%)
20-50mm	102 (34%)
>50	10 (3%)
unknown	
Tumour Grade	
1	75 (25%)
2	173 (58%)
3	52 (17%)
Local Therapy	
WLE+Axilla	97 (32%)
No Radiotherapy	3 (3%)
Radiotherapy	94 (97%)
Mastectomy + Axilla	198 (66%)
No Radiotherapy	178 (90%)
Radiotherapy	20 (10%)
unknown	
WLE or Mastectomy only	5 (2%)
Systemic Therapy	
EndoTherapy	
None	
Tamoxifen	298(99%)
ATAC trial	3 (<1%)
unknown	
Chemotherapy	
Yes	70 (23%)
No	230 (77%)
unknown	
ER-Allred Score	
<3	
≥3	300 (100%)
PgR-Allred Score	
<3	101 (34%)
≥3	199 (66%)
HER2 (IHC)	
0	279 (93%)
2	13 (4.3%)
3	8 (2.7%)
Combined Endocrine Receptor (CER)	
Negative	
Low	158 (53%)
high	142 (47%)
Combined Outcome Score	
2	5 (2%)
3	49 (16%)
4	120 (40%)
5	84 (28%)
6	22 (7%)
7	9 (3%)
8	11 (4%)
COS category	
Low (0)	174 (58%)
High (1)	126 (42%)

Table 4-5 Patient and tumour characteristics in ER+/ endocrine treated cohort

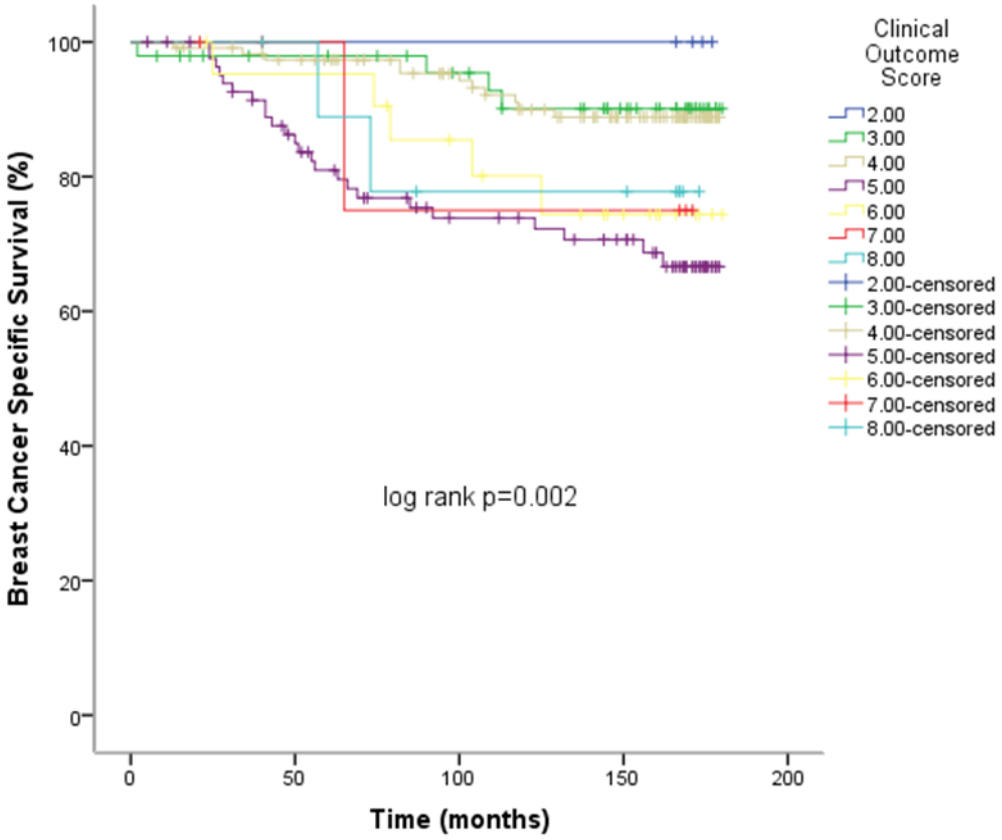
Follow Up Details for Clinical Outcome Score ER+ endocrine treated Cohort (n=300)	
Breast Cancer Specific Survival	n=290
Range	2-180 months
Mean survival	128 months
Median survival	152 months
Deaths (any)	116 (40%)
Breast Cancer related Deaths	48 (16.5%)
Early Recurrence (events censored at 5 years)	n=300
Range	0-5 years (0-60 months)
Mean DFS	4.6 years (55months)
Median DFS	5 years
Recurrence type	
No	271 (90%)
Local	2 (<1%)
Distant	22 (7.5%)
Site not documented	5 (1.5%)
Late Recurrence (10 years follow up)	n=291
Range	2-128 months
Mean DFS	94 months
Median DFS	109 months
Recurrence type	
No	229 (79%)
Local	10 (3%)
Distant	31 (10%)
Site not documented	21 (7%)

Table 4-6 Follow up details in ER+/endocrine treated cohort

4.4.2 COS and outcome in ER+ Endocrine treated patients

In ER+ endocrine treated patients the COS scores and classification into low and high risk was significantly associated with 15 year breast cancer specific survival, 10 year recurrence and early (5 year) recurrence.

Examining the distribution of scores, scores 2-4 remain separate from higher scores, dividing the cohort into low and high risk. Interestingly score 5 appears to have a worse outcome than scores 6-8, although many more patients score 5 and this may reflect difference in patient number. No patient in this ER+ endocrine cohort scored very high (9 or 10). The COS scores and outcome in terms of breast cancer specific survival is shown in figure 4-7, p=0.002 HR 1.4 (CI 1.1-1.7) along with patient numbers in each score.



Clinical Outcome Score	No. Patients n=300	Significance
2	5 (2%)	
3	49 (16%)	p=0.5
4	120 (40%)	p=0.8
5	84 (28%)	**$p=1.8 \times 10^{-4}$**
6	22 (7%)	p=0.49
7	9 (3%)	p=0.44
8	11 (4%)	p=0.445
Overall		**p=0.002**

Figure 4-7 COS and Survival in ER+/ endocrine treated cohort

At 15 years in ER+ endocrine treated patients, low COS scores had significant improvement in breast cancer specific survival compared to high COS. There were 15 deaths in low COS group with a mean survival time of 170 months (range 165-175). For high COS, there were 33 deaths and mean survival time was 145 months (range 135-166), $p=1.7x10^{-5}$ (HR 3.5, CI 1.9-6.4).

In terms of recurrence both early and late recurrence rates were significantly higher in patients with high COS. At 5 years, there 6 events (1 local, 4 distant, 1 site not documented) in the low COS group with a mean DFS of 4.89 years (58.7 months, range 57.6-59.8) compared to 23 events (1 local, 18 distant, 4 site not documented) in high COS, mean DFS 4.6 years (55.2 months, range 53.3-57), log rank $p=1.9x10^{-5}$ (HR 5.6, CI 2.3-13.9). At 10 years, there were 24 events in low COS, mean DFS 119 months (range 115-122) and 34 in high, mean DFS 102 months (range 95-109), log rank $p=6x10^{-5}$ (HR 2.7 CI 1.6- 4.5). Figure 4-8 demonstrates the Kaplan Meier curves for ER+ endocrine treated patients and COS for breast cancer specific survival, early and late recurrence.

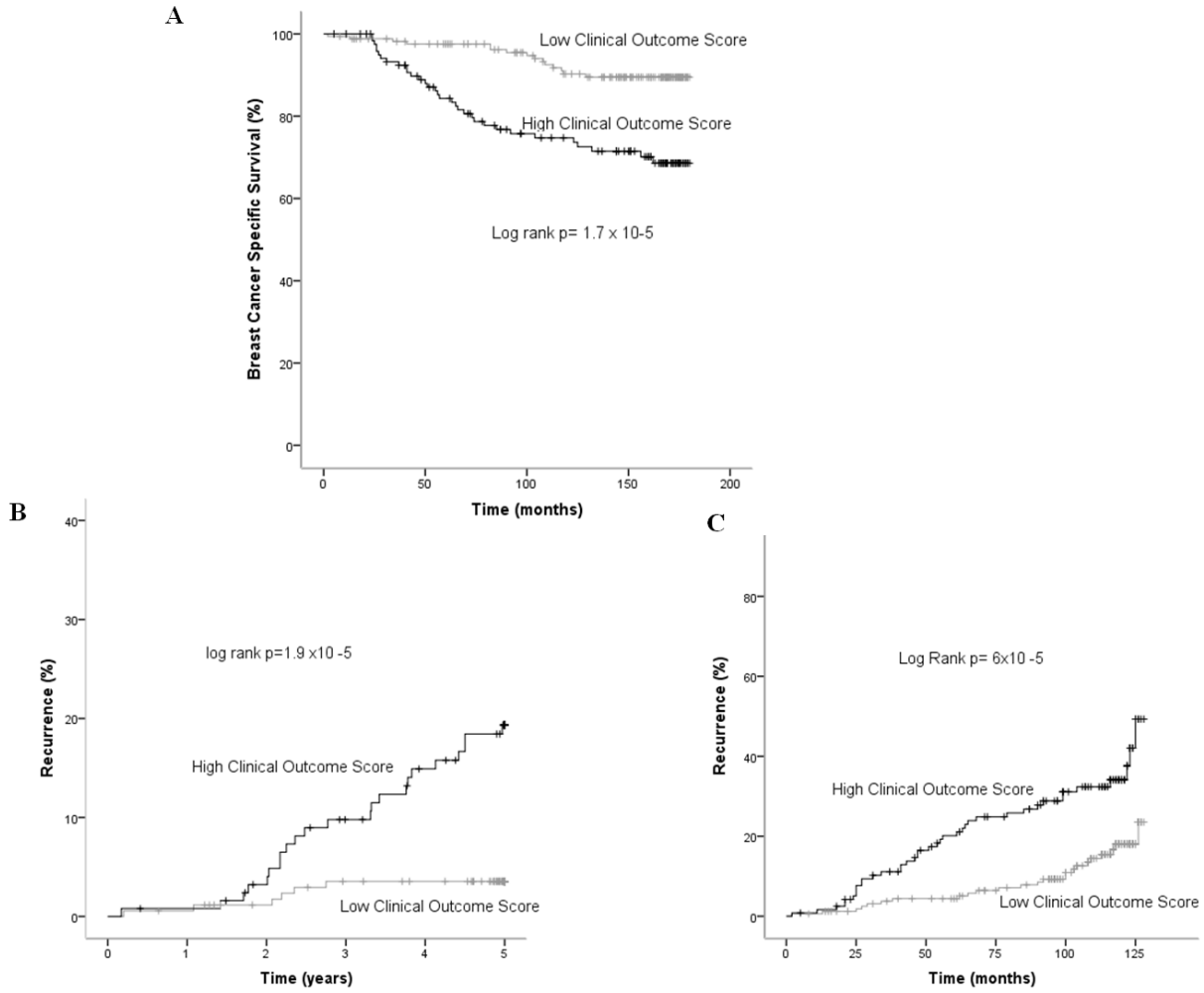


Figure 4-8 Low and High COS in ER+ endocrine treated cohort

Kaplan Meier survival curves for A) COS and breast cancer specific survival. B) COS and early recurrence. C) COS and 10 year recurrence

4.4.3 Grade, tumour size and nodal involvement in ER+ endocrine treated patients

Recognised prognostic factors include grade, nodal status/ involvement and tumour size. Due to the advent of breast screening more breast cancers are being detected at an earlier stage and tumours are smaller in size with less nodal involvement. It is accepted practice that patients with tumours considered high risk (grade 3, or large size or >3 nodes) involved should be considered for additional adjuvant chemotherapy if performance status and patient preference are acceptable. In ER+ breast cancer Grade 1, node negative and size less than

20mm are favourable prognostic factors, and if tumours are HER2 negative most cases will be treated with adjuvant endocrine therapy alone, when the tumour is considered to have a high chance of being endocrine responsive. Intermediate grade 2, size 20-50mm and nodal disease (1-3+) are not helpful in adjuvant therapy decision making.

In our cohort of ER+ endocrine treated early breast cancer, tumour grade, nodal status (node negative, 1-3+ and >3nodes +) and tumour size were analysed for 5 year recurrence, 10 year recurrence and breast cancer specific survival at 15 years, figure 9. As expected all three factors were prognostic. Mean DFS times and breast cancer specific survival data for all three factors are given in table 4-7. Most patients in this cohort had either node negative disease, or node light (1-3+) and tumours less than 50mm (patient numbers in each category shown in table 4-5).

	Recurrence at 5 years			**Recurrence at 10 years**			**15 yr Breast Cancer Specific Survival**		
	DFS (months)	Log rank	HR	**DFS** (months)	Log rank	HR	Survival (months)	Log rank	HR
Nodal status									
0	58.5	p= $6x10^{-5}$	HR 2.4	117	p= $4x10^{-7}$	HR 2.2	168	p= $2\ x10^{-8}$	HR 2.4
1-3+	56.5			109			159		
>3+	53.2			90			122		
Grade									
1	58.4	p=0.044	HR 2.0	118	p=0.012	HR 1.6	167	p=0.009	HR 1.8
2	57			112			161		
3	56.4			102			143		
Size (mm)									
<20	57.7	p=0.039	HR 1.8	114	p= $4x10^{-4}$	HR 1.8	165	p= $8\ x10^{-5}$	HR 2.3
20-50	56.6			109			153		
>50	53.9			81			109		

Table 4-7 Traditional Prognostic factors and outcome in ER+ endocrine treated cohort

In univariate analysis, nodal status was the most powerful prognostic factor at all time points. Nodal involvement gives information on both tumour burden and instrinsic tumour aggressiveness. It is noteworthy that the curves for node light (1-3+) and >3nodes + are initially almost indistinguishable and only diverge at 2.5 years (30 months) and thereafter are clearly separate.

Figure 4-9 and the survival times (table 4-7) clearly demonstrate the prognostic significance of each factor, and helps illustrate the difficulty clinicians have in patients with intermediate grade, size and 1-3 nodes, as whilst these tumours do significantly better than tumours considered high risk, they are still disadvantaged compared to low risk.

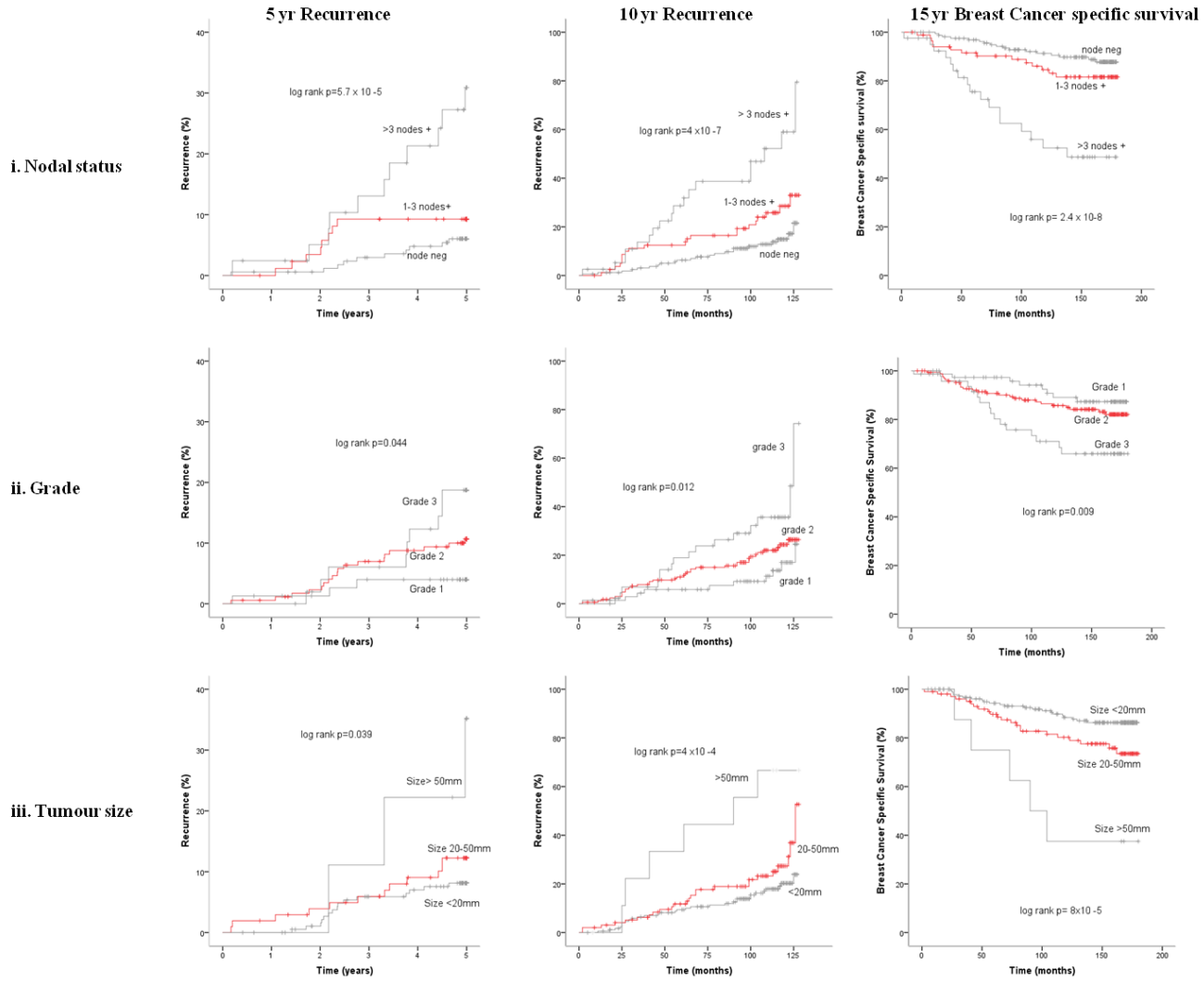


Figure 4-9 Recognised prognostic factors and the intermediate 'challenging' group in ER+ endocrine treated cohort

Kaplan Meier Survival Curves for Nodal status (node negative, 1-3+, >3 nodes+), Grade and tumour size at 5 years, 10 years and 15 years. The intermediate 'clinical challenging' group for each prognostic factor is highlighted in red.

4.4.4 COS aids identification of increased risk in ER+ endocrine treated patients that have grade 2, lymph node light or tumour size 20-50mm.

The clinical outcome score was analysed in all ER+ endocrine treated patients that had grade 2 disease (n=173), nodes 1-3+ (n=87) and tumour size 20-50mm (n=102). For each factor low clinical outcome score was significantly associated with improved early DFS, improved 10 year DFS and breast cancer specific survival. In addition the clinical outcome score may identify ER + patients normally considered lower risk with node negative (n=168) or tumours less than 20mm in size (n=188), at increased risk of poorer outcome.For early recurrence (events censored at 5 years) in ER + endocrine treated patients with 1-3 involved nodes, 52 patients had low COS and 2 events. The mean DFS time was 4.87 years (58.4 months, range 55.2-60 months). 35 patients had a high COS and there were 6 events, mean DFS was 4.47 years (53.6 months, range 48-57.6), log rank p=0.037, HR4.7.

In grade 2 patients, 95 had low COS with 3 events, the mean DFS time was 4.89 years (58.7 months, range 57.4- 60 months). 78 patients had a high COS and there were 14 events, the mean DFS time was 4.56 years (54.7 months, range 51.6-57.5), log rank p=0.001, HR 6.0.

In tumours 20-50mm, 55 patients had low COS, there were 3 events and mean DFS time was 4.79 years (57.5 months, range 54-60). 47 patients had a high COS, with 9 events and mean DFS time was 4.6 years (55.2 months, range 51.6-58.8), log rank p=0.038, HR 3.6.

Interestingly, in ER+ endocrine treated patients COS may predict increased risk in tumours<20mm and node negative disease. Both of these factors, in the absence of other markers of increased risk are relative indications for endocrine therapy alone. 188 patients had tumours <20mm, the majority (n=114) had low COS and there were 3 events, mean DFS was 4.93 years (59.2 months, range 58.5-60). High COS (n=74) had 11 events, mean DFS was 4.6 years (55.2 months, range 52.8-57.6), log rank p=0.001, HR 6.1. In node negative

ER+ breast cancer (n=168), 95 patients had low COS and there was 2 events, mean DFS time was 4.9 years (58.8 months, range 57.6-60) and 73 patients had high COS, with 7 events and mean DFS time 4.79 years (57.5months, range 55.2-58.8), log rank p=0.032, HR 4.7.

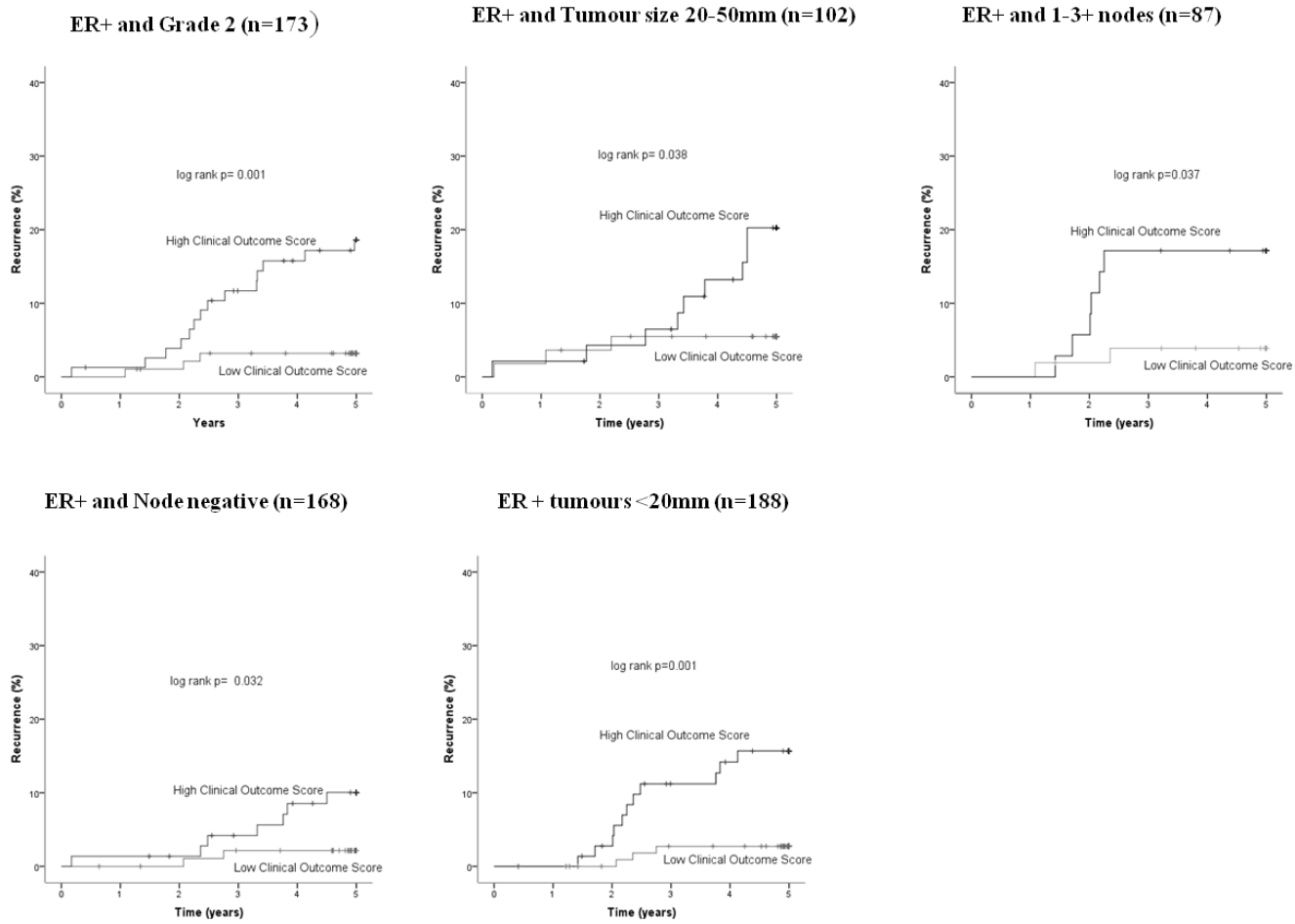


Figure 4-10 High COS predicts increased risk of early recurrence in intermediate prognostic categories

Kaplan Meier Survival curves for early (5 year) recurrence. High and low COS was compared in all ER+ endocrine treated patients with Grade 2 disease, Tumour size 20-50mm and lymph nodes 1-3+. In addition high COS predicts increased risk in tumours <20mm and lymph node negative disease.

For late recurrence at 10 years, in ER+ endocrine treated patients with grade 2 disease, the low COS group (n=89) had 12 events, mean DFS time was 119 months. In the high COS (n=78) group there were 24 events and mean DFS time was 102 months, log rank p=0.003, HR 2.7. In patients with tumour size 20-50mm, in the low COS group (n=52) there were 7 events and mean DFS was 117 months, compared to 18 events in high COS group (n=45) and mean DFS was 99 months, log rank p=0.002, HR 3.7. In patients with 1-3 nodes involved, low COS (n=49) had 9 events and mean DFS was 116 months, compared to high COS (n=32) with 12 events and mean DFS time 98 months, log rank p=0.040, HR 2.4.

At 10 years, in node negative ER+ endocrine treated breast cancer (n=159), the clinical outcome score appears to identify patients at increased risk. Low COS (n=89) had 6 events and mean DFS time was 123 months, compared to high COS (n=70) in which there were 16 events and mean DFS time was 112 months, log rank p=0.002, HR 3.9. It is notable, that patients with node negative cancer and a high COS had a shorter mean DFS time compared to node positive low scorers.

At ten years for recurrence, in patients with ER+ endocrine treated breast cancer with tumour size <20mm COS did not significantly predict outcome (log rank p=0.1), however for breast cancer specific survival (see below) in this group low COS had a significant survival advantage. The Kaplan Meier curves for 10 year recurrence are shown in figure 4-11.

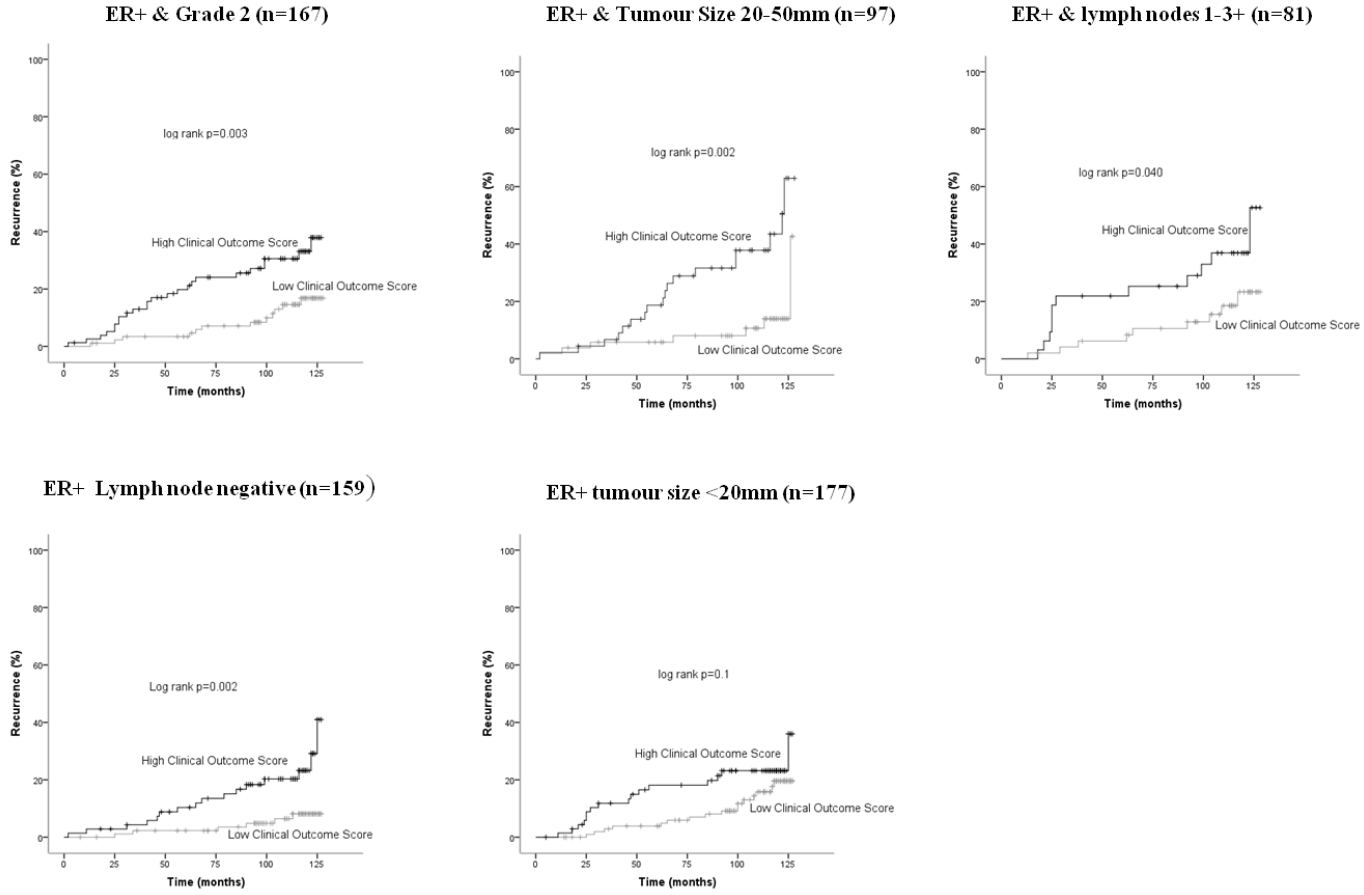


Figure 4-11 High COS predicts increased risk of late recurrence in intermediate prognostic categories

Kaplan Meier Survival curves for late (10 year) recurrence. High and low COS was compared in all ER+ endocrine treated patients with Grade 2 disease, Tumour size 20-50mm and lymph nodes 1-3+. In addition high COS predicts increased risk in lymph node negative disease.

In terms of survival, in grade 2 ER+ endocrine treated patients, low COS (n=90) had 7 events and mean survival time was 171 months (range 165-177 months). High Cos (n=77) had 19 events and mean survival time was 147 months (range 134-160 months), log rank p=0.002, HR 3.7. For patients with 1-3 nodes involved, low COS (n=51) had 5 events and mean survival time was 169 months (range 160-178 months), compared to high COS (n=33) with 9 events and mean survival time 144 months (range 124-164 months), log rank p=0.024,

HR3.3. In tumours 20-50mm, low COS (n=54) had 6 events and mean survival time was 165 months (range 154-176 months) compared to high COS (n=47) in which there were 16 events and mean survival time was 139 months (range 122-156 months), log rank p=0.004, HR 3.6.

In node negative ER+ breast cancer treated with endocrine therapy (n-161), low COS was significantly associated with improved survival, p=0.003, HR 4.5. In low COS group (n=89) there were 4 events and mean survival time, was 174months (range 169-178). This was the longest survival time in all the sub group analysis. In high COS node negative (n=72), there were 13 events and mean survival time was 159 months (149-169). It is notable that mean survival time was shorter for node negative cases with high COS (159 months) compared to node positive cases with a low COS (169 months).

In ER+ endocrine treated breast cancer and tumour size <20mm (n=180), low COS (n=108) had 8 events and mean survival time was 171 months (range 166-176 months) compared to high COS (n=72) in which there were 13 events and mean survival time was 154 months (range 141-166 months), log rank p=0.017, HR 2.8. Similar to node negative with high COS, tumours <20mm with a high COS had a shorter mean survival time (154 months) compared to larger tumours (20-50mm) with a low COS (165 months), suggesting that in small early ER+ breast cancer the clinical outcome score, representing biological aggressiveness, may be more informative of risk than the tumour burden. The Kaplan Meier survival curves are shown in figure 4-12.

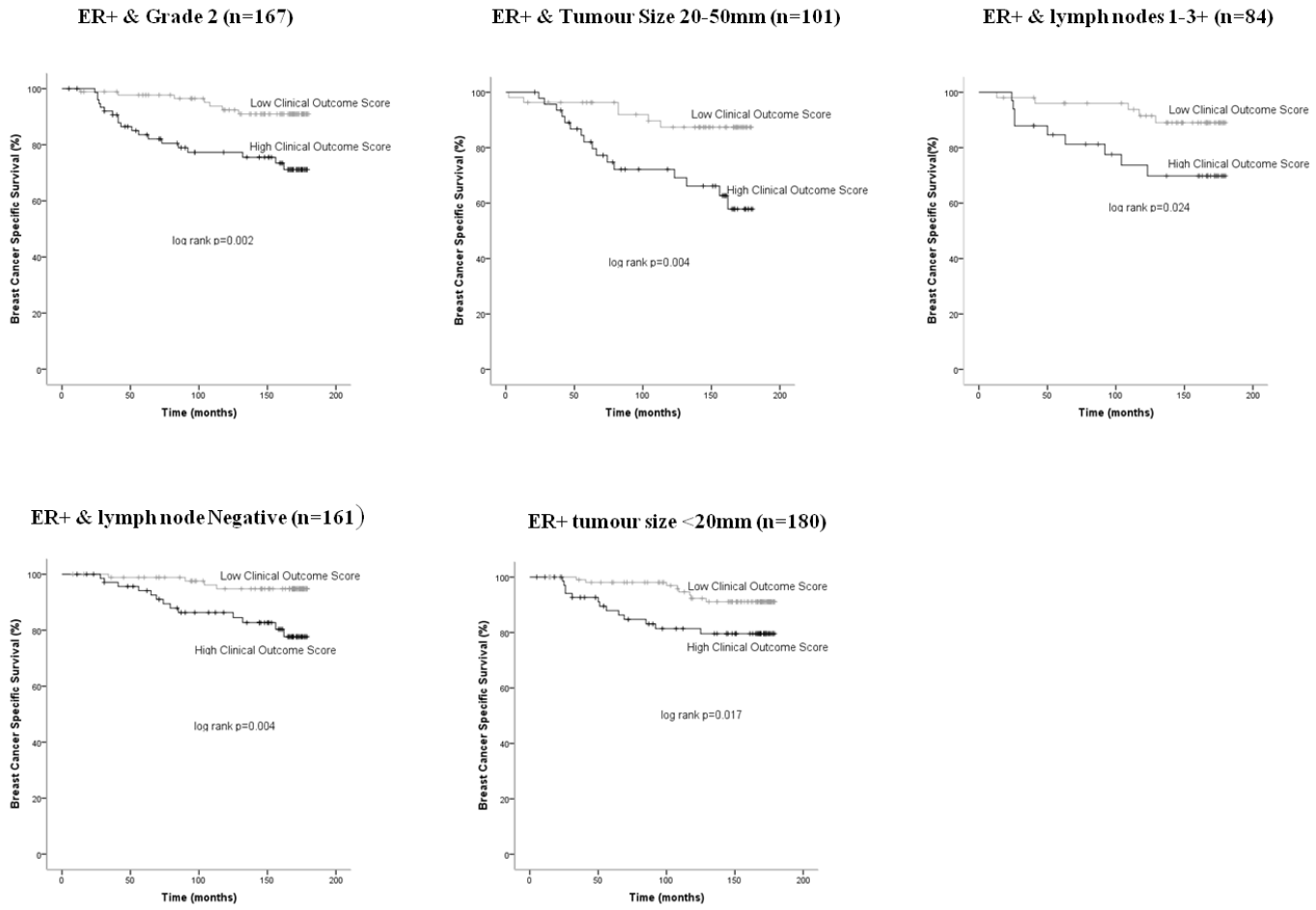


Figure 4-12 High COS predicts increased risk reduced survival in intermediate prognostic categories

Kaplan Meier Survival curves for breast cancer specific survival at 15 years. High and low COS was compared in all ER+ endocrine treated patients with Grade 2 disease, Tumour size 20-50mm and lymph nodes 1-3+. In addition high COS predicts increased risk in lymph node negative disease and tumours <20mm.

4.4.5 Distribution of Prognostic and predictive Factors in Low and High COS

The frequencies of recognised pathological and prognostic factors, and the novel combined endocrine score, in the cohort of ER+ endocrine treated patients divided into low and high COS are detailed in table 4-8. Between the 2 groups there is fairly even distribution of Allred ER scores, lymph node status and tumour size. Notable differences in frequencies are in the Allred PgR scores, the combined endocrine receptor category, grade and age. In addition all low COS are HER 2 IHC 0. In terms of grade, whilst grade 3 is very uncommon in low COS

it accounts for nearly 40% of high COS, there is a fairly even distribution of grade 2 between the groups.

Factor	Low Clinical Outcome Score (n=174)	High Clinical Outcome Score (n=126)
Allred ER score		
3	10 (6%)	13 (10%)
4	6 (3.5%)	6 (5%)
5	8 (5%)	6 (5%)
6	106 (61%)	68 (54%)
7	29 (16%)	18 (14%)
8	15 (9%)	15 (12%)
Allred PgR score		
0	11 (6%)	24(19%)
2	22 (13%)	44 (34%)
3	6 (3.5%)	12 (10%)
4	4 (2%)	10 (8%)
5	9 (5%)	9 (7%)
6	84 (48%)	17 (13.5%)
7	23 (13%)	2 (1.5%)
8	15 (9%)	8 (6%)
Combined Endocrine		
Receptor	0 (0%)	0 (0%)
0 (<1.5)	57 (33%)	101 (80%)
1 (1.5-5.5)	117 (67%)	25 (20%)
2 (>5.5)		
Nodes		
0	95 (55%)	73 (58%)
1-3+	52 (30%)	35 (28%)
>3+	24(14%)	16 (13%)
missing	3 (1%)	2 (1%)
Grade		
1	74 (42.5%)	1 (<1%)
2	95 (54.5%)	78 (62%)
3	5 (3%)	47 (38%)
Tumour Size		
<20mm	114 (65%)	74 (59%)
20-50mm	55 (32%)	47 (37%)
>50mm	5 (3%)	5 (4%)
HER 2 IHC score		
0	174 (100%)	105 (83%)
2		13 (10%)
3		8 (6%)
Age		
≤50	47 (27%)	11 (9%)
>50	127 (73%)	115 (91%)

Table 4-8 Distribution of prognostic factors in ER+ endocrine treated patients with low and high COS

4.5 Results IV - Clinical Outcome score in ER+/HER2- Endocrine treated patients

4.5.1 Clinical Outcome Score in ER+/ HER 2negative Early Breast cancer treated with endocrine therapy

Within ER+ endocrine treated cohort 279 patients scored 0 by IHC for HER2. An IHC score of 3 defines HER 2 positivity and an IHC score of 2 is equivocal and usually further FISH analysis is performed. The rationale for including score 2 in the COS formula was that it represents increased expression and activation of this cell signalling pathway and provides information of tumour biology.

The Clinical Outcome score was analysed in all ER+ HER 2 negative (using IHC score 0 to define negative). Low scores were significantly associated with improved outcome, in terms of early recurrence, late recurrence and breast cancer specific survival in this cohort, figure 4-13.

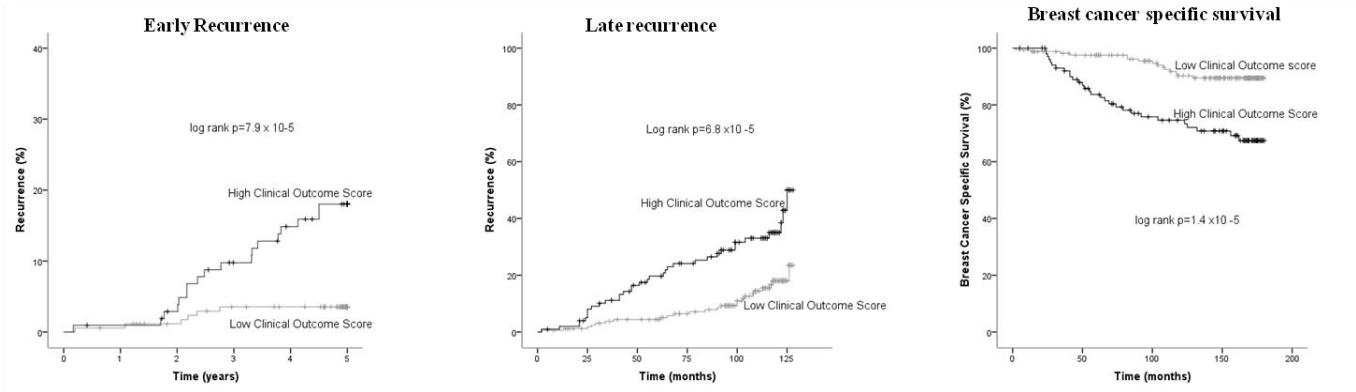


Figure 4-13 Low and High COS in ER+/HER2 negative endocrine treated patients

Kaplan Meier survival curves for low and high COS in ER+/HER2 negative endocrine treated patients (n=279). Low COS was associated with improved DFS at 5 years ($p=7.9x 10^{-5}$), improved DFS at 10 years ($p=6.8 x10^{-5}$) and improved breast cancer specific survival ($p=1.4 x10^{-5}$).

At 5 years, in ER+ HER2 negative early breast cancer patients treated with endocrine therapy, low COS (n=174) there were 6 early events and mean DFS 4.89 years (58.7 months, range 57.6-59.8 months). High COS (n=105) had 18 early events and mean DFS time 4.6 years (55.2 months, range 52.8-57.5 months), log rank $p=7.9x10^{-5}$, HR 5.4 (CI 2.2-13.7).

At 10 years, in the low COS group there were 24 events, mean DFS time was 119 months and high COS, there were 34 late events, with a mean DFS time of 102 months, log rank $p=6.8\ x10^{-5}$, HR 2.7 (CI 1.6-4.7).

In ER+ HER2 negative breast cancer, low COS was associated with statistically significant improved breast cancer specific survival, there were 15 related breast cancer deaths and mean survival time was 170 months (range 165-175 months). In high COS there were 29 breast cancer related deaths, and mean survival time was 144 months (133-156), $p=1.4x10^{-5}$, HR 3.7 (CI 2.0-7.0)

As for ER+ endocrine treated, in ER+/HER2 negative endocrine treated patients low COS was associated with significant improved outcome in terms of 5, 10 year recurrence and breast cancer specific survival when analysed in the intermediate ('challenging') prognostic sub groups: grade 2 patients, size 20-50mm and lymph nodes 1-3 positive . The statistical power of COS was similar to the log rank for ER+ subgroups detailed above. Examining the time to event, these were again similar however, not unexpectedly ER+/HER2 negative endocrine treated patients (compared to all ER+ endocrine treated patients) had slightly longer time to events, representing that HER2 negative ER+ breast cancer is more indolent. Therefore low COS appears to be an excellent predictor of good outcome in all prognostic marker subgroups. In ER+/HER2 negative patients it was noted again that in tumours <20mm with high COS had shorter time to event than patients with tumours 20-50mm with low COS. Similarly, node negative patients with high COS had shorter time to events than node positive

with low COS. Raising the question, is tumour biology more important than tumour burden? Further analysis of the prognostic power of each factor alone and in combination with the clinical outcome score was undertaken.

4.5.2 Cox Regression Model- Risk associated with each prognostic factor in ER+/HER2 negative endocrine cohort

Cox regression model was applied to assess risk at 2 time points, 5 years DFS and 15 year breast cancer specific survival (table 4-9).

Factor	5yr DFS	15yr Breast Cancer Specific Survival
NODAL STAGE	(p=0.002)**	(p=3.3x10^{-7})**
0		
1-3+	HR 1.4 (CI 0.5-4) p=0.5	HR1.7 (CI 0.8-3.5) p=0.157
>3+	HR 5 (CI 2-12.8), p=0.001**	HR 5.7 (CI 2.8-11.7), p=1.2x10^{-6}**
GRADE	(p=0.084)	(p=0.015)*
1		
2	HR 2.3 (CI 0.6-7.9), p=0.19	HR 1.4 (CI 0.6-3.17) p=0.385
3	HR 4.4 (CI 1.1-17.2), p=0.031*	HR 3.2 (CI 1.3-7.8), p=0.009**
Tumour Size	(p=0.140)	(p=0.015)*
<20mm		
20-50mm	HR 1.7 (CI 0.7-4) p=0.183	HR 2.1 (CI 1.1-3.9), p=0.017*
>50mm	HR 3.8 (CI 0.8-17) p=0.077	HR 6 (CI 2-17), p=0.001**
COS		
Low		
High	HR 5.4 (CI 2-13), p=4x10^{-4}**	HR 3.6 (CI 1.9-6.7), p=5x10^{-5}**

Table 4-9 Univariate Cox regression model of risk associated with prognostic factors in ER+/HER2 negative endocrine treated cohort.

** significant; ** highly significant. HR (Hazard ratio) with confidence interval detailed.*

All factors appear to be time dependent, most marked for tumour size and grade, which is not significantly associated with early risk in this cohort. High risk tumours, >3 nodes+, Grade 3 and tumours >50mm were highly significant for survival, however, the HR demonstrate the difficulty clinicians have within ER+/HER2 negative early breast cancer. Although nodal stage and grade are overall highly significant, 1-3 nodes positive or grade 2 is not

significantly associated with risk at either end point. Interestingly tumour size categorical risk was significant at 15 years. High clinical outcome score (COS) was highly significantly associated with early risk and 15 year breast cancer specific survival.

Multivariate analysis was performed. At 5 years with outcome being DFS, COS HR 1.9 (CI 1.27-3, p=0.002) and lymph node HR 2.5 (CI 1.49-4.3, p=0.001) were independent significant variables. At 15 years with outcome being breast cancer specific survival in multivariate analysis, size, lymph node stage and COS were independent significant variables. High COS was associated with the greatest risk, COS HR 4.5 (CI 2.4-8.6, $p=5x10^{-6}$), lymph node stage HR 2.6 (CI 1.7-4, $p=7x10^{-6}$) and tumour size HR 1.9 (CI 1.1-3.3, p=0.014).

4.5.3 Tumour Burden and Tumour Biology in ER+/HER2 negative Endocrine Treated Breast Cancer

Tumour size and lymph node involvement represent anatomical extend of disease (tumour burden) in early breast cancer. As tumour burden increases the likelihood of occult systemic metastases increases, and whilst tumour burden may represent biological aggression (increased growth, metastatic potential) it can also be as a result of time elapsed. Differentiating whether the nodal stage or tumour size is a consequence of time in situ or instrinsic biological aggression is a challenge. As noted above low COS is significantly associated with improved outcome in grade 2, tumour size <20mm or 20-50mm and lymph node negative or node light (1-3+) ER+ (and ER+/HER2 negative) early breast cancer. COS was therefore analysed in combination with nodal stage and tumour size in ER+/HER2 negative endocrine treated breast cancer.

i. Nodal Stage and Clinical Outcome Score

Breast Cancer specific survival was used as the end point. For all ER+/HER2 negative endocrine treated early breast cancer patients, in node negative tumours (n=155) there were 16 events and mean survival time was 168 months (range 162-173), in patients with 1-3 nodes positive (n=77) there were 13 events, and mean survival time was 160 months (range 150-170), the difference between node negative and 1-3 nodes positive was not significant (p=0.153). In tumours with >3 nodes positive (n=38) there was 15 events, the mean survival time was 124 months (range 103-145). Overall nodal status was highly significant, $p=3x10^{-7}$, HR 2.3 (CI 1.6-3.4).

In low COS, ER+/HER2 negative endocrine treated patients overall mean survival times were longer. In node negative tumours (n=89) had only 4 events and mean survival time was 174 months (range 169-178), tumours with 1-3 nodes positive (n=51), there were 5 events and the mean survival time was 169 months (range 160-178). In tumours with >3 nodes positive and a low COS (n=24), there was 6 events and mean survival time was 149 months (range 129-170), log rank p=0.003, HR 2.7 (CI 1.4-5.1).

In high COS, ER+/HER2 negative endocrine patients, all node stages had overall shorter mean survival times, most marked in patients with >3 nodes involved. In node negative tumours (n=64) there was 12 events, and mean survival time was 159 months (range 148-170). In tumours with 1-3 nodes positive (n=25), there was 8 events and mean survival time was 141 months (range 117-164). Although there was not a significant difference (p=0.193) examining the Kaplan meier curve, it is likely that lack of patient number influences this. In >3 nodes positive (n=12), high COS mean survival time was 71 months (range 37-105 months), $p=3x10^{-7}$, HR 2.9 (CI 1.7-4.7). It is notable that this is over 50% less than low COS patients (71 months versus 149 months). In addition, despite having smaller number of

patients per group in each lymph node category high COS had double the number of events compared to low.

Importantly, low COS in ER+/HER2 negative endocrine treated patients with either lymph node negative or lymph node light disease appears to identify a patient group with excellent overall survival. However, low COS with >3 nodes positive is still associated with a significantly poor outcome, tumour burden still matters.

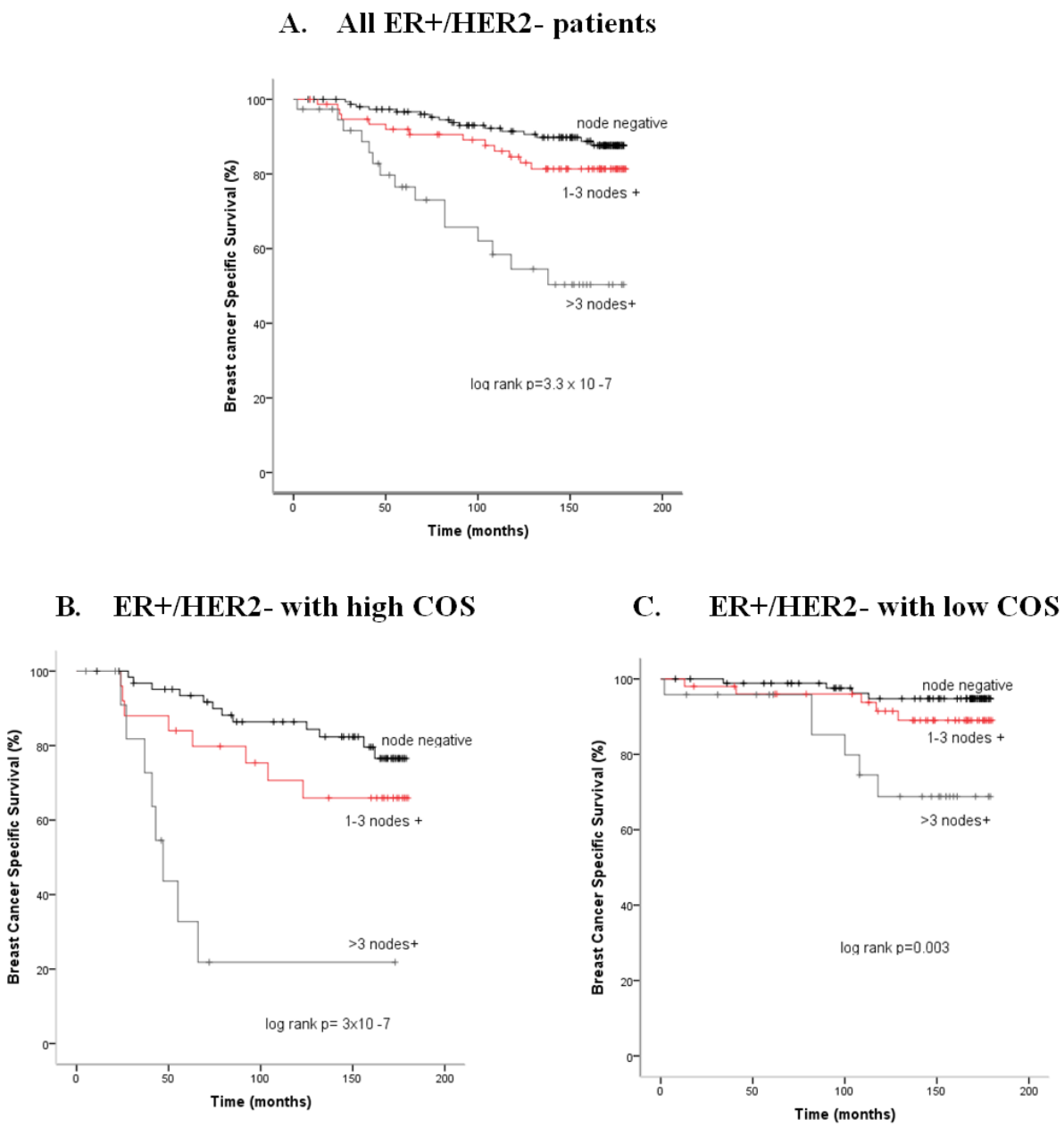


Figure 4-14 Influence of COS in ER+/HER2 negative endocrine patients and lymph node stage.

Kaplan Meier demonstrating that tumour burden is still important. A). Prognostic significance of nodal stage in all ER+/HER2- endocrine treated patients (n=270) B). ER+/HER2- endocrine treated patients with high COS (n=102). C). ER+/HER2- endocrine treated patients with low COS (n=174) >3 nodes still carries significant risk however low COS patients with <3 nodes involved has an excellent prognosis.

ii. Tumour size and Clinical Outcome Score

For ER+/HER2 negative endocrine treated early breast cancer patients, in tumours <20mm (n=172) there were 20 events and mean survival time was 165 months (range 159-171), in patients with tumours 20-50mm (n=95) there were 21 events, and mean survival time was 153 months (range 142-164). The difference between tumours <20mm and tumours 20-50mm was significant (p=0.012, HR 2.2). In tumours >50mm (n=8) there was 4 events, the mean survival time was 114 months (range 68-160). The difference between tumours 20-50mm and greater than 50mm was not significant (p=0.055) however overall tumour size in all ER+/HER2 negative endocrine treated patients was significant, p= 0.001, HR 2.3 (CI 1.4-3.8).

In low COS, ER+/HER2 negative endocrine treated patients overall mean survival times were longer. In tumours <20mm (n=108) had only 8 events and mean survival time was 171 months (range 166-176), tumours 20-50mm (n=54), there were 6 events and the mean survival time was 165 months (range 154-176). There was no statistical difference between tumours <20mm and tumours 20-50mm (p=0.399). In tumours with >50mm and a low COS (n=5), there was 1event and mean survival time was 157 months (range 119-195), log rank p=0.427. Tumour Size was not significant in patients with low COS.

In high COS, ER+/HER2 negative endocrine patients, all patients had overall shorter mean survival times, most marked in patients with tumours >50mm. In <20mm tumours (n=61) there was 11 events, and mean survival time was 154 months (range 141-168). In tumours 20-50mm (n=40), there was 15 events and mean survival time was 135 months (range 117-154). The difference between tumours <20mm and tumours 20-50mm was significant (p=0.037, HR2.2) All patients with tumours >50mm (n=3) and a high COS had an event. The mean survival time was 57 months (range 10-103), the difference between tumours 20-50mm

and greater than 50mm was significant (p=0.002, HR 2.2). Overall in high COS group tumour size was highly significant p=$6x10^{-5}$ (HR 2.8 CI 1.5-5.3).

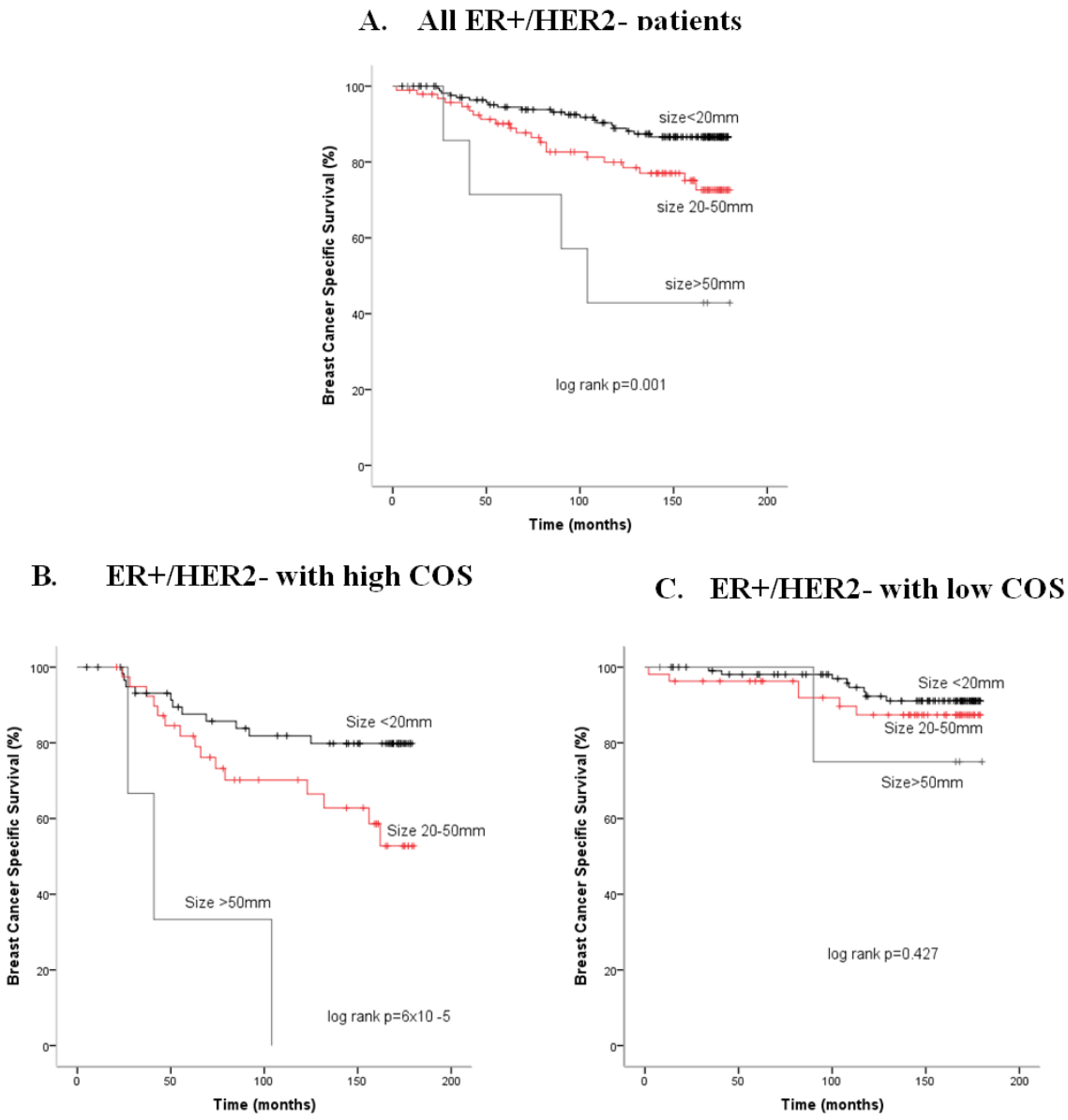


Figure 4-15 Influence of COS in ER+/HER2 negative endocrine patients and tumour size

Kaplan Meier survival curves demonstrating the prognostic impact of tumour size and impact of the COS classification. A). All ER+/HER2– endocrine treated patients, there was a significant difference between tumours <20mm and tumours 20-50mm (p=0.012). B). ER+/HER2- with high COS, tumour burden important. C) ER+/HER2- with low COS, tumour burden (size) not significant.

4.5.4 Effect of chemotherapy

The results demonstrate that within a cohort of ER+/HER2 negative early breast cancer patients treated with endocrine therapy- high COS is associated with significant risk of poor outcome. Our aim was to produce a scoring system that identifies ER+/ HER2 negative breast cancer patients with increased risk with and therefore aide selection of patients that may benefit from adjuvant chemotherapy. As a secondary analysis, we examined the cohort to investigate retrospectively if patients with high COS benefited from chemotherapy. Within the ER+/HER2 negative endocrine treated cohort, 22% (n=64) patients received adjuvant chemotherapy. The choice of chemotherapy regimens, duration and timing of chemotherapy agents was not known, however the patients were diagnosed between 1995-1998 when anthracylines containing regimens or CMF were commonly used in early breast cancer patients. Prior to analysing whether chemotherapy benefited ER+/HER2 endocrine treated patients with high COS, COS was analysed in this cohort excluding patients who had received chemotherapy (n=218). High COS in all ER+/HER2 negative endocrine *only* patients was significantly associated with poorer outcome, in terms of recurrence at 5 years (p=0.008, HR 4.2), 10 years (p=0.006, HR 2.4) and breast cancer specific survival (p=0.001, HR 3.7)

The effect of chemotherapy was analysed in the cohort of ER+/HER2 negative endocrine treated patients with low COS. The addition of chemotherapy did not benefit this group. LOW COS not receiving chemotherapy (n=127) had 9 events and mean survival time was 170 months (range 165-170), low COS patients receiving chemotherapy (n=40) had 6 events, with a mean survival time of 156 months (range 152-176), log rank p=0.188.

In ER+/HER2 negative endocrine treated patients with high COS, chemotherapy resulted in a significantly poorer outcome. High COS patients not receiving chemotherapy (n=83) had 19 events and mean survival time was 152 months (range 141-164), compared to high COS

patients receiving adjuvant chemotherapy (n=21) with 10 events and mean survival time was 113 months (range 83-143), p=0.010, Kaplan Meier survival curves, figure 4-16.

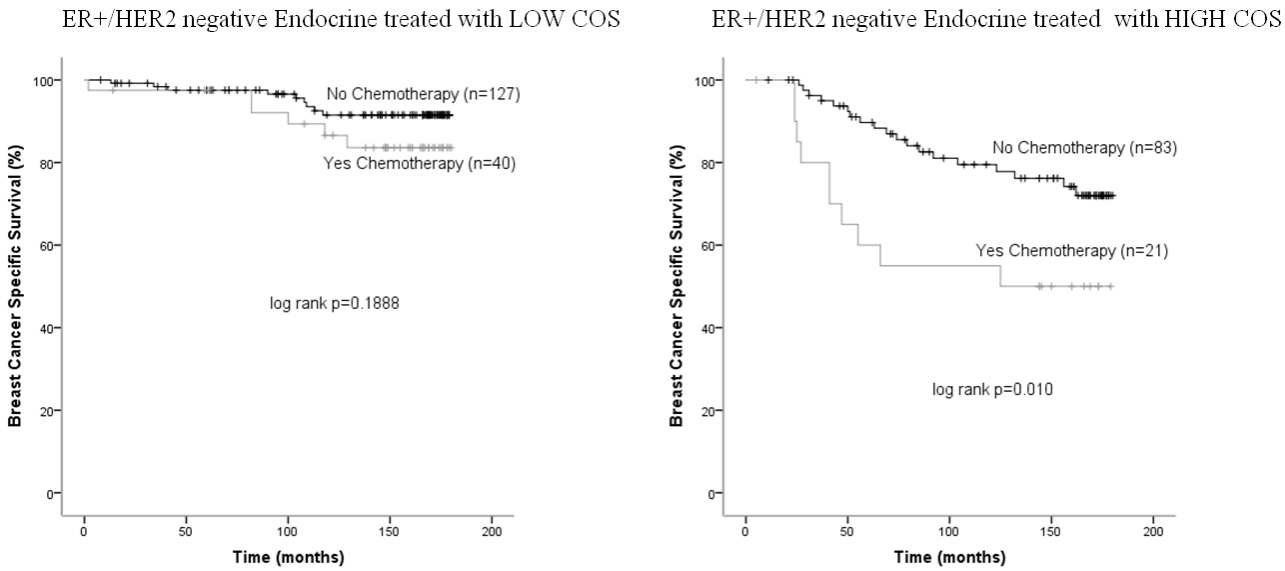


Figure 4-16 Chemotherapy in ER+/HER2- endocrine treated patients with low and high COS

In ER+/HER2- endocrine treated patients with low COS, addition of adjuvant chemotherapy conferred no benefit. In high COS patients the addition of chemotherapy was associated with poor outcome.

The poor outcome in high COS patients treated with chemotherapy is likely multi-factorial and may result from differences in other prognostic factors, such as tumour size or nodal involvement. The effect of chemotherapy was analysed in low and high COS group in each prognostic category and detailed in table 4-10.

Sub-Group (ER+/HER2- endocrine treated)	Chemotherapy	Number of events & (mean survival time-months)	significance
Tumour size 20-50mm			
Low COS	No (n=34) Yes (n=20)	3 (168) 3 (159)	p=0.6
High COS	No (n=31) Yes (n=9)	10 (144) 5 (105)	p=0.2
Tumour Size <20mm			
Low COS	No (n=89) Yes (n=19)	5 (172) 3 (164)	p=0.2
High COS	No (n=51) Yes (n=10)	8 (157) 3 (134)	p=0.2
Lymph node negative			
Low COS	No (n=85) Yes (n=4)	4 (-)* 0 (-)	p=0.6
High COS	No (n=58) Yes (n=6)	10 (161) 2 (143)	p=0.35
1-3 nodes +			
Low COS	No (n=32) Yes (n=19)	4 (163) 1 (177)	p=0.3
High COS	No (n=18) Yes (n=6)	6 (142) 2 (127)	p=0.9
Grade 2			
Low COS	No (n=68) Yes (n=27)	5 (169) 7 (173)	p=0.8
High COS	No (n=58) Yes (n=9)	13 (152) 4 (106)	p=0.06

Table 4-10 Chemotherapy and clinical outcome in ER+/HER2 negative endocrine treated analysed in prognostic sub groups

Survival analysis in ER+/HER2 negative endocrine treated patients examining prognostic sub groups and COS category to investigate effect of adjuvant chemotherapy. (-) no survival times calculated as all events censored.*

Adjuvant chemotherapy in ER+/HER2 negative endocrine treated patients when divided into low COS and high COS and analysed by tumour size, lymph node stage and grade was not associated with survival benefit. Surprisingly, high COS patients receiving adjuvant chemotherapy had shorter mean survival times in all sub-groups analysed (not statistically significant), although numbers in each group are small. These results suggest that chemotherapy (regimens not known) provides no additional benefit to ER+/ HER2 negative

endocrine treated patients, and actually if patients have high clinical outcome scores may be associated with shorter breast cancer specific survival.

4.6 Discussion

The advent of gene expression microarray analysis, including the identification of the molecular instrinsic subtypes and development of prognostic signatures has brought to the fore, that breast cancer is heterogenous and tumour biology influences patient outcome and response to adjuvant treatment. The oestrogen-signalling pathway, growth factor signalling pathways and tumour proliferation are heavily represented in the numerous gene profiles now published. This exploratory study was based on a pragmatic (and cost effective) approach to assessing tumour biology utilizing information available in the routine pathological report of all breast cancer specimens as surrogate markers for oestrogen signalling pathway, growth factor signalling and proliferation. Using a simple summation formula, combining Grade, HER2, the Combined Endocrine Receptor (CER) and age, results in the Clinical Outcome Score (COS). This retrospective study demonstrates in ER positive early breast cancer treated with endocrine therapy, when COS is considered in combination with markers of tumour burden, patients with nearly 100% survival can be identified. Importantly low COS appears to identify patients with grade2, lymph node light or negative disease, and intermediate tumour size who had excellent outcome and suggests endocrine therapy alone can be used and supporting the safe omission of chemotherapy.

A pragmatic approximation of gene array analysis was recently accepted by the St Gallen Consensus Conference panel [28]. The panel supported the principle of using instrinsic tumour subtypes as a basis for selecting patient therapy, their rationale being the wealth of literature that supports breast cancer heterogeneity, and differing response to therapies within the subtypes[28]. It was recognised that gene array analysis would not be possible in all patients in the immediate future and supported surrogate approximations of defining

molecular subtype using IHC and in situ hybridization techniques [126, 142-144] to guide therapeutic decision making. In this study we have similarly, adopted conventional IHC markers and utilised them to calculate an 'IHC equivalent outcome score' in ER+ breast cancer, based on meta-analysis which has demonstrated commonality of genes representing steroid hormone activation, epidermal growth factor system and proliferation in the different gene prognostic scores [39]. Cuzick et al [124] recently reported the IHC4 score, this uses four IHC markers (ER, PgR, HER2 and Ki67) in combination to calculate risk and compared this to the prognostic information provided by Oncotype Dx^{TM} for patients enrolled in the ATAC trial, they demonstrated that these four markers would at least be equivalent to Oncotype Dx^{TM}, and validated their data in a separate cohort. Cuzick 's study [124], and other large IHC based trial data [105, 139] provide good evidence that with appropriate attention to detail and robust quality control procedures, quantitative and reproducible data can be obtained using conventional IHC techniques. In our own study the IHC data was performed in a single laboratory employing stringent quality control measures thus reducing testing variation. Our data supports that with such quality control measures, IHC analysis of ER, PgR and HER-2 gives valuable prognostic information that is quantifiable. It would be interesting to examine an equivalent COS scoring system using the histoscore of ER and PgR to calculate a CER, Cuzick et al utilized this scoring method in their IHC study (dividing the calculated score which is in the range of 0-300 by 10 to give more manageable calculations).

COS utilises tumour histological grade as the substitute marker of proliferation. Meta-analysis supports that detection of tumour proliferation activity is the most important factor in gene prognostic signatures [39] and nuclear Ki67 labelling index is the favoured IHC proliferation marker in breast cancer [28] based on a number of quality studies demonstrating its value both as a potential prognostic/ predictive marker in breast cancer and key role in differentiating luminal A and B subtypes[142]. In this study, we anticipated utilising Ki67

however on independent analysis it was not found to be statistically significant despite testing a number of cut-off values that guidelines have proposed adopting [28, 52]. It is recognised that Ki67 testing proposes a significant challenge, due to assay variability and reproducibility. In a recent review examining the role of Ki67 in breast cancer, it was suggested that although a very promising and exciting marker, caution should be taken with premature widespread adoption until its exact role is defined given problematic variability and contradictory study findings[145]. Tumour histological grade is recognised as a marker of proliferation and is an accepted prognostic marker in breast cancer. Previously it has been subject to reproducibility suspicion, however following the Nottingham modifications (a more objective criteria) intra-laboratory reproducibility has improved[8], although there is still a challenge classifying grade 2.

This exploratory study has definite limitations. It is retrospective and in a fairly dated cohort (nearly all patients were treated with tamoxifen rather than AIs and details of chemotherapy regimen were not known but almost certainly will not have routinely received new generation agents such as taxanes). Despite these issues and its limitation in patient numbers, we report highly statistical significance even in sub group analysis indicating that COS may differentiate the categories of patients in which adjuvant therapy decision making is most problematic. The linear relationship between COS scores 2-10 and outcome are very exciting. We would certainly urge for this to be validated, it is very reproducible and simple to calculate therefore validation in a second cohort should not be problematic. Ideally the COS score require validation with comparison with gene microarray prognostic signature, such as PAM 50 or Oncotype Dx^{TM}.

COS is intended to represent tumour biology and augment prognostication using traditional prognostic factors. As a result of breast screening more newly diagnosed breast cancers are increasingly both small in size and node negative. It is likely that in the future more emphasis

will be on tumour biology rather than tumour burden. Importantly in this exploratory analysis COS appears to differentiate biologically more indolent tumours from aggressive tumours in both node negative patients and tumours less than 20mm. In fact, within this cohort both node negative and tumours less than 20mm with high COS had shorter DFS times than node positive or larger tumours with low COS. The observation that low COS when analysed in combination with traditional prognostic factors indicate very low risk suggests that this may be a 'good marker', and may reassure clinicians regarding the safe omission of adjuvant chemotherapy. A very topical question though is what constitutes safe omission. The optimal management of ER+/HER2 negative early breast cancer is controversial, with a main area of controversy being the threshold to recommend adjuvant chemotherapy [28]. A 'belt and braces' approach is often employed and subsequently we have a tendency to over treat. Although the justification for over treatment comes from over 40 years of clinical research which demonstrates the beneficial effects of adjuvant chemotherapy, and as noted by the steadily declining breast cancer mortality rates observed over the last two decades are, which are at least in part, due to widespread application of this strategy [146]. In addition, the most recent Oxford overview, a meta-analysis including over 100,000 early breast cancer patients reported that modern chemotherapy regimens reduce breast cancer mortality by one third compared to no chemotherapy and this applies to all women, irrespective of age, nodal status, size of tumour and ER status[110]. Although, the benefits reported in the Oxford overview represent population wide benefits and do not consider the molecular heterogeneity of breast cancer. It is likely that it will still be a number of years before prospective data is available that will ultimately reassure clinicians regarding the safe omission of chemotherapy in ER+ patients with challenging prognostication, the ongoing TAILORx (Trial Assigning IndividuaLized Options for Treatment (Rx) trial and MINIDACT (Microarray in Node Negative Disease May Avoid ChemoTherapy) will provide high-level evidence for the role of

tumour biology and prognostic scores in identifying ER+ patients who may and those who may not benefit from chemotherapy.

Another area of concern is the relative chemo-insensitivity of ER+ breast cancer [130]. In this study as a secondary analysis we reviewed benefit of chemotherapy in ER+/HER 2 negative breast cancer with both high and low COS. Worryingly, the results indicate that chemotherapy imparted no benefit within these groups. Although this was a very crude analysis with limited data regarding the types, duration and timing of treatment and we interpret this with caution. However, there is an increasing level of evidence suggesting that ER+ breast cancer is relatively chemo-resistant, especially biologically more indolent ER+ tumours. Focusing research effort into sensitivity of the subtypes of ER+ breast cancer and chemo-sensitivity, elucidating the mechanisms of endocrine resistance, or new strategies targeting ER+ breast cancer are all strategies underway to address this issue.

In conclusion, the Clinical Outcome Score (COS) calculated using conventional biomarkers and the Combined Endocrine Receptor (CER) is a simple and potentially easily reproducible scoring system that may identify ER+/HER- endotherapy treated patients most at risk of poor breast cancer outcome. Further testing in larger cohorts is warranted.

5 The Sodium Iodide Symporter (NIS) in ER positive breast cancer

5.1 Introduction

5.1.1 The Sodium Iodide Symporter

Sodium iodide symporter (NIS or SLC5A5, solute carrier family 5, member 5) [147] is normally expressed in the thyroid and lactating breast [148]. NIS is a transmembrane glycoprotein that delivers iodide into the thyroid gland for thyroid hormone production. In the lactating breast NIS functions to secrete iodide into the infant's milk [149]. NIS is expressed on the basolateral membrane of the lactating mammary alveolar cells [150] and accumulates iodine from the bloodstream into milk. The iodide is then used for thyroid hormone biosynthesis, essential for the infant's normal brain development. Expression of breast NIS is induced by oxytocin secreted from the posterior pituitary and its expression enhanced by elevated levels of serum prolactin and oestrogen present in the postnatal period [150]. In vivo experiments demonstrate that breast tissue in non-lactating female mice do not express NIS unless oxytocin treatment is administered, however in ovariectomized mice oxyctocin treatment is not sufficient for NIS expression, and oestradiol (E2) supplementation was required for functional NIS expression [149], suggesting that ovary function and endogenous oestrogens are important mediators of NIS expression in the lactating breast.

In thyroid cancer NIS is exploited for both diagnostic and therapeutic application. Since NIS confers highly efficient iodide accumulation in cells, its expression in thyroid cancer cells allows uptake of radioactive substrates of NIS, such as iodide (^{123}I ^{124}I and ^{131}I) and pertechnetate ($^{99m}TcO_4^-$). In addition, as normal thyroid NIS expression is under the hormonal control of thyroid stimulating hormone (TSH), in de-differentiated thyroid cancers that have reduced or absent NIS expression, administering TSH (or withdrawing thyroid hormone

supplementation post thyroidectomy to increase serum TSH) will induce tumour NIS expression in most cases, enabling radio-iodine treatment [151].

5.1.2 NIS expression in breast cancer

The discovery that NIS is expressed in the majority (70-80%) of breast cancers [149, 152], but not to a significant level in normal (non-lactating) breast tissue has meant that NIS is a potentially exploitable target for radio-iodine therapy in breast cancer. Whilst the majority of breast cancers express NIS, functional uptake of iodide is usually reduced or absent [153, 154].The correlation of $^{99m}TcO_4^-$ uptake and NIS mRNA expression in 25 patients with early breast cancer demonstrated that only 4 out of 25 tumours with NIS mRNA expression had functional uptake [154]. The disparity between NIS expression and function in breast cancer has been attributed to impairments in either (or both) transcription (level of NIS expression) and translation. In breast cancer the NIS protein is expressed predominantly in the intracellular space, while NIS is on the basolateral membrane in lactating mammary tissue [152]. Impairments in protein synthesis or protein modifications and NIS trafficking to the plasma membrane may be impaired in some breast cancers, as it is in some thyroid cancers [155].

Enhancement of endogenous NIS expression in breast cancer has been proposed as an approach that would allow ^{131}I therapy. NIS, however is normally expressed in the thyroid gland, and to a lesser amount, sites such as stomach and salivary glands [148], so selective induction of NIS in the target breast cancer would be required, necessitating a better understanding of regulatory processes involved in NIS expression in breast cancer. Compared to thyroid cancer in which the key regulators of NIS expression and function have been elucidated, in breast cancer these factors are poorly understood. In vitro and animal models suggest that the regulation of NIS in breast cancer has important differences to the regulation of NIS in thyroid cancer. There is strong evidence in vitro that the ER and

downstream cell signalling pathways are important regulators of NIS expression and function in breast cancer.

5.1.3 NIS regulation in Breast Cancer

Retinoids, active metabolites of vitamin A comprise both naturally occurring and synthetic compounds that have been used in animal models and humans as differentiation agents for various types of cancer. Retinoic acid (RA) is a robust inducer of endogenous NIS expression in breast cells in vitro. RA significantly induces NIS expression and active iodide uptake in several ER positive human breast cancer cell lines, including MCF-7, T47D, and BT474 [156-158]. RA fails to induce endogenous NIS expression and active iodide uptake in ER negative cell lines, suggesting that the ER is an important regulator of NIS expression. RA-responsive NIS expression was found to be correlated with the presence of a functional ER (using pS2 as a reporter gene of ER function) [159]. This same group found that suppression of endogenous ER gene in MCF7 cells by RNA interference down regulated RA induced NIS expression [159].

The actions of RA are mediated through two families of nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR). The classic (genomic) retinoid pathway involves the ligand activated nuclear receptors, RAR and RXR. Both RAR and RXR have three isoforms, α, β, and γ. RAR-RXR heterodimers bind to RA or RX- response elements and activate transcription. Non-genomic actions are less well characterised but involves crosstalk between the RAR-RXR heterodimer and signal transduction pathways in the cytoplasm, such as PI3K/AKT and MAPK pathways. Activation of signal transduction pathways have been implicated by several studies of NIS expression in breast cancer.

i. Phosphatidylinositol 3-kinase/ AKT signalling pathway

Mutations in genes that constitute the phosphatidylinositol 3-kinase (PI3K) pathway occur in >70% of breast cancers. PI3K is a major signalling hub downstream of HER2 and other receptor tyrosine kinases. PI3K activates Akt, serum/glucocorticoid regulated kinase (SGK), phosphoinositide-dependent kinase 1 (PDK1), mammalian target of rapamycin (mTOR), and several other molecules involved in cell cycle progression and survival. PTEN (Phosphatase and tensin homolog), encoded by the PTEN gene is a tumour suppressor gene with a phosphatise protein product which acts as a negative regulator of the PI3K-Akt pathway.

In addition to its pro-survival and growth-promoting roles, the PI3K pathway interacts with ER directly and indirectly. ER phosphorylation at Ser167 by Akt increases oestrogen-induced, tamoxifen-induced, and ligand-independent ER transcriptional activity [160]. Akt is a family of 3 closely related, highly conserved cellular homologues (AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ). The encoded proteins are serine/threonine protein kinases and belong to the protein kinase B (PKB) family. Akt kinases are activated in a PI3K kinase dependant manner. Akt is first phosphorylated at Thr ^{308} but for full activation additional phosphorylation at Ser ^{473} is necessary [161]. Once activated, Akt activates the ER [160] or substrates that directly or indirectly regulate apoptosis. High levels of activated Akt are associated with poor survival in ER positive tamoxifen treated breast cancer [162].

Induction of NIS expression in MCF7 cells treated with RA is regulated in part by the PI3K/Akt pathway and crosstalk with the RAR. RA activates Akt, within the first 10 minutes of RA treatment in MCF7 cells and treatment of cells with an Akt inhibitor or Akt knockdown with siRNA abolished RA induced NIS expression [163]. The regulatory subunit of PI3K, p85, directly interacts with RAR isoforms. Co-immunoprecipitation studies have demonstrated the association between p85 and the RARβ/RXRα heterodimer [163]. Since

loss of function analysis demonstrates the requirement of both RARβ and p85, the crosstalk between RARβ and PI3K signalling may mediate NIS induction by RA.

Activation of PI3K/ Akt pathway has also been implicated in NIS cell trafficking and lack of functional iodide uptake [164]. PI3K activation in MCF7 cells leads to expression of underglycosylated NIS lacking the cell surface trafficking necessary for iodide uptake ability. This was demonstrated in MCF7 cells with RA induced endogenous NIS expression and following transient transfection with exogenous NIS. In addition a correlation between NIS expression and upregulation of PI3K signalling (as indicated by phosphorylation and nuclear translocation of Akt) was found in human breast cancer tissue microarray[164].

ii. MAPK signalling cascade

The mitogen activated protein (MAP) kinases (MAPKs) are widely expressed protein kinase intracellular signalling molecules involved in cell division and mitosis. Extracellular regulated kinase 1/2 (ERK1/2, p42/44), Janus kinase (JNK), p38 MAPK signalling pathways are distinct serine-threonine kinase cascades each consisting of 3 enzymes: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. Many different stimuli can activate the MAPK pathway, including growth factors, cytokines and stress. Activation of this signalling cascade is common to many cancer cells. p44/42 pathway is typically activated via ligand binding to RTK and GRB2 (growth factor receptor bound protein 2)/SOS (son of sevenless), activating Ras. Activated Ras promotes Raf-1 phosphorylation and activation, which in turn activates MAPK. The pathway regulates proliferation, apoptosis, metastasis and angiogenesis. Activation of this MAPK pathway also phosphorylates and activates ER in a ligand independent manner [165-167].

Inhibitors of insulin like growth factor receptor 1(IGF-1 receptor), JNK and p38 MAPK have been demonstrated to significantly reduce NIS mRNA expression and iodide uptake in MCF7

cells in RA stimulated cells [168] suggesting that MAPK signalling is important in NIS expression and function. p38 MAPK signalling pathway is typically activated by cytokines and stress and regulates a number of cell processes similar to the p44/42 pathway such as proliferation, differentiation and migration. Four p38 isoforms, α, β, γ, δ are found in mammalian cells. RA stimulates phosphorylation of p38 isoforms, α and β in MCF7 cells through a small GTPase Rac1. Overexpression of p38β , as well as Rac1, significantly enhance the RA induced NIS expression and iodide uptake[169].

5.1.4 Study Aims

The aims of this study was to further probe the relationship between ER positive breast cancer and NIS expression and examine the interactions between ER and downstream cell signalling pathways as potential regulators important for NIS expression and function.

In order to address these aims, ER+ and ER- human breast cancer cell lines were transfected with the h-NIS gene, allowing us to establish if NIS expression and function were associated with ER expression. In order to further probe this relationship ER was then silenced in ER+ breast cancer cell lines and effects on NIS expression and function again recorded.

In addition, correlations between ER and NIS expression were also established in two separate cohorts of human breast cancer tumour specimens. Cohort one consisted of 50 frozen breast cancer specimens and was utilised to establish ER and NIS expression at the mRNA level. Results from cohort one demonstrated NIS was expressed in ER positive tumours only which lead to the development of cohort two which consisted of tumours from 300 ER+ early breast cancer tamoxifen treated patients. This cohort was utilised to assess the correlations between NIS and ER expression at the protein level and also to establish if NIS expression was associated with clinical outcome measures. In addition, this cohort allowed

correlations to be established between levels of NIS expression and expression of members of the MAP kinase and PI3K/Akt pathways that were already available.

5.2 Materials and Methods

5.2.1 Cells and Cell Culture Conditions

Four human breast cancer cell lines were used in this study. ER positive breast cancer cell lines: MCF7, T47D and ER negative breast cancer cell lines: MDA-MB 231, MDA-MB 453 all purchased from American Type Culture Collection (ATCC, Manassas, USA). Cells were maintained in Dulbecco's modified Eagles medium (DMEM) (Invitrogen, UK), supplemented with 10% fetal bovine serum, penicillin/streptomysin (100U/mL), fungiozone (2µg/mL) and L-glutamine (200mM). Cells were cultured at 37ºC in a 5% CO_2 atmosphere.

5.2.2 hNIS Plasmid transfection

Plasmid pcDNA3-hNIS, figure 5-1, described previously by the Radiation Oncology Group, University of Glasgow [170] was transfected into all four breast cancer cell lines as described by Carlin et al [170]. Briefly, cells were seeded at 5 $x10^3$ per well in six-well plates, and allowed to grow to 50% confluence, before being transfected with 3µg plasmid DNA (pcDNA3-hNIS) using Effectine lipid transfection reagent (QIAGEN, West Sussex, UK. Cat no 301425) according to the manufacturer's instructions. After 24 hours, geneticin G-418 sulphate (0.5 mg/ml) was added to select for transduced cells. Transfectants were maintained in identical conditions to the parental cells, with the addition of geneticin at each passage.

5.2.3 Knockdown of the ER (siRNA interference)

Short interfering RNA (siRNA) duplexes specific for the human ERα gene (NCBI reference sequence NM_000125) were specifically designed based on the Rosetta algorithm and purchased from Sigma-Aldrich Ltd (Dorset, UK). Cells were transfected with ER siRNA or

non- interfering scrambled siRNA using Lipofectamine^{TM}2000 (Invitrogen, Paisley, UK), according to the manufacturer's protocol.

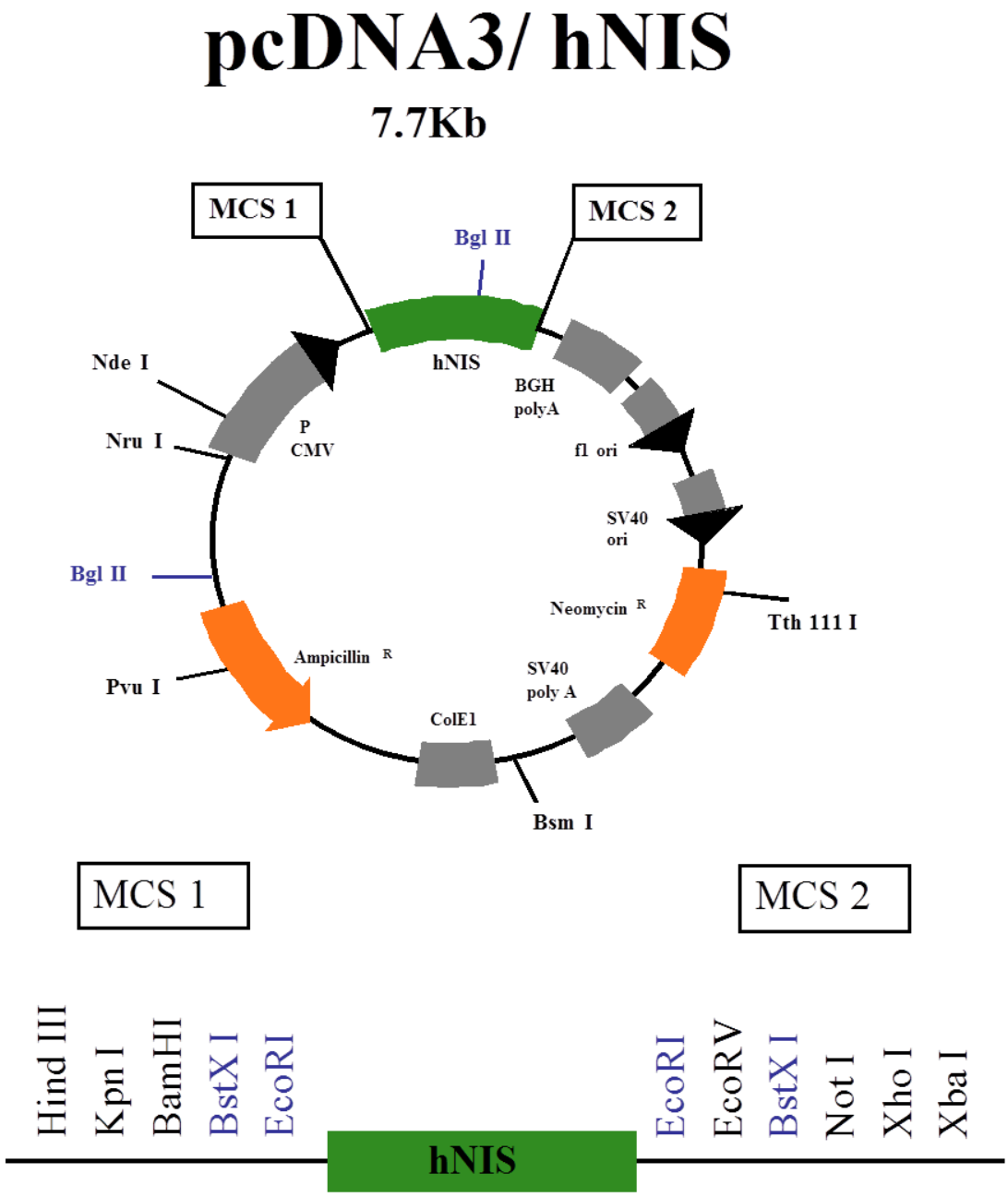


Figure 5-1 Plasmid pcDNA3-hNIS

hNIS cDNA inserted into the EcoRI site of pcDNA3 plasmid contained within the multiple cloning sites (MCS)

5.2.4 NaI^{125} uptake

Uptake experiments were adapted from previous work by Carlin et al. [170] and Weiss et al., [171]. 10^5 cells were seeded into 6-well plates containing 4 mL of standard culture medium. The transient oestrogen receptor (ER)-silencing of the adequate cell lines was performed over the next two days. Uptake was then initiated by incubation for 1h at 37°C in 1mL 0.1% BSA (in PBS) containing 37 KBq $Na^{125}I$ and 10μM NaI. The negative controls were performed in the presence of the NIS transporter inhibitor perchlorate ($NaClO_4$ 1μM). Iodide uptake was terminated by the removal of the uptake mix and washed three times in ice-cold 0.1% BSA (in PBS). Radioactivity was then solubilised by incubating for 1h at 4°C in 1mL of 10% tricarboxylic acid (TCA). Supernatant was finally harvested in centrifuge tubes and radioactivity was assessed using a Packard Cobra II gamma counter. The specific uptake was obtained by subtracting the inhibited uptake from the non-inhibited uptake. Control plates were seeded along with the experiment plates and the cells were counted to determine the specific uptake per 10^5 cells.

5.2.5 RNA extraction from cell lines

Total RNA was extracted from cell lines using the RNeasy Mini Kit (QIAGEN) as per manufacturer's instructions. RNA was quantified using a biophotometer.

5.2.6 Patient tumour Samples

The study was granted approval by the local ethics committee for both the cohort used to determine mRNA expression (reverse transcriptase real time PCR (RT–PCR) cohort) and the cohort used to determine protein expression (immunohistochemistry (IHC) cohort).

The RT–PCR cohort contained 73 frozen invasive tumour samples taken from breast cancer patients at the time of primary tumour resection. All patients were diagnosed with invasive breast carcinoma between 1987 and 2005 in the Greater Glasgow area.

The IHC cohort is completely distinct from the PCR cohort with no overlapping patients. All patients in the IHC cohort were diagnosed with primary operable breast cancer between 1980 and 1999 and received standard adjuvant treatment according to protocols at the time of diagnosis. Only patients with full clinical data available were included in analysis. All tissue samples were taken at the time of surgical resection, assessed and determined by a pathologist. These were used for tissue microarray (TMA) construction, as described previously [172].

5.2.7 Human Tissue Processing and RNA extraction

After surgical resection of the primary tumour, representative parts of malignant tissue were identified by a pathologist, snap frozen and stored in liquid nitrogen at -80ºC until processed.

Total mRNA was extracted from 50 to 75mg of breast tissue using the TRIZOL (Invitrogen, Paisley, UK) method according to manufacturer's protocol. RNA quantity and quality was assessed by UV spectrometry (GeneQuant machine, GE Healthcare, Little Chalfont, UK) and by examination of rRNA bands after agarose gel electrophoresis. Only samples that showed both 18S band and a stronger expressed 28S band were used. The RNA samples were assessed for expression of ER, NIS and reference gene GAPDH by reverse transcription and real-time PCR.

5.2.8 Primers and Probes

Primer and probe sequences were designed from published sequence for the human oestrogen receptor α (hER) (NCBI reference sequenceNM_000125) using the ABI prism PrimerExpress^{TM} v2.0 software and BLAST searches (http://www.ncbi.nlm.nih.gov) carried out to confirm specificity of the nucleotide sequences chosen. Both primer and probe were custom synthesised (VHBIO Ltd). The sense (forward) primer corresponded to bases 1422-

1439 of hER (5’-AGCACCCAGTGAAGCTACT-3’). The antisense (reverse) primer was complimentary to bases 1561-1578 (5’-TGAGGCACACAAACTCCT-3’). These primers generated a PCR product of 156 base pairs. The internal probe corresponded to bases 1518-1542 (5’-TGGCTACATCATCTCGGTTCCGCA-3’). The probe was labelled with the fluorescent reporter dye 6-carboxyfluoroscein (FAM) at the 5’ end and the quencher molecule 6-carboxytetramethylrhodamine (TAMRA) at the 3’end.

Similarly, primer and probe sequences were designed from the published sequence for the human sodium iodide symporter (hNIS) (NCBI reference sequence NM_00453), and custom synthesised as above (VHBIO Ltd). The sense primer corresponding to bases 696-715 (5’-ACCTACGAGTACCTGGAGAT-3’) and the antisense primer complimentary to bases 814-832 (5’-AGCCCGGTCACTTGGTTCA-3’). These primers generated a PCR product of 137 base pairs. The internal probe corresponded to bases 759-782 of the hNIS sequence (5’-ATTGTAGCCACGATGCTGTACACC-3’). This probe was labelled with fluorescent reporter dye FAM at the 5’end, and the quencher molecule TAMRA at the 3’end.

Progesterone Receptor (PgR) (NCBI reference sequence NM_000926) primer and probe sequences were adapted from de Cremoux et al [173], and custom synthesised as above (VHBIO Ltd). The sense primer (5’-GAACCAGATGTGATCTATGCAGGA-3’) and the antisense primer (5’CGAAAACCTGGCAATGATTTAGAC-3’), the primers generated a PCR product of 122 base pairs. The internal probe (5’ACCTGACACCTCCAGTTCTTTGCTGACAAG-3’) was labelled with fluorescent reporter dye FAM at the 5’end, and the quencher molecule TAMRA at the 3’end.

The house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard for all real-time PCR reactions. GAPDH PCR was carried out using the

specifically designed primers and probe kit, TaqMAN GAPDH control reagents. This was obtained from Applied Biosystems (Cheshire, UK), part number 402869.

5.2.9 Standard curve generation and quantitation of test samples

A standard curve was constructed, which was used to produce an exact quantitation of starting number of copies of target sequence. To construct the standard curve, firstly a PCR reaction was carried using TaqMan- generated PCR primers, but using standard (ie. non-TaqMan) PCR reagents. The resulting PCR products were then purified. The PCR product was quantified spectrophotometically at A_{260}, and converted to number of molecules/µl, using the equation in figure 5.3 [174]. TaqMan PCR of standards and unknown samples was then carried out, and the ABI 7700 sequence detection software determined the initial amounts of unknown samples by direct comparison of their C_t value with the C_t values of known standards.

ER, PgR, hNIS and GAPDH- specific PCR products were obtained by reverse transcribing and PCR amplifying 1µg of total RNA obtained from human breast cancer cell lines. ER and PgR were obtained from by reverse transcribing and PCR amplifying 1 µg of RNA from human breast cell line MCF7, using the same primer probes employed for quantitative real time PCR. The resulting PCR products were then purified using an S-400 spin column (Pharmacia Biotech, Uppsala, Sweden) and quality assessed by agarose gel electrophoresis and ethidium bromide staining. The PCR products were quantified spectrophotometrically and then serially diluted to maintain a constant total DNA concentration. The standard curve used for quantitation of the real time PCR reaction was constructed using 10^1 to 10^8 copies of the MCF7 ER and PgR products obtained from the reaction described above.

Similarly, hHIS specific PCR products were obtained by reverse transcribing and PCR amplifying 1µg of total RNA obtained from the hNIS transfected MCF7 cell line. The

resulting PCR product was purified and assessed as above, and a standard curve used for quantitation of real time PCR reaction was constructed using 10^1 to 10^8 copies of the hNIS sequence.

$$\text{No. of molecules /µl} = \left(\frac{A_{26}}{13.2 \times S} \right) \times \frac{N}{10^{12}}$$

A_{26}= absorbance at 260nm,

S **= size of DNA in kilobases**

N **= Avogadro's number: 6.022×10^{23}**

Figure 5-2 Calculation of initial number of molecules for generation of a standard curve

This calculation is used to determine the number of molecules in a sample of known DNA concentration. It is based on the determination of concentration of double stranded DNA, which establishes the molarity (in pmol/µl) of a DNA sample from its absorbance at 260nm.

1 mole contains 6.022×10^{23} molecules (Avogadro's number), allowing calculation of the initial number of molecules in a DNA sample [174].

5.2.10 Real-time RT-PCR amplification

Real-time RT PCR was carried out using the commercially available TaqMan Gold RNA PCR kit (Perkin-Elmer Applied Biosystems, Warrington). Briefly, 1µg of total RNA was reverse transcribed in a 50µl reaction volume, containing 5.5mM $MgCl_2$, 5µl 10x TaqMan RT buffer, 2mM dNTP (500uM each nucleotide), 20 units of RNAse Inhibitor, 2.5µM oligo $d(T)_{16}$ and 62.5 units MultiScribe Reverse Transcriptase. 2.5µl of the resulting solution, containing cDNA template, was added to an amplification reaction mixture of total volume 25µl consisting of 5.5mM $MgCl_2$, 2.5µl 10x TagMan buffer, 200µM dATP, dCTP, dGTP and

400µM UTP, 0.625 units of AmpliTag Gold, 0.25 units of AmpErase uracil N-glycosylase (UNG) and 100nM each of both primers and probe.

The thermal cycling conditions consisted of an initial incubation for 2 mins at 50ºC, followed by 10 min at 95ºC. Thermal cycling was then carried out at 95ºC for 15 sec, followed by 60ºC for 1 minute, for 40 cycles. Real time PCR amplification of ER cDNA was carried out exactly as above, however thermal cycling was carried out at 95ºC for 15 sec, followed by 57ºC for 1 minute, for 40 cycles.

Each assay included a standard curve (10^1 to 10^8 copies) and a no template control, along with the cDNA templates obtained from the reverse transcription step. PCR reactions were performed using an ABI prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, USA), which measured the fluorescent signal generated by the PCR reaction.

5.2.11 Western Blotting

MC7-hNIS transfected cells and MDA-MB 231 cells treated were lysed in RIPA buffer (50 mM Tris pH7.6, 150 mM sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 1 mM sodium ortho-vanadate and 1:100 Calbiochem protease inhibitor cocktail set 1) and centrifuged at 12 000 rpm for 10 min, the supernatant removed and protein concentration determined using BCA/$CuSO_4$ assay. Deglycosylation was performed as described by Beyer et al [175] using Peptide N-Glycosidase F (PNGase-F) (New England Biolabs, Ipswitch, MA). 40 µg of protein per well was resolved by 4-12% gradient Bis-Tris gel electrophoresis (Invitrogen, UK); proteins were transferred to nitrocellulose membranes (Millipore, UK), which were blocked for 1 hour in 5% BSA and probed with primary antibodies: anti-NIS (1:750) at 4^0C overnight. Membranes were then incubated with secondary anti-rabbit antibodies (1:0000) and visualized with ECL kit

(Amersham, UK). Where necessary, the membranes were stripped by incubating with Re-Blot Plus stripping buffer (Chemicon, UK) before re-probing with other antibodies including anti-Actin (1:250 Santa Cruz, USA) to confirm equal protein loading.

5.2.12 Immunohistochemistry

The hNIS polyclonal rabbit antibody [175], previously kindly gifted to the Department of Radiation Oncology, Beatson Institute from Professor Jhiang, Ohio was utilised for IHC analysis of 300 breast cancer tumour specimens taken from patients and IHC controls (including 5 normal breast and negative controls, including smooth muscle, normal lung and pancreas) . Prior to performing immunohistochemistry, antibody specificity was confirmed by western blotting (figure 5-4), demonstrating a ~ 90kDa molecular weight in MCF7-hNIS transfected cells and a ~50kDa molecular weight protein in MCF7-hNIS transfected cells following deglycosylation. Tissue sections were dewaxed and rehydrated through graded alcohols and then subjected to heat induced antigen retrieval by pressure steaming in preheated 10mM citrate buffer for 5 mins. Immunostaining was then performed; sections were first treated with hydrogen peroxide and then blocked using horse serum, followed by incubation in primary antibody (1: 300 dilution anti hNIS for 1 hour). DakoCytomation EnVision was applied for 30 mins and sections incubated with DAB (1:50 dilution). Finally, sections were counterstained, dehydrated and mounted. Positive and negative (isotype matched antibody) control slides were incorporated in each run. IHC for ER and PgR was performed in Glasgow Royal Infirmary diagnostic pathology laboratories as per diagnostic protocols.

Tissue staining intensity was scored by consultant histo-pathologist Dr Tamsin Doig, using a weighted histoscore method [176] also known as the Hscore system [177]. Histoscores were calculated from the sum of (1 x % cells staining weakly positive) + (2 x % cell staining moderately positive) + (3 x % cells staining strongly positive) with a maximum of 300. Each

cellular location was separately assessed with a weighted histoscore assigned to any membrane, cytoplasm and nucleus staining. The histoscores for each core were then averaged. Where one core was missing the remaining core(s) scores were used.

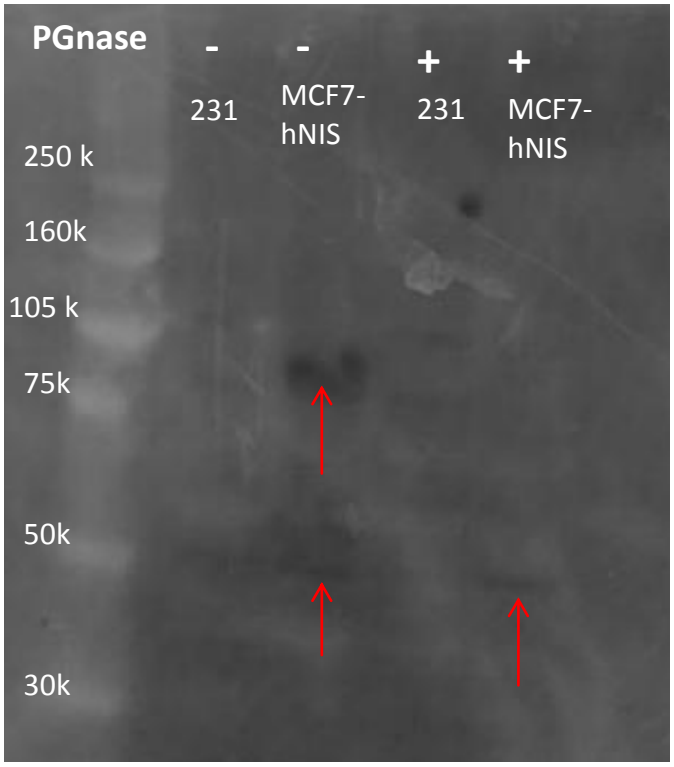


Figure 5-3 Western Blot

NIS is a glycoprotein and has varied degrees of glycosylation. In MCF7-hNIS transfected cells not subject to deglycosylation, anti-hNIS detected a ~90kDa band (there is a suggestion of a ~50kDa band here as well). MCF7-hNIS transfected cells were deglycosylated andthe resulting band was ~50 kDa.

5.2.13 Statistical Analysis

Correlations were calculated using both Spearman's Correlation and Pearson's Correlation methods. Univariate outcome analysis was performed using Kaplan Meier method and calculation of hazard ratios (HR) for both univariate and multivariate analysis performed using Cox's proportional-hazards model, a stepwise backward procedure was used to derive a final model of variables that had a significant independent relationship with patient outcome.

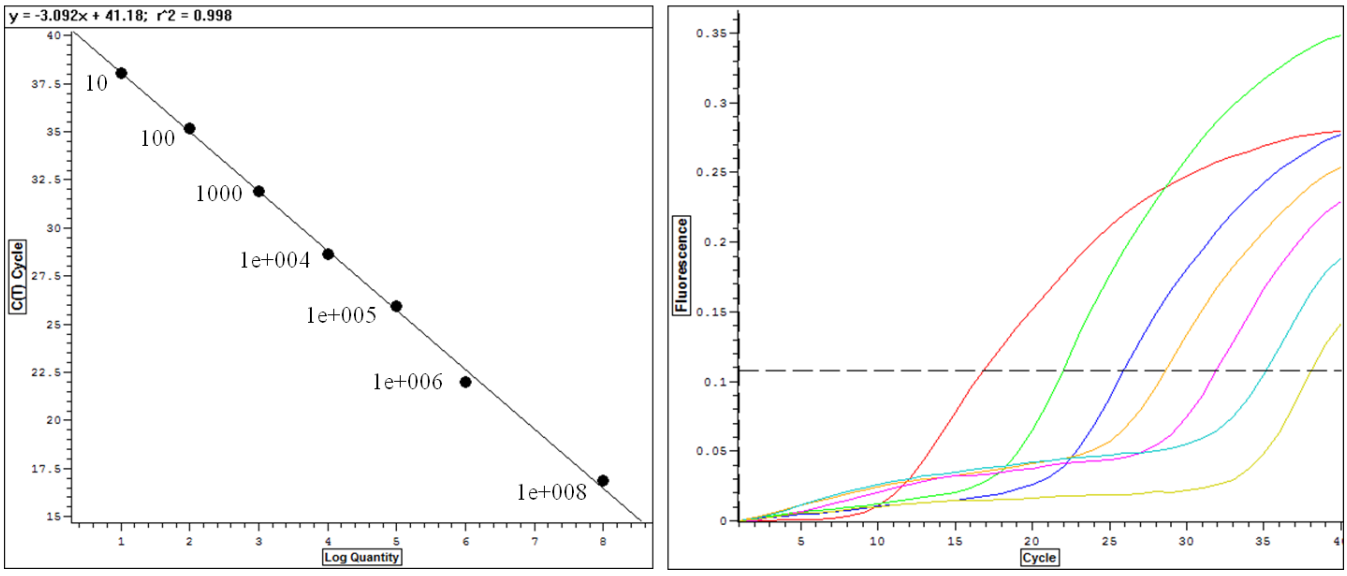
All statistical analysis was performed using SPSS software version 19 (SPSS Inc., Chicago IL, USA).

5.3 Results I

5.3.1 Standard Curve generation for accurate quantitation of ER, PgR and hNIS

A range of quantities of ER, PgR, NIS and GAPDH- specific PCR products were amplified using the real-time PCR method to establish corresponding C_t values. These were plotted against the log of the initial quantity of substrate to produce standard curves. This exemplifies the high sensitivity and accuracy of amplification over a large concentration range, figs 5.4-5.7. RNA obtained from cell lines and patient tumour samples were reverse transcribed and PCR amplified using the real time methodology. Samples were assayed three times (although in a small number of patient samples, this was not possible as not enough RNA was available).

A **B**



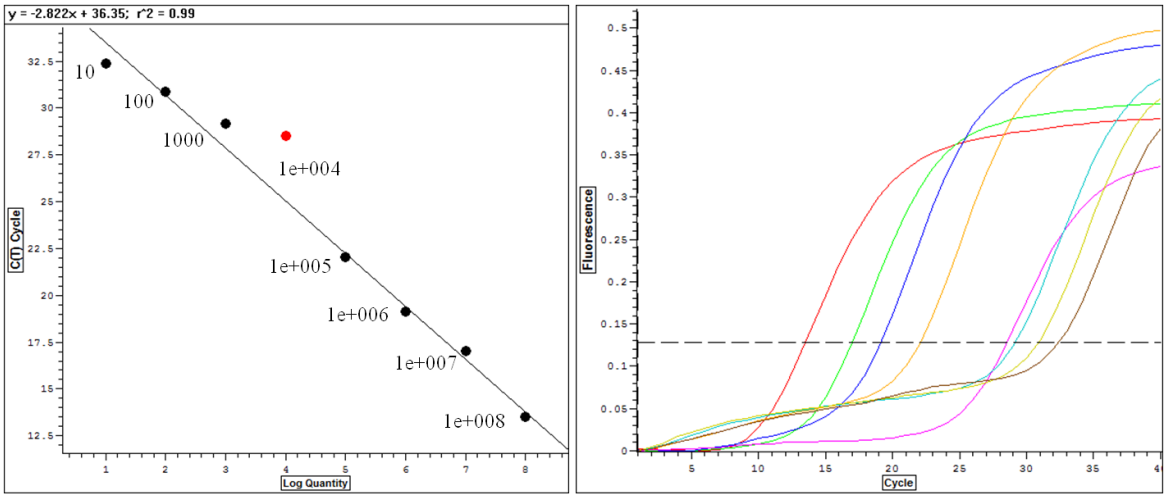
● standards
Slope: -3.09
Y- intercept: 41.18
Coefficient: 0.998

ER Copy number	C_t
1e+008	16.85
1e+006	21.98
1e+005	25.98
1e+004	28.66
1000	31.93
100	35.18
10	38.06

Figure 5-4 ER Standards

Standards were obtained by PCR amplification of cDNA from the cell line MCF7. The ER-specific PCR product was the quantified spectrophotometrically, serially diluted and amplified using the real time PCR method. A) The C_t values were plotted against the initial (log) quantity of substrate to produce a standard curve (r=0.998). B)The plots from left to right, correspond to 10^8 to 10 ER sequence copies.

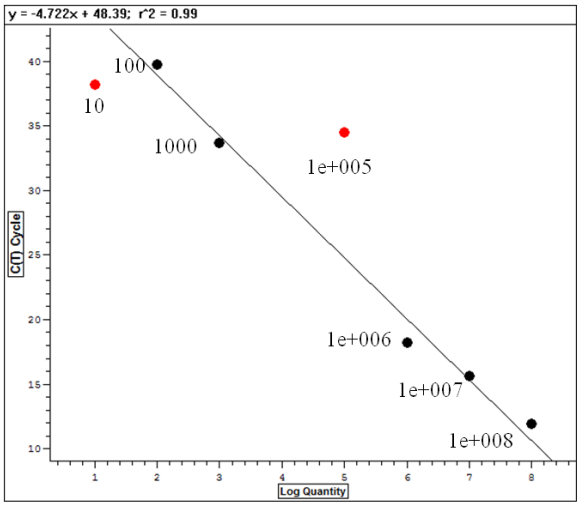
A similar procedure was used to quantify PgR (figure 5-5) and NIS (figure 5-6) and the reference GAPDH sequence.

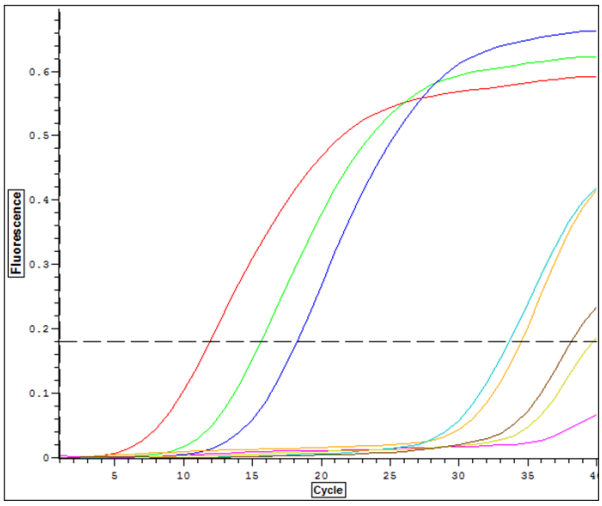


• standards
Slope: -2.9
Y- intercept: 36.5
Coefficient: 0.99

PgR Copy number	C_t
1e+008	13.50
1e+007	17.01
1e+006	19.15
1e+005	22.07
1e+004	28.54
1000	29.16
100	30.88
10	32.40

Figure 5-5 PgR standards





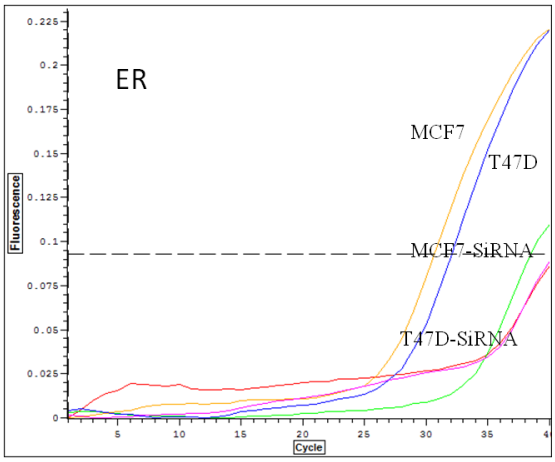
standards
Slope: -4.72
Y- intercept: 48.39
Coefficient: 0.99

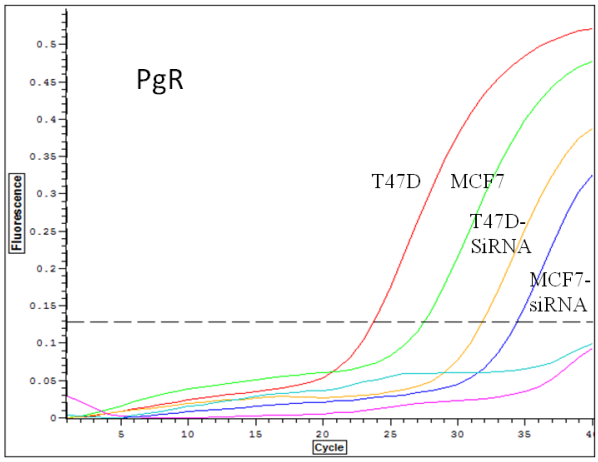
NIS Copy number	C_t
1e+008	11.71
1e+007	15.44
1e+006	18.04
1e+005	33.45
1e+004	na
1000	34.32
100	39.46
10	38.89

Figure 5-6 NIS standards

5.3.2 PgR expression reduced by siRNA specific ER knockdown

PgR is an oestrogen regulated gene, and its synthesis in normal and breast cancer cells requires oestrogen and the ER[45].The working hypothesis is that tumour PgR expression represents an intact oestrogen- ER response pathway[51]. Knock down of the ER was confirmed with real time RT-PCR quantification of the ER and PgR expression in MCF7 and T47D cell lines, fig 5-7 and 5-8.





Cell line	ER Copy number	C_t
MCF7	828	30.6
MCF7-SiRNA	0.32	38.5
T47D	200.5	32.1
T47D-SiRNA	0	>40

Cell line	PgR Copy number	C_t
MCF7	1415	27.4
MCF7-SiRNA	4.9	34.4
T47D	2.9e+004	23.4
T47D-SiRNA	40.4	31.8

Figure 5-7 Real time RT-PCR quanitification following ER knockdown using siRNA

Quantitative real time RT PCR measuring the ER and PgR (marker of ER function) in MCF7 cells and T47D cells in parental lines and following siRNA treatment to ensure knock down of ER. Reduction in PgR mRNA expression suggests downregulation of ER function.

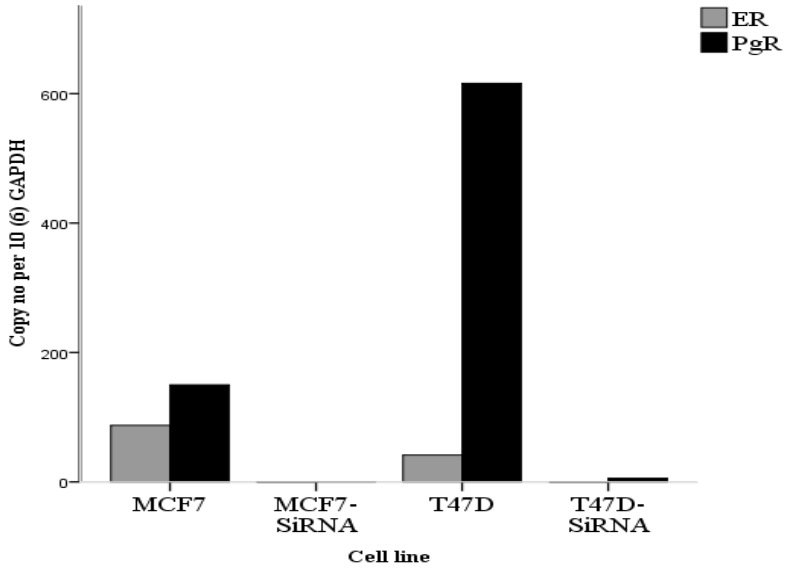


Figure 5-8 Knockdown of ER using siRNA and reduction in both ER and PgR mRNA expression level

mRNA expression level of ER and PgR in ER+ cell lines (MCF7 and T47D)- normal and following treatment with siRNA specifically targeting the ER.

5.3.3 In vitro model- assessment of NIS function associated with ER status of cells

5.3.3.1 $Na^{125}I$ uptakes in parental and hNIS-transfected breast cancer cell lines compared with NIS and ER expression

cDNA encoding the human NIS (hNIS) protein was transfected into two ER negative cell lines (MDA-MB 231 and MDA-MB 453) and two ER positive cell lines (MCF7 and T47D) using the eukaryotic expression vector pcDNA3, under the control of the CMV promoter and selected for the presence of the geneticin-G418-sulphate resistance gene.

Real time RT-PCR, with specificity for NIS, ER and GAPDH sequences, fig 5-9A, suggest that parental ER positive cell lines MCF7 and T47D have very low levels of endogenous NIS expression whereas ER negative breast cancer cell lines MDA-MB231 and MDA-MB453 have no endogenous NIS transcription. All four cell lines have increased NIS gene expression as a result of pcDNA3-hNIS transfection. Copy numbers of NIS per$1x10^6$ GAPDH are very

low in all transfected cell lines, table 5-1. Iodide uptake was assessed in the parental cell lines and hNIS transfected cell lines by incubating cells with 10µM NaI containing trace $Na^{125}I$ (specific activity = 1µCi/µmol) for 1 hour. Cell uptake was also measured in the presence of 1mM perchlorate (NaClO4), a competitive inhibitor of active sodium iodide transporter (NIS), figure 5-9B. All cell lines, parental and transfected had trace amounts of passive iodide uptake as demonstrated by small amounts of detectable cell iodide in the presence of perchlorate. Only MCF7 and T47D NIS transfected cell lines were able to actively accumulate iodide, figure 5-10. These results demonstrate hNIS transfection results in specific, perchlorate-inhibitable iodide uptake in ER positive cell lines MCF7 and T47D but not in ER negative cells transfected with hNIS. This suggests that the ER status or the ER phenotype of a cell is associated with NIS function.

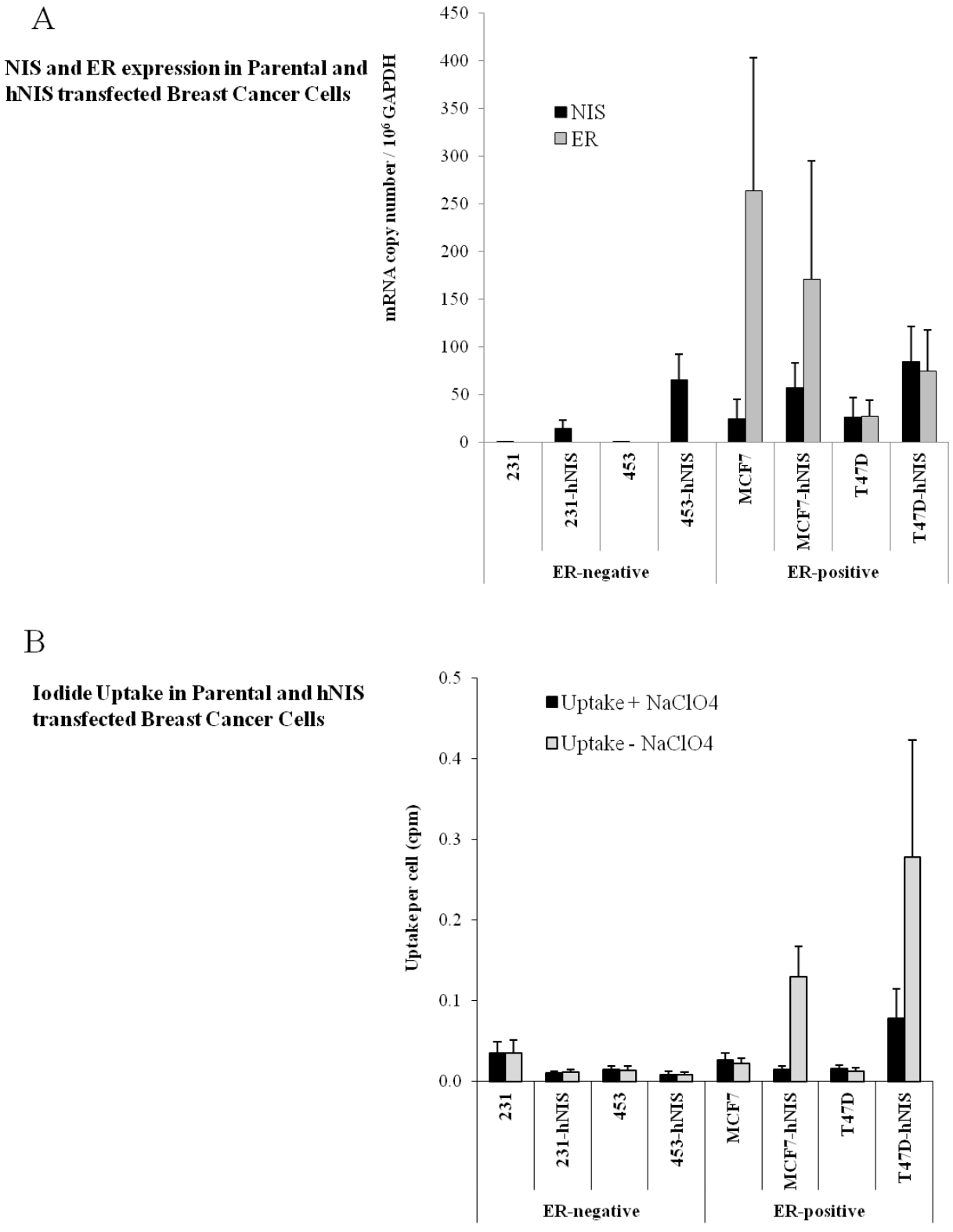


Figure 5-9 Uptake of Na ^{125}I and mRNA expression level of ER and NIS in parental and transfected cell lines

Cell Line	NIS copy no/ $1x10^6$ GAPDH (±Std error of mean)	ER copy no/ $1x10^6$ GAPDH (± Std error of mean)	$Na^{125}I$ Specific Uptake (cpm$x10^3$ per 10^5 cells)
MDA-MB 231	0 ±0.1	0	0.014 ± 0.014
MDA-MB 231-hNIS	15 ± 8	0	0.013 ± 0.005
MDA-MB 453	0 ± 7	0	0
MDA-MB 453-hNIS	65 ± 27	0	0.055 ± 0.003
MCF7	24 ± 20	263 ± 139	0
MCF7-hNIS	57 ± 26	170 ± 124	11.4 ± 3.8
T47D	26 ± 21	28 ± 16	0
T47D-hNIS	84 ± 36	74 ± 43	20.1 ± 18.6

Table 5-1 NIS and ER copy number relative to GAPDH expression and $Na^{125}I$ uptake in parental and hNIS transfected cell lines

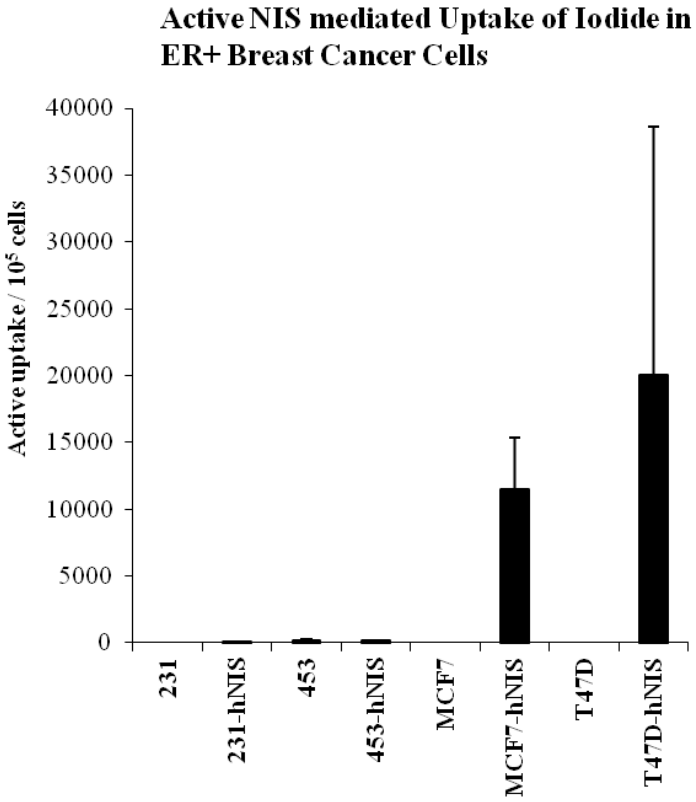


Figure 5-10 Active $Na^{125}I$ uptake in ER+ hNIS transfected cells

Active uptake is easily calculated by subtracting iodide uptake in the presence of inhibitor (i.e. passive) from iodide uptake without inhibitor (i.e. active and passive)

5.3.3.2 ER positive-hNIS transfected breast cancer cell lines treated with ER targeted siRNA– comparison of $Na^{125}I$ uptakes and ER/NIS gene expression.

The hNIS transfected ER positive cell lines (MCF7 and T47D) were treated with ER specific siRNA duplex to knockdown the ER and non-specific scrambled siRNA duplexes as a control. Real time RT PCR confirmed ER knock down in ER siRNA-MCF7-hNIS and three fold reduction in ER expression in T47D-hNIS cells. NIS expression was confirmed in all cell lines. Fig 5-11 A

Iodide uptake in the presence and absence of 1mM perchlorate (NaClO4) was assessed in scrambled and siRNA-hNIS transfected MCF7 & T47D cell lines, figure 5-11B. All cell lines, retained the ability to actively accumulate iodide that was inhibitable with perchlorate, table 5-2. These results suggest firstly that the level of ER expression as determined by real time RT PCR does not influence NIS function. Secondly, compared to ER negative cells transfected with hNIS (MDA-MB 23-hNIS and MDA-MB 453-hNIS), that failed to actively accumulate iodide, MCF7-hNIS transfected cells with ER knockout can accumulate iodide. This suggests that it is the ER cell phenotype, rather than the ER *per se*, that is important to NIS function. Growth factor signalling pathways that have bidirectional crosstalk with the ER have been demonstrated to be important to NIS regulation[164], and ER knockout or knock down may enhance their signalling.

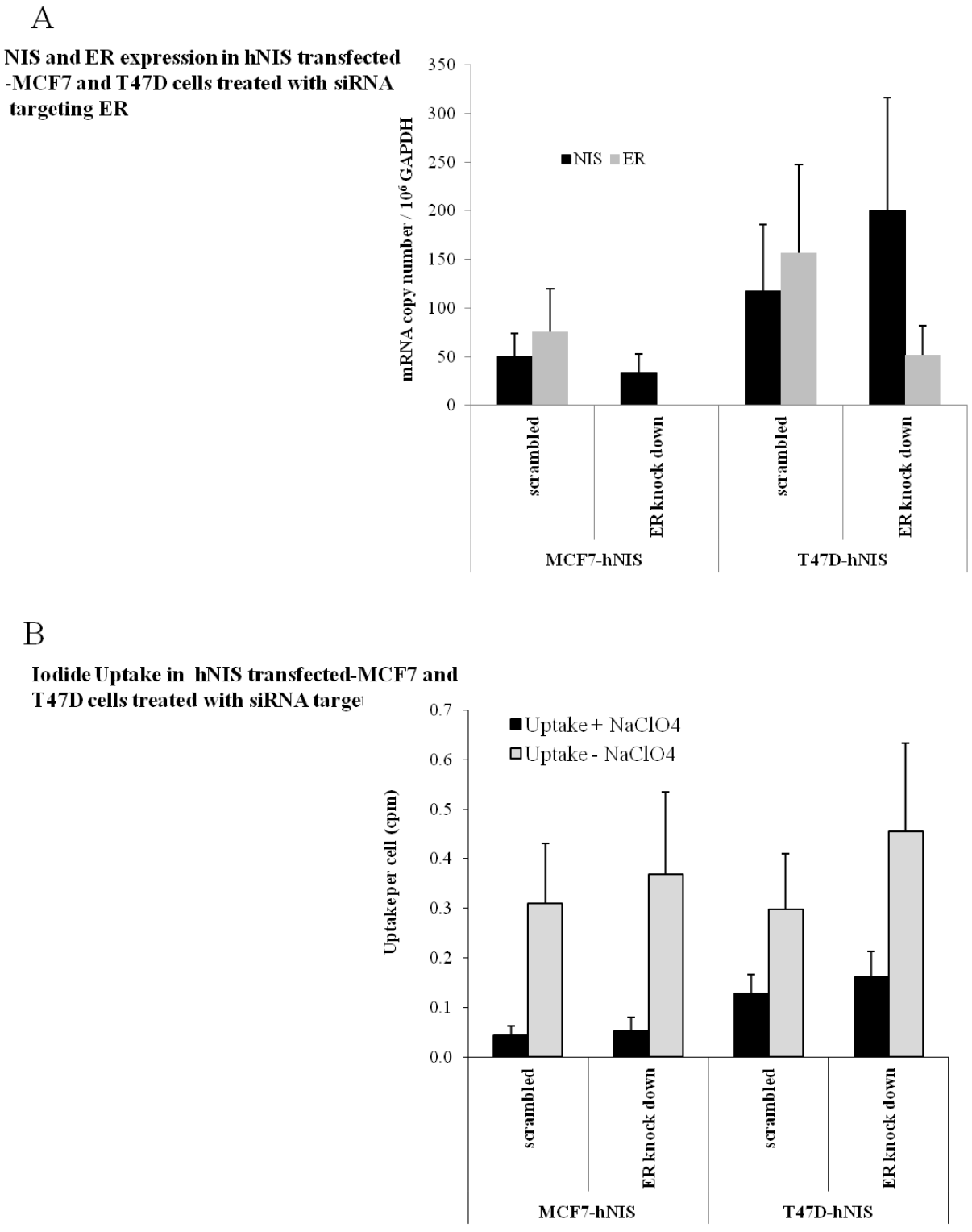


Figure 5-11 Uptake of Na ^{125} I and mRNA expression level of ER +/hNIS transfected cell lines following treatment with siRNA to knockdown ER

ER+ /hNIS transfected cell lines were treated with siRNA specific for the ER, scrambled (non-interfering siRNA) treatment was performed as a control. Iodide uptake and expression level of NIS and ER were analysed in both scrambled and siRNA treated cells.

Cell Line	NIS copy no/ $1x10^6$ GAPDH (±Std error of mean)	ER copy no/ $1x10^6$ GAPDH (± Std error of mean)	$Na^{125}I$ Specific Uptake (cpm$x10^3$ per 10^5 cells)
MCF7 Scrambled	50 ± 23	76 ±48	26.6 ± 11.7
MCF7-SiRNA	33 ± 19	0.1 ±0.1	31.5 ± 16.0
T47D Scrambled	117 ± 68	156 ± 90	16.9 ± 13.4
T47D-SiRNA	200 ± 115	52 ± 30	29.3 ± 18.

Table 5-2 NIS and ER copy number relative to GAPDH expression and $Na^{125}I$ uptake in hNIS transfected cell lines following siRNA treatment

Iodide uptake and real time RT PCR quantification of NIS and ER in ER+/hNIS transfected cells treated with siRNA targeting the ER (scrambled as a control)

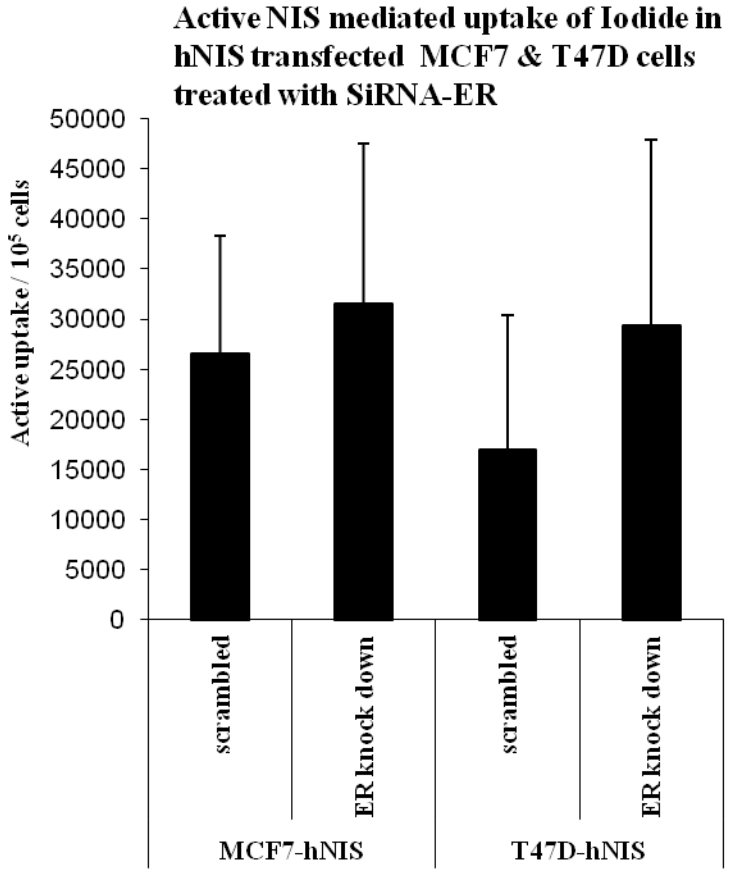


Figure 5-12 Active $Na^{125}I$ uptake in ER+ hNIS transfected cells treated with ER specific siRNA

5.4 Results II. NIS expression in cohort of mixed ER negative and ER positive breast cancer patients

To further investigate the hypothesis that NIS expression in human breast cancer is related to hormone receptor positive breast cancer, 73 frozen breast cancer tumour specimens from patients with ER positive, ER negative or ER unknown breast cancer was analysed. RNA was extracted from frozen tumour specimens and reverse transcribed and PCR amplified using real time methodology to assay ER, NIS and GAPDH expression. Of the 73 specimens analysed 50 (68%) were evaluable for the study.

5.4.1 Patient and tumour characteristics

Patients were diagnosed in Greater Glasgow between 1986 and 2006. The ER status, positive or negative, determined by IHC on the primary tumour was known for 62% (n=31) of the tumours analysed. Table 5-3 details the patient and tumour characteristics of the 50 specimens analysed. Accurate survival and recurrence follow up data was available for 72% of patients (n=36) the range of follow up was 5months-247 months, mean 86 months and median follow up 68 months.

	Number of cases	% cases
Age		
≤ 50	12	24%
>50	38	76%
Type		
Ductal	32	64%
unknown	18	36%
Grade		
1	1	2%
2	20	40%
3	26	52%
unknown	3	6%
Size(mm)		
<20	8	16%
20-50	32	64%
>50	8	16%
unknown	2	4%
Nodal status		
negative	13	26%
positive	30	60%
unknown	7	14%
ER status		
negative	8	16%
positive	23	46%
unknown	19	38%
Outcome		
Alive	19	38%
Breast Cancer Death	15	30 %
Non Breast Cancer Death	13	26%
Unknown	3	6%

table 5-3 Patient and tumour characteristics in Patient Cohort 1 (real-time RT PCR analysis)

n=50	NIS copies per 10^6 GAPDH	ER copies per 10^6 GAPDH
No of tumours with zero (non-detectable)	11 (22%)	4 (8%)
Range of copy number	0- 7538	0-58054
Median copy number	7.75	210
Percentiles		
0-25%	0-1.25	0-26
25-50%	1.25-7.75	26-210
50-75%	7.75-50	210-2128
75-100%	>50	>2128

Table 5-4 Distribution of NIS and ER mRNA expression levels in Patient cohort 1

5.4.2 Real time quantitation of ER and NIS

The real time PCR procedure revealed a wide range of NIS and ER gene expression levels in patient breast cancer specimens. 39 tumours (78%) had detectable NIS expression although expression level among tumours with detectable NIS was very low relative to GAPDH. Most (60%) of tumours expressed less than 10 copies of NIS per $1x10^6$ GAPDH (n=30), the median value was 7.75. In contrast, tumour gene expression of ER was higher, 46 tumours (92%) of the cohort had detectable ER expression and the majority of tumours (78%) expressing greater than 10 copies of ER per $1x10^6$ GAPDH. For the entire cohort tumour NIS expression was significantly correlated with tumour ER expression, Spearman's correlation coefficient 0.642 (p=<0.001), figure 5-13

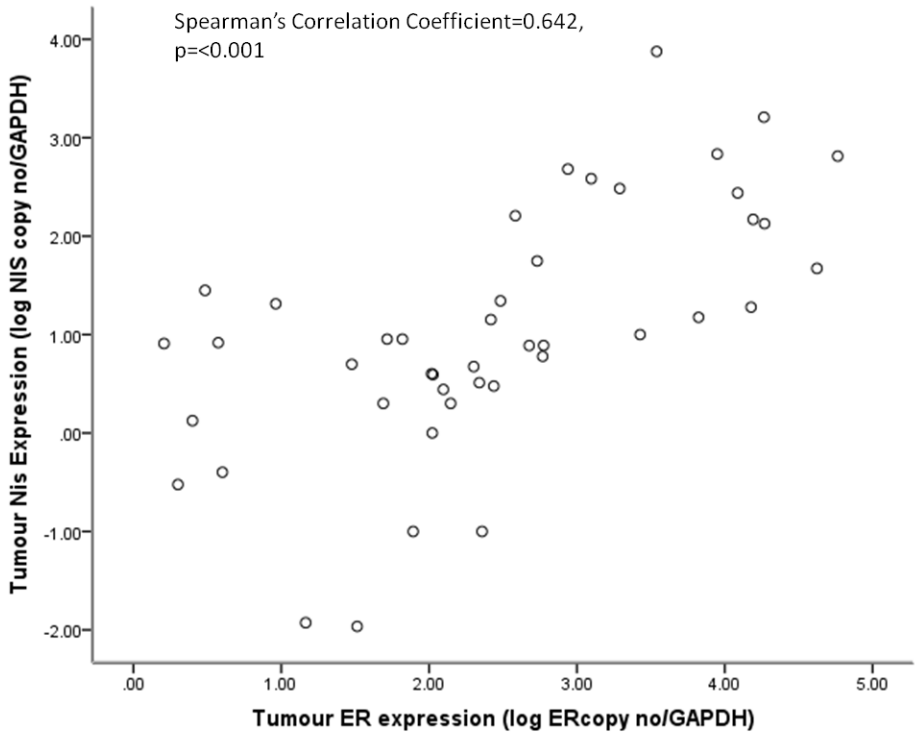


Figure 5-13 Scatter plot demonstrating significant correlation between ER and NIS mRNA expression level in breast cancer specimens (patient cohort 1)

5.4.3 Defining High and Low Expression in Tumour samples

The upper quartile copy number for NIS (>49.25) was considered too low, relative to $1x10^6$ GAPDH expression to confidently represent tumour positivity, therefore a threshold of 100 NIS copies/ GAPDH defined NIS positive expression (n=11, 22% of cohort). No established cut-off exists to define ER positive from negative using real time PCR. The median value was (210 copies per $1x10^6$ GAPDH) was therefore utilised. The majority of the cohort (n=39, 78%) had low NIS expression (<100 NIS copies/$1x10^6$ GAPDH). 18% (n=9) had 100-1000copies NIS copies/$1x10^6$ GAPDH and 5% (n=2) had very high NIS expression (>1000copies/$1x10^6$ GAPDH). 50% (n=25) of tumours had negative ER expression (<210 copies/$1x10^6$ GAPDH). Within ER positive tumours (n=25), the majority (n=14) had very high expression (>1000copies/$1x10^6$ GAPDH), table 5.5. The majority (60%, n=15) of ER positive tumours determined by real time PCR were documented ER positive, 28% (n=7) were unknown ER status, and 3 tumours (7%) were reclassified as positive.

ER copies per $1x10^6$ GAPDH	**NIS copies per $1x10^6$ GAPDH**
<210 copies Low (n=25, 50%)	<100 copies Low (n=39, 78%)
210copies-1000copies High (n=11, 22%)	100-1000 copies High (n=9, 18%)
>1000 copies Very high (n=14, 28%)	>1000 copies Very high (n=2, 4%)

Table 5-5 Definition of Low and High mRNA expression level in patient cohort 1

All NIS positive tumours were ER positive. In ER positive tumours, 44% (n=11) expressed NIS (>100copies/10^6 GAPDH)

5.4.4 Patient outcome and tumour NIS and ER expression

Within this cohort tumour high NIS expression was significantly associated with poor outcome. High NIS expression was associated with a significantly shorter breast cancer specific survival, NIS positive tumours (n=10) had 7 events and a mean survival of 74 months (range 42-106 months), compared to low NIS expressing tumours (n=26), with 9 events and a mean survival time of 161 months (range 119-204 months), log rank p=0.040. For breast cancer recurrence, in tumours with high NIS (n=8), there were 7 events and mean time to event was 68 months (range 23-113 months). Low NIS (n=23) had 4 recurrence events and mean time to event was 156 months (range 127-184 months), log rank p=0.002. Kaplan Meier curves are shown in fig 5.14. In multivariate analysis, when combined with tumour size, lymph node status and tumour grade, NIS expression was independently significant for recurrence (HR 5.2, p=0.036).

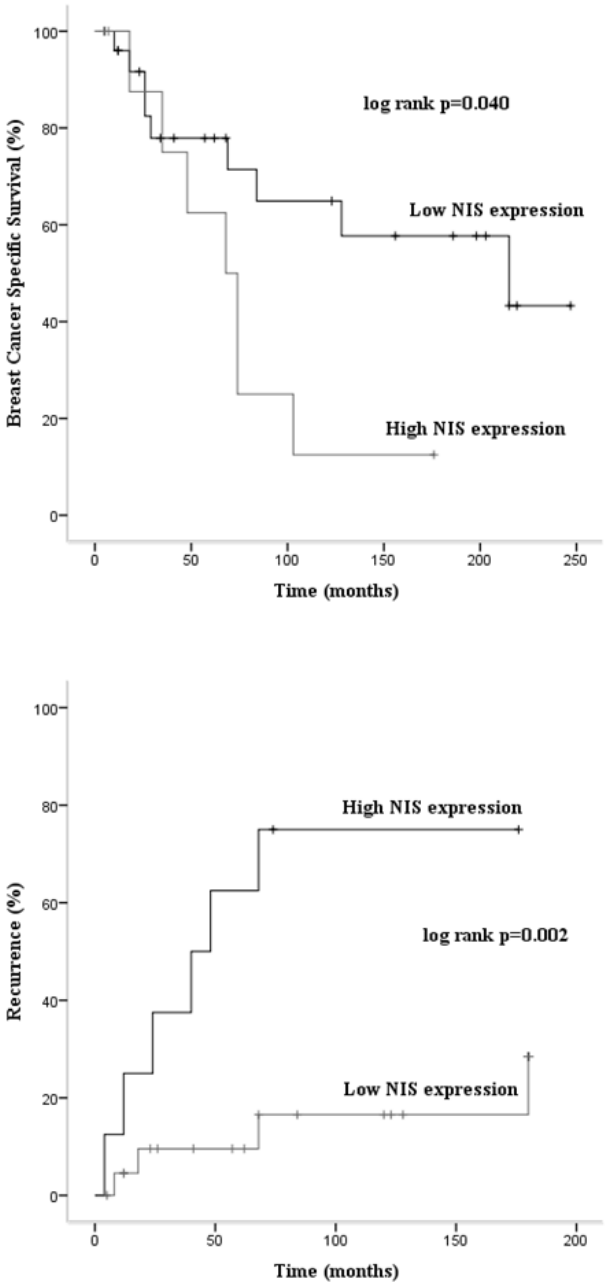


Figure 5-14 Kaplan Meier Survival Curves for Low and High NIS expression in patient Cohort 1

5.4.5 Characteristics of NIS positive tumours

All tumours with positive NIS expression were ER positive (ER >210 copies/10^6 GAPDH). Over 90% were ductal carcinomas and all tumours were grade 2 or 3. There was an equal distribution of tumour size within patients with positive NIS expression, and 72% (n=8) had

lymph node involvement, table 5.6, although no statistically significant correlations existed with tumour size, nodal stage or grade.

	Percent of patients (number of patients)
ER positive (>210 ER copies/10^6GAPDH)	100% (n=11)
Tumour Type	
Ductal	91% (n=10)
Unknown	9.1% (n=1)
Grade	
1	0% (n=0)
2	64% (n=7)
3	36% (n=4)
Size	
<20mm	36% (n=4)
20-50mm	36% (n=4)
>50mm	18% (n=2)
unknown	9% (n=1)
Lymph node Stage	
Negative	28% (n=3)
0-3 nodes+	36% (n=4)
>3nodes+	36% (n=4)

Table 5-6 High NIS expressing breast cancer tumours and distribution of recognised prognostic indices (patient cohort 1)

5.5 Results III NIS expression in cohort of ER positive early breast cancer patients- an Immunohistochemical analysis

5.5.1 Clinical and pathological Characteristics

As it was demonstrated using cohort one that all tumours expressing NIS at the mRNA level were ER+ it was necessary to further probe this relationship at the protein level. This relationship was probed utilising a pre-existing tissue microarray consisting of 300 ER+ early breast cancers. Clinical and pathological characteristics for the cohort of patients with ER+ Early Breast Cancer (n=300) that were analysed for NIS expression are detailed in table 5-7

	Cohort (n=300)
Age	
≤50	51 (17%)
>50	249 (83%)
Nodal Status	
0	140 (47%)
1-3+	83 (28%)
>3	58 (19%)
unknown	19 (6%)
Tumour Size	
<20mm	111 (37%)
20-50mm	164 (55%)
>50	19 (2%)
Tumour Grade	
1	68 (23%)
2	143 (48%)
3	79 (26%)
unknown	10 (3%)
PgR-Allred Score	
Unknown	20 (6%)
0	87 (29%)
3	14 (5%)
4	20 (7%)
5	26 (9%)
6	25(8%)
7	70 (23%)
8	38 (13%)
Herceptest	
unknown	4 (1%)
0	222 (74%)
1	42 (14%)
2	13 (4%)
3	19 (6%)
Chemotherapy	
Yes	81 (27%)
No	219 (27%)
Survival- Mean (range), years	6.4 (0.1-16.7)
Breast Cancer Related Deaths	62 (21%)
Recurrence- Mean (range), years	5.9 (0-16.7)
Recurrence-Any	
No	214 (71%)
Yes	85 (28%)
Duration of Tamoxifen- Mean, years	4.6
Recurrence on Tamoxifen	
No	230 (77%)
Yes	70 (23%)

Table 5-7 Patient and tumour Characteristics- cohort 2 (IHC analysis)

5.5.2 Localisation of NIS in normal breast, thyroid and ER positive breast cancer

In addition to the 300 breast tumours analysed 5 normal breast specimens were also analysed for NIS, normal breast tissue demonstrated weak cytoplasmic staining with some luminal membrane accentuation. Positive NIS staining was demonstrated in human thyroid, fig 5-8. Negative controls, including smooth muscle, normal lung and pancreas demonstrated no staining.

The vast majority of (>95%) ER+ breast cancer specimens demonstrated NIS staining, with more that half (60%) being observed to have cytoplasmic staining only. It was also observed that the cytoplasmic pattern appeared to have large "granules", suggesting that NIS may be associated with an organelle. Nuclear staining was also observed in approximately one quarter (23%) of tumours, this was almost exclusively in association with NIS cytoplasmic staining. Membranous staining was uncommon (6%), only being observed in the luminal membrane of better differentiated tumours fig 5-9. Table 5-6 details the cellular staining and location of NIS in ER positive breast cancer.

Cellular location of NIS in ER+ Breast Cancer tumours	**No of cases (%) (n=300)**
Cytoplasm only	200 (67%)
Cytoplasm and nuclear	69 (23%)
Cytoplasm and luminal membranes	15 (5%)
Nuclear only	12 (4%)
Membranous only (including luminal)	3 (1%)

Table 5-8 Cellular location of NIS in ER+ Breast Cancer Tumours as determined by IHC (cohort 2)

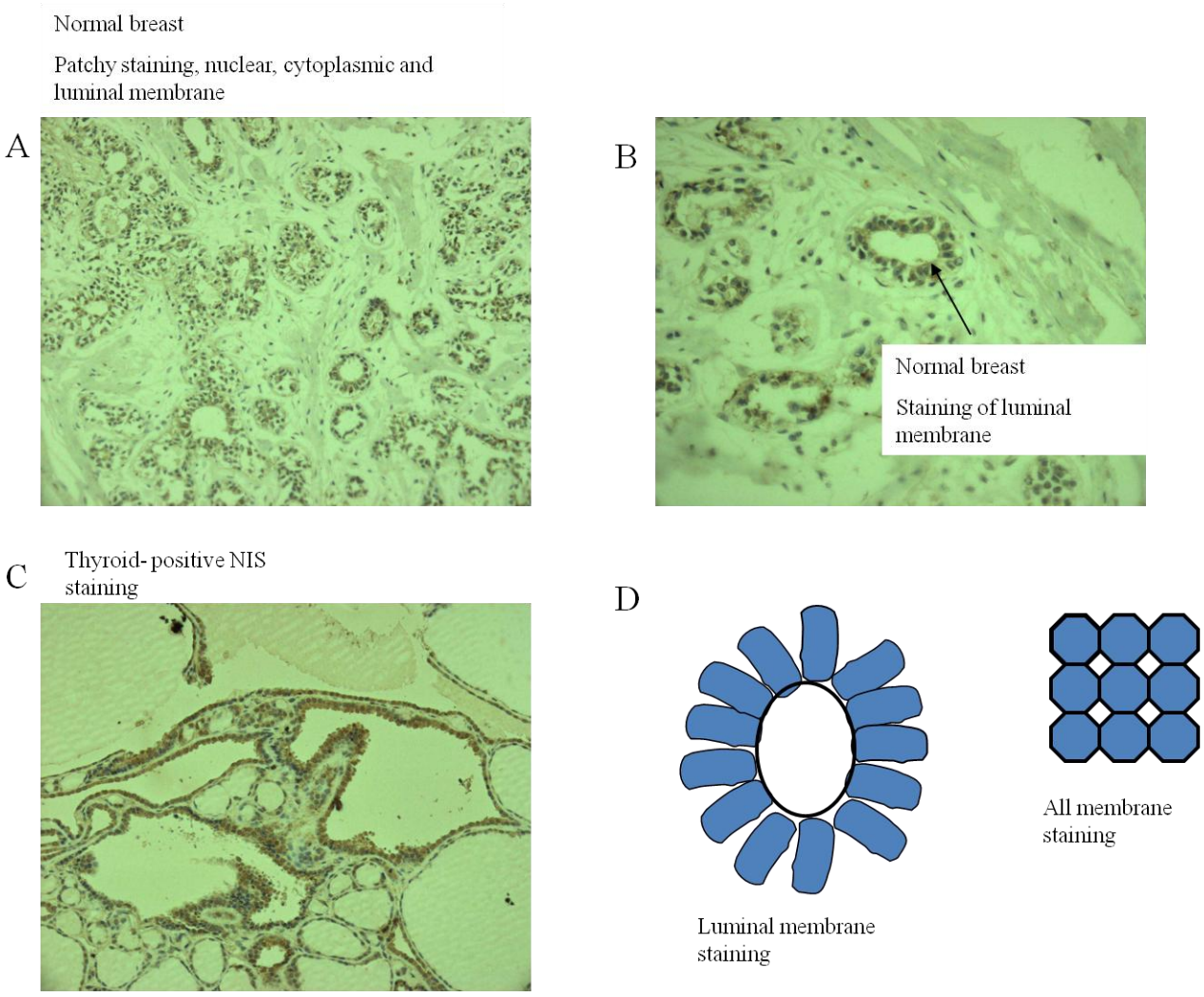


Figure 5-15 Immunohistochemical detection of NIS protein expression in Normal Breast and thyroid

A-B) normal breast specimens C) thyroid (included as a positive control) D) schematic representation of differentiation between luminal membrane and all membrane staining pattern

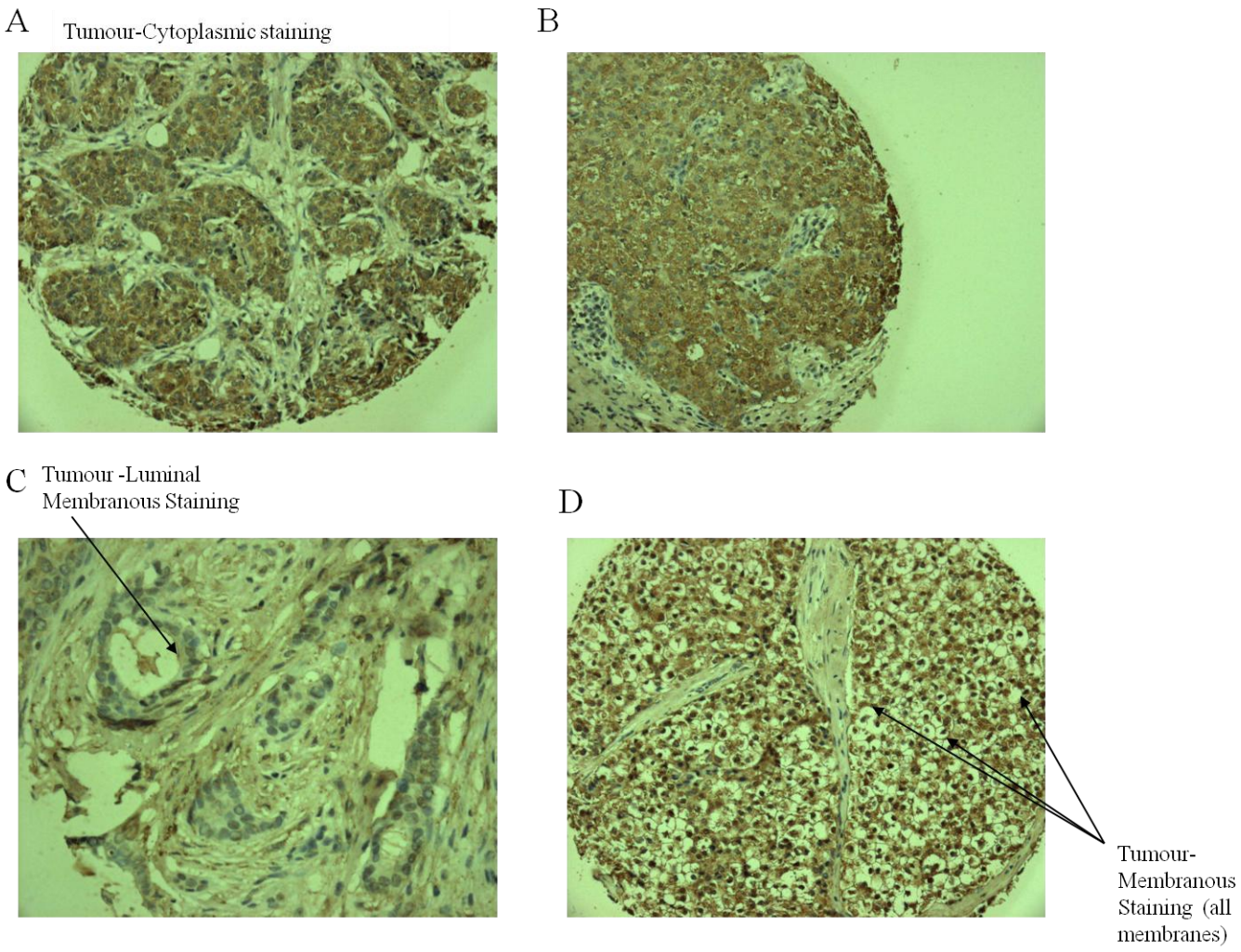


Figure 5-16 Immunohistochemical detection of NIS protein expression in ER+ Breast Cancer Specimens (cohort 2)

NIS staining in breast cancer specimens- A-B) cytoplasmic C) luminal membrane staining and D) all membrane staining

5.5.3 Level of NIS expression

A total of 300 ER positive tumour samples (ER positive defined as a histoscore greater than or equal to 1, which represents ≥1% of tumour cells staining positive) were analysed for NIS, only 2 tumours (<1%) had no NIS expression. The median histoscore of tumours with NIS expression was 107 (interquartile range 50-165). Fig 5-17 demonstrates the histogram of NIS histoscores in ER+ breast cancer. A histoscore of 50 (lowest interquartile score) defined the cut-off for low NIS expression and high NIS expression. There was no statistically

significant correlation between ER expression level and NIS expression level within this cohort.

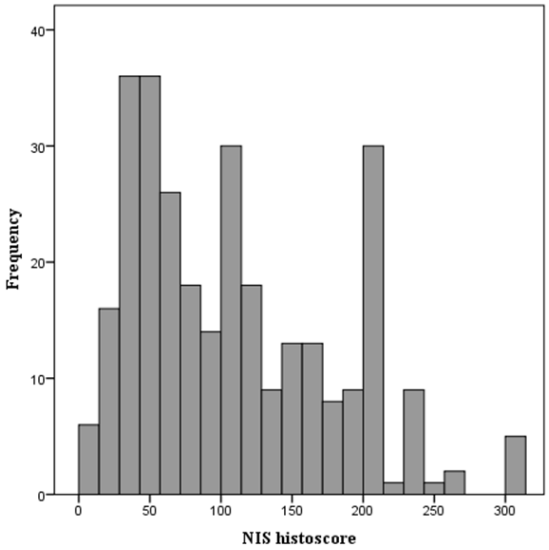


Figure 5-17 Histogram demonstrating the range of NIS histoscores in ER+ breast Cancer (Cohort 2)

5.5.4 NIS Expression Correlates with Signal transduction Pathways

Expression of key proteins involved in the signal transduction pathways PI3K/Akt and Ras/Raf/ MAPK had previously been analysed in this ER positive Tamoxifen treated cohort [178]. The entire cohort of ER positive patients was analysed and NIS was significantly associated with a large number of key proteins, most notably within the Ras/Raf/MAPK pathway, table 5-, demonstrate the Spearman's correlation coefficient and significance. All correlations were of similar value and significance when Pearson's Correlation was performed (data not shown).

Protein	Spearman Correlation Coefficient with NIS	Significance
Ras		
K-Ras cytoplasm	cc =0.192	p =0.001
K-Ras nuclear	cc = 0.147	p=0.013
Raf-1		
Raf cytoplasm	cc=0. 340	$p=1.2 \times 10^{-8}$
Raf nuclear	cc=0.189	p=0.002
^{259} Raf cytoplasm	cc= 0.383	$p=5 \times 10^{-11}$
^{259} Raf nuclear	cc=0.202	p=0.001
^{338} Raf cytoplasm	cc=0.345	$p=7.5 \times 10^{-9}$
^{338} Raf nuclear	cc=0.269	$p=8.4 \times 10^{-6}$
p44/42 MAPK		
MAPK cytoplasm	cc= 0.459	$p=9 \times 10^{-16}$
MAPK nuclear	cc=0.154	p=0.011
MAPK cytoplasm	cc=0.259	$p=1.3 \times 10^{-5}$
MAPK nuclear	cc=0.274	$p=4.1 \times 10^{-6}$

Table 5-9 NIS correlations with members of Ras/Raf/ MAPK pathway

Protein	Spearman Correlation Coefficient with NIS	Significance
Ⓟ^{473}Akt cytoplasm	cc=0.252	$p=1.5 \times 10^{-5}$
Ⓟ^{308}Akt cytoplasm	cc= 0.196	p=0.009
PTEN cytoplasm	cc=0.392	$p=1.4 \times 10^{-11}$
mTOR	cc=0.317	$p=6.9 \times 10^{-8}$

Table 5-10 NIS correlations with members of PI3K/Akt pathway

Protein	Spearman Correlation Coefficient with NIS	Significance
Ⓟ^{118}ER membrane	cc=0.229	$p=6.7 \times 10^{-5}$
Ⓟ^{167}ER membrane	cc=0.242	$p=6.2 \times 10^{-5}$

Table 5-11 NIS correlation with activated ER at the membrane

NIS was found to significantly correlate with K-Ras which is an excellent activator of Raf-1. Both active (Ⓟ^{338}Raf) and inactive (Ⓟ^{259}Raf) were significantly associated with NIS in the cytoplasm. NIS was also significantly associated with p44/42 MAPK in the cytoplasm. Activated p44/42 MAPK phosphorylates the ER at serine 118 and 167 and results in ligand

independant activation of the ER [165-167]. NIS was significantly associated with Ⓟ^{118} ER and Ⓟ^{167} ER at the membrane. These results suggest that in vivo activation of the p44/42 MAPK pathway mediated by Ras/Raf and ligand independant activation of ER may be involved in NIS regulation in ER positive breast cancer. In addition NIS was significantly associated with fully activated Akt (Ⓟ^{473}Akt) and mTOR which is activated by the PI3K/Akt pathway, suggesting that this signal transduction pathway may regulate NIS. Interestingly, NIS was significantly associated with PTEN, an inhibitor of PI3K/Akt.

5.5.5 NIS and patient survival- entire ER+ Cohort

For the entire cohort of ER positive tamoxifen treated patients, high NIS was associated with poor patient outcome, tumours with low NIS expression (n=82) there were 8 events and mean survival time was 12.68 years (range 11.4-14.0) compared to high NIS expression (n=218) in which there were 54 events and mean survival time was 12.67 years (range 11.7-13.6). Although statistically significant, p=0.005, the mean survival times were similar and the Kaplan Meier survival curve has cross-over, fig5-18A.

5.5.6 NIS and patient survival- Subgroup analysis, influence of PgR

In ER positive breast cancer, the progesterone receptor (PgR) represents a functional/intact ER signalling pathway. Loss or down regulation of PgR is associated with impaired response to endocrine therapy and increased biological aggressiveness. There is increasing evidence that complex cell signalling and cross talk between growth factor signalling pathways and the ER (both genomic and non-genomic) contribute to PgR downregulation [59]. Subgroup analysis was performed and NIS expression was analysed in terms of patient outcome in ER+ tumours with negative, low and high PgR expression.

NIS expression in ER+/PgR negative tumours (n=87) was associated with a significant poor outcome. Low NIS expressers (n=27) had 1 event and a mean survival time 13.5 years (range

12.4-14.3 years) compared to high NIS expressers (n=60) in which there was 21 events, and mean survival time was 8.3 years (range 7.3-9.3 years), log rank p=0.008. Kaplan Meier survival curve is shown in fig 5-18B

NIS expression was not associated with outcome in ER+ tumours with high PgR (defined as Allred PgR score ≥6) (n=133). Over 70% (n=95) of ER+/high PgR expressed high NIS in which there were 18 events and mean survival time 11.0 years (range 10.1 -11.9 years), in ER+/high PgR low NIS expressers (n=38) had 7 events and mean survival time was 11.4 years (range 9.3-13.4 years), log rank p=8.16, fig 5-18C. ER+/high PgR tumours with a positive or indeterminate herceptest (≥2+) were excluded (n=19), NIS expression was not associated with outcome in ER+/high PgR/HER2- tumours.

In ER+ tumours with low PgR (defined as Allred PgR score ≤5) (n=147), low NIS expressers (n=40) had 1 event and mean survival time was 13.5years (range 12.9-14.1 years) compared to high NIS expressers (n=101), in which there were 30 events and mean survival time 10years (range 9.1-11.0 years), log rank p=0.003, fig 5-18D. ER+/low PgR tumours with a positive or indeterminate herceptest (≥2+) were excluded (n=15). ER+/low PgR/HER2- tumours with high NIS (n=98) had 26 events, compared to zero events in low NIS expressers (n=34), log rank p=0.002.

5.5.7 NIS and recurrence- Subgroup analysis, influence of PgR

In terms of breast cancer recurrence in ER+/ high PgR tumours NIS expression was not associated with increased risk, although the majority of tumours expressed high NIS. ER+/high PgR with low NIS (n=38) had 9 events, the mean disease free survival (DFS) time was 9.8 years (range 8.4-11.2 years) and ER+/high PgR with high NIS expression (n=95) there was 25 events, mean DFS time was also 9.8 years (range 8.6-11 years).

NIS expression was associated with increased risk of recurrence in ER+/low PgR tumours. Low NIS expressers (n=40) had 3 events, mean DFS time was 10.7 years (range 9.9-11.6) and high NIS expressers (n=106) there were 34 events and mean DFS time was 9.6 years (range 8.5-10.6 years), log rank p=0.007, fig 5-19. Excluding HER2+ cases did not influence the results

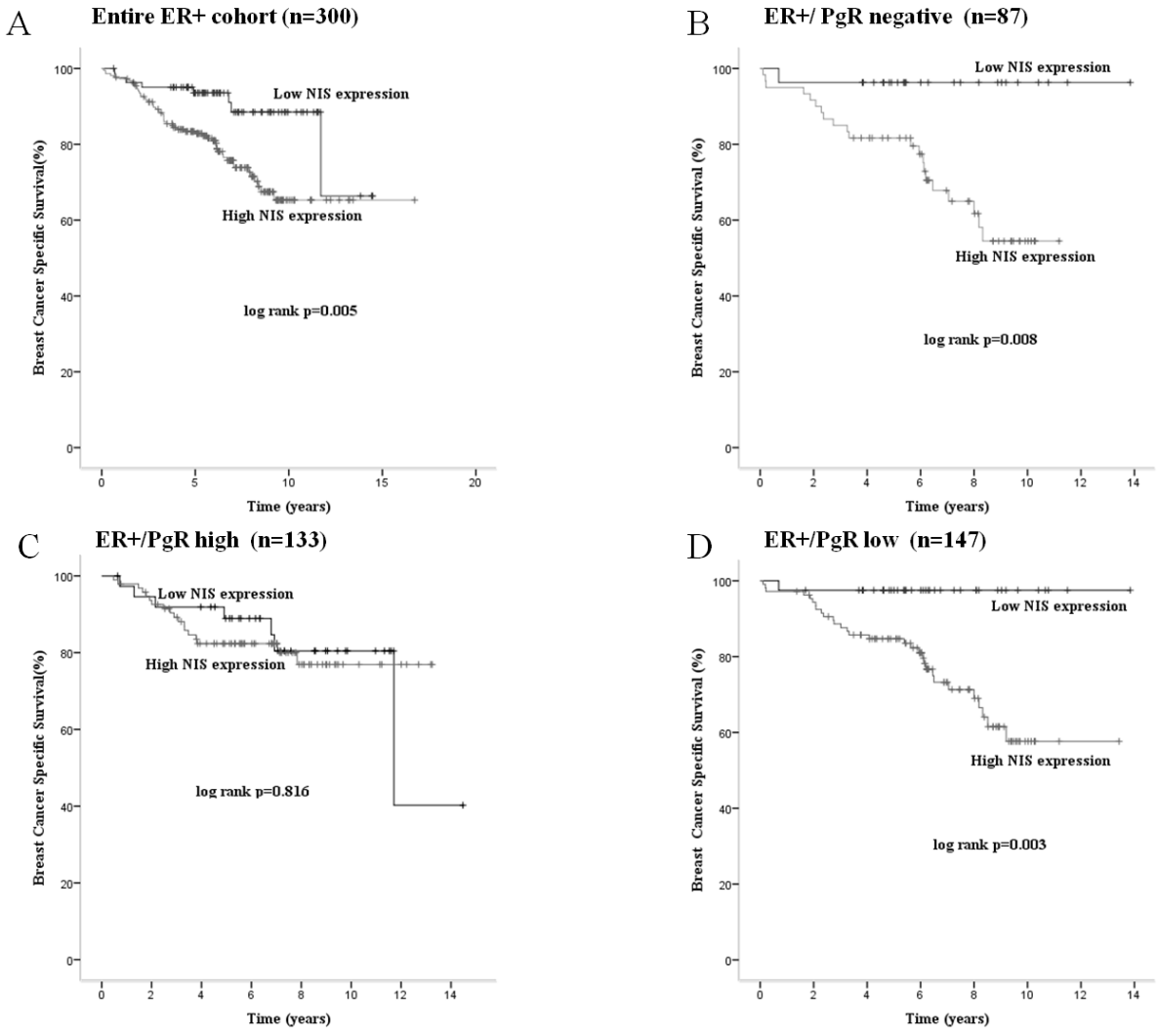


Figure 5-18 High NIS associated with poor breast cancer specific survival

High NIS expression was significantly associated with poor outcome in the entire ER+ cohort (p=0.005) (A), and in ER+/PgR negative (p=0.008) (B). In ER+ tumours with high PgR (defined as Allred score≥6) NIS was not associated with outcome (p=0.816) (C). In ER+ with low PgR (PgR Allred <6), NIS was significantly associated with poor outcome (p=0.003)

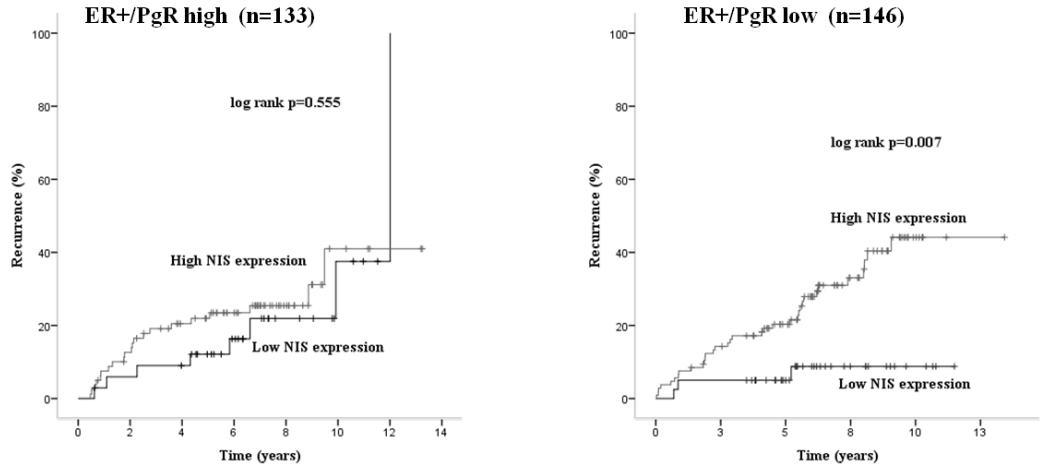


Figure 5-19 NIS expression and recurrence in ER+/PgR low and ER+/PgR high

5.5.8 Factors influencing NIS expression and poor patient outcome

High NIS expression is associated with a significant survival disadvantage and increased risk of recurrence in this cohort of early ER+ breast cancer and this appears to be most significant in ER+ with low PgR IHC scores, a biologically more aggressive subtype with increased likelihood of endocrine resistance. NIS is a transmembrane glycoprotein and not recognised as a protein involved in tumour virulence, in fact in thyroid cancers NIS expression is associated with well differentiated tumours and may be reduced as a result of tumour de-differentiation. Factors associated with high NIS expression influencing poor patient outcome were sought.

Influence of recognised prognostic indices

The outcome disadvantage associated with high NIS expression was most significant in ER+/low PgR group. Recognised prognostic indices were compared for low and high NIS in ER+/low PgR tumours, table 5-12. High NIS expression was significantly associated with nodal stage (p=0.012), grade (p=0.015) and tumour size (p=0.005). ER+/low PgR with low NIS had a higher frequency of invasive lobular carcinoma type.

Prognostic Factor	ER+/low PgR- Low NIS expression (n=40)	ER+/low PgR- High NIS expression (n=107)
Tumour Type		
Ductal	31 (77.5%)	95 (89%)
Lobular	5 (12.5%)	5 (5%)
Other special type	4 (10%)	7 (6%)
Nodal stage		
Negative	23 (62.5%)	45 (42%)
1-3+	8 (20%)	29 (27%)
>3+	6 (15%)	23 (21%)
unknown	1 (2.5%)	10 (9%)
Grade		
1	14 (35%)	22 (21%)
2	21 (52%)	48 (45%)
3	5 (12.5%)	35 (32%)
Tumour Size		
<20mm	19 (47.5%)	37 (34%)
20-50mm	18 (45%)	63 (59%)
>50mm	2 (5%)	4 (4%)
Unknown	1 (2.5%)	3 (3%)

Table 5-12 Distribution of recognised prognostic factors in ER+/low PgR cohort with high and low NIS

5.5.9 Changes in cell signalling protein expression associated with NIS expression

Within this cohort of ER+ breast cancer, significant correlations between NIS expression and p44/42 MAPK pathway members and members of PI3K/AKT pathway have been identified. We have also found that NIS is associated with poor outcome, most significant in ER+/low PgR. These results suggest that enhanced non-genomic ER signalling may be contributing to NIS regulation.

Alterations in the expression level of proteins involved in the p44/42 MAPK and PI3k/AKT pathways between ER+ tumours with high and low NIS expression were examined using the Kruskal-Wallis test. Five key proteins (Raf, p44/42MAPK, AKT, PTEN and mTOR) were identified to have statistically significant increased expression associated with high NIS expression, fig 5-20.

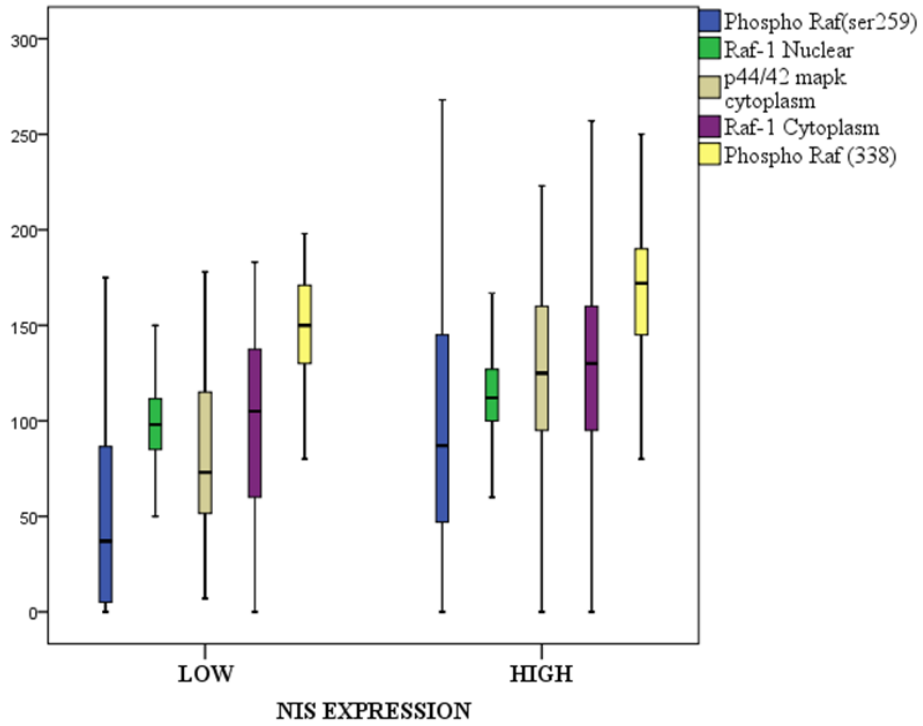
p44/42 MAPK pathway Expression

Tumours with high NIS expression had a statistically higher expression levels of Raf-1, both nuclear and cytoplasmic ($p=0.001$ and $p=0.005$ respectively), cytoplamic ^{229}Raf ($p<0.001$) activated ^{338}Raf ($p<0.001$) and p44/42 MAPK ($p<0.001$) compared to tumours with low NIS expression.

PI3K/Akt Pathway Expression

Tumours with high NIS expression had a statistically higher expression levels of activated cytoplasmic Ⓟ^{473} Akt ($p=0.024$), nuclear and cytoplasmic PTEN (a PI3K/Akt inhibitor) ($p=0.002$ and $p=0.000$ respectively) and mTOR ($p=0.019$) compared to tumours with low NIS. In the ER+/low PgR group, differences in Ⓟ^{473} Akt expression between low and high NIS expressing tumours were more marked than the entire cohort, with high NIS expressing tumours having higher Ⓟ^{473} Akt expression ($p=0.009$) compared to low NIS.

P44/42 MAPK pathway



PI3K/Akt pathway

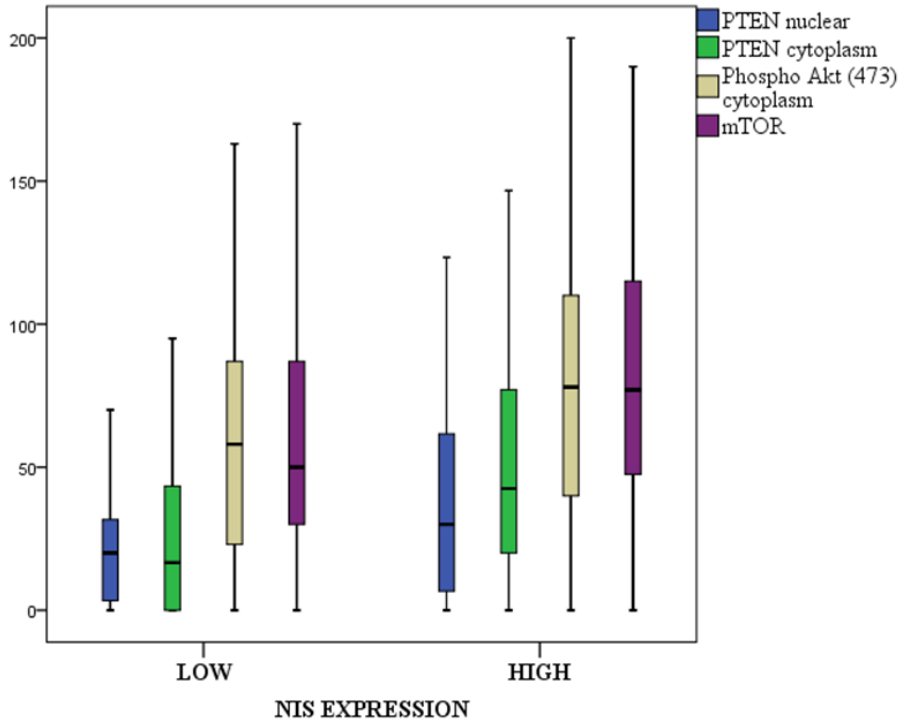


Figure 5-20 Tumours with high NIS have significantly higher levels of protein expression involved in MAPK and PI3K/Akt pathway.

5.6 Discussion

NIS expression in breast cancer is a potentially exploitable therapeutic and diagnostic target. A major obstacle however is lack of NIS function despite its detectable presence in the majority of tumour cells. Uncovering the regulation of NIS in breast cancer may identify key regulatory processes involved in both its expression and function and enable strategies to be developed for the utilisation of radioiodide. In this study based on previous, predominantly in vitro reports regarding NIS regulation and iodide uptake in breast cancer, the role of the ER in NIS expression and function was investigated.

In this study cDNA encoding the human NIS (hNIS) protein was transfected into ER positive and ER negative breast cancer cell lines using the eukaryotic expression vector pcDNA3, under the control of the CMV promoter. Increased hNIS gene expression was confirmed in all hNIS transfected cell lines yet only ER positive cell lines (MCF7 and T47D) demonstrated functional uptake. The finding that only ER positive cell lines can functionally uptake radioiodide following induction of NIS expression is well established in breast cells treated with retinoids. Retinoids, including retinoic acid (RA) and its isomers tRA, 9-cis RA and 13-cis RA are robust inducers of NIS expression and function in breast cancer cell line studies, although this is confined to ER+ cell lines [156-158]. RA- responsive NIS expression was found to be correlated with the presence of a functional ER (using pS2 as a reporter gene of ER function) [159]. Retinoic acid receptors (RAR) are type II nuclear hormone receptors and like the ER, a type I nuclear hormone receptor, has both genomic and non genomic mechanism of action. The primary role of both the RAR and ER is as transcription factors, directly regulating gene expression. Hua et al [179] using chromatin immunoprecipation and expression analysis demonstrated that RAR binding throughout the genome is highly coincident with ER binding, resulting in widespread crosstalk of RAR and the ER, the relationship between the receptors appears antagonistic with competitive binding at or near

overlapping cis-regulatory elements, it is possible that in vitro studies in which RA induced functional NIS expression is limited to only ER+ cell lines may be an effect of the relationship between RAR and ER and the importance of the ER in NIS regulation may be confined to RA induced NIS expression. In this study functional NIS expression although confined to ER+ cell lines was induced by transfection with a hNIS containing plasmid transfection, without RA treatment, suggesting that the ER (or ER signalling) independently is important for NIS function and not just in concert with RA stimulation. In addition, as both ER+ and ER- cell lines expressed NIS mRNA, it suggests that cellular interactions or modifications pertinent to the hormonal status of the cell at the post transcriptional stage are involved.

NIS gene transfers have been successfully performed in a variety of breast cancer tumour models, both in vitro and in animal models by either plasmid- mediated transfection [180] or virus mediated gene delivery [181-183]. Dwyer et al [181] infected T47D and MDA-MB 231 cells with a hNIS containing replication deficient virus under the control of the MUC-1 promoter, infected T47D but not, infected ER negative MDA-MB 231demonstrated functional iodide uptake, although this may be because of variable endogenous MUC-1 expression in the cell lines analysed [184]. Montiel-Equihua et al [182] infected ER+ cells: ZR75-1, MCF7 and ER- cells: MB-435, SKBR3 with a novel replication incompetent virus, AdSERE, in which an oestrogen-responsive promoter directed the expression of hNIS. In addition an adenovirus containing hNIS under the control of a CMV promoter was infected (AdCMV-hNIS). AdSERE failed to induce NIS mediated uptake in ER negative cells, however in contrast to this current study, all cell lines infected with AdCMV-hNIS could accumulate radioiodide above the baseline. MB-435 and SKBR3 are both ER-/PgR-/HER2+ cell lines [185], in contrast to MDA-MB 231 and MDA-MB 453 which are triple negative breast cancer cell lines suggesting that growth factor receptor (HER2) signalling may also be

important in NIS regulation. In addition, Ryan et al [186] recently reported RA induced NIS expression in the ER negative cell line SK-Br-3, although a functional assay was not performed.

Alotaibi et al [159] previously reported that silencing of the ER resulted in decrease levels of both basal NIS expression and RA induced NIS expression (function was not assessed) in MCF7 cells using a semi-quantitative assay for NIS expression. In this study knockdown of the ER in ER+/hNIS transfected cell lines did not influence NIS expression or function. These results suggest firstly that the level of ER expression does not influence NIS function. Secondly, compared to ER negative cells transfected with hNIS that fail to actively accumulate iodide, in ER+ /hNIS transfected cells, ER knockdown can accumulate iodide. Suggesting that the ER cell phenotype, rather than the ER *per se*, is important to NIS function. Perhaps growth factor signalling pathways that have bidirectional crosstalk with the ER and that have been demonstrated to have a role in NIS regulation [164, 168] may be key regulators of NIS in ER+ cells and ER knock down may enhance their signalling through crosstalk.

To further investigate the relationship between the ER and NIS in vivo two separate breast cancer patient cohorts were analysed for NIS and ER expression. The first cohort of patients included patients with ER+ and ER- breast cancer. ER and NIS expression was analysed in 50 frozen tumour samples using quantitative real time RT-PCR , importantly a significant correlation between NIS and ER expression was identified (cc. 0.642, p=<0.001), supporting a role for the ER in NIS regulation in vivo. Ryan et al[186] recently reported a significant correlation between the ER and NIS expression using similar methods in breast cancer specimens, fibroadenomas and normal tissue. Interestingly the linear relationship between ER and NIS was highest in ER+/HER2 positive breast cancers, supporting the role of growth factor receptor signalling and downstream cell pathway activation in ER+ tumours expressing

NIS. In this present study 78% of tumour samples analysed had detectable NIS expression, although levels of NIS expression were very low, the majority (60%) had less than 10 copies of NIS/ one million copies of housekeeping gene GAPDH, the levels of NIS expression detected in patient tumour samples were much lower than the NIS expression levels recorded for our hNIS- transfected cell lines. For outcome analysis a cut-off was selected to define high NIS expression, applying this only 22% of patient samples had significant expression of NIS and all tumours with high NIS expression were ER positive. Although NIS was expressed at much lower amounts in ER negative tumours in the absence of functional assay or an assay to detect protein presence it can't be assumed that very low levels of mRNA expression are not relevant within the tumour cell. Quantitative real time RT PCR is a relatively new technique not routinely performed, therefore standardising assay methodology between studies and establishing thresholds to define significant levels of expression correlated to protein functional studies and protein detection will be required to uniform results and aide interpretation between independent studies. Importantly, outcome analysis in this cohort of 50 breast cancer patients demonstrated that high NIS expression was associated with poor patient outcome. High NIS expression had significantly shorter mean survival time and time to recurrence. NIS was independently significant in multivariate analysis. Suggesting that in breast cancer high NIS expression is a marker of poor outcome.

In the second patient cohort composed of 300 tumours of early ER+ breast cancer patients NIS expression was analysed by semi-quantitative IHC. The advantage of IHC is that protein cellular location can be recorded. Initial reports suggested that NIS expression was confined to malignant breast specimens [149], however in this current study normal breast tissue demonstrated some weak patchy staining. Low levels of NIS expression has been reported in normal breast using quantitative RT-PCR[186] and IHC [152], although studies including our own are limited by small number of normal specimens analysed. This potentially will be an

obstacle, as the principles behind the potential exploitation of NIS in breast cancer are based on the selective endogenous expression in breast cancer tumours, future studies will benefit from analysing larger number of normal breast tissue and elucidation of regulatory mechanisms in normal breast. In keeping with previous reports[149, 152] we observed the majority of breast cancer tumours to have detectable NIS protein expression, in fact in this cohort of ER+ breast cancer 95% of tumours analysed had detectable NIS protein. We also report a high incidence of intracellular NIS with only 6% of tumours having detectable staining at the luminal membranes, this is lower than previous reports [175]. The specificity of NIS antibodies and their cross reactivity has been suggested to contribute to the high incidence of intracellular NIS detected by IHC [175, 187], useful strategies were suggested to enable confirmation of specificity of antibody[175] which were adhered to in this current study, although a different antibody detecting a separate epitope of NIS was not studied. The prevalent view, however is that the high incidence of intracellular NIS expression is due to cell surface trafficking impairments and this also accounts for lack of functional activity of NIS. Knostman et al [164]have reported that PI3K activation in MCF7 cells results in expression of underglycosylated NIS lacking cell surface trafficking necessary for iodide uptake and other in vitro studies have indicated the p38 MAPK pathway in NIS regulation. We report a significant correlation between NIS expression and protein expression involved in signal transduction pathways PI3K/Akt and p44/42 MAPK, supporting their role in NIS regulation. In addition we report significant alterations in key pathway member expression levels between breast tumours with low and high NIS expression, tumours with high NIS had increased levels of signal transduction pathways members further supporting their role in NIS regulation in ER+ breast cancer.

Gene expression profiling and the identification of the molecular intrinsic subtypes has brought to the fore that breast cancer includes a number of phenotypically distinct disease

processes and that within ER+ cancer is a heterogeneous group of diseases. The ER signalling pathway is a complex network with many levels of control including extensive crosstalk with growth factor signalling and cell signalling pathways. PgR expression represents a functionally intact ER signalling pathway and evidence suggests that down regulation of PgR results from enhanced non-genomic ER signalling involving cross talk with growth factor receptor and downstream signalling cascades[46]. It has been postulated that ER+/PgR- tumours may represent a distinct tumour phenotype of its own [45]. We report that increased NIS expression is associated with poor patient outcome in ER+ tumours with low or absent PgR expression however NIS was not associated with outcome in ER+ tumours with high levels of PgR. Together with the correlations identified between p44/42 MAPK pathway and PI3K/Akt pathway, these results strongly suggest that non-genomic ER signalling is likely important in NIS regulation in ER+ breast cancer. Our in vitro work suggested that the ER *per se*, may be not be essential to induction of NIS expression and function. Renier et al [188] recently examined NIS expression using IHC in triple negative breast cancer specimens (ER-/PgR-/HER2-) and reported NIS expression in 65% of cases (15 out of 23 tumour specimens analysed), they reported functional NIS uptake as demonstrated using scintigraphy methods in one tumour. Ryan et al [186] categorised tumours analysed for NIS expression by the epithelial subtype and reported NIS expression in luminal A, B, HER2 and basal types. It is likely that NIS expression is not 'exclusive' to ER+ breast cancer, and activation of growth factor receptor signalling and cell signalling pathways important in tumour progression and aggression are the key regulators. It is possible that between the molecular instrinsic subtypes NIS regulation will differ, therefore examining NIS expression in relation to the subtype and examining alterations in key cell signalling pathway expression pertinent to subtype virulence will be important to gain more insight into the regulatory pathways involved in NIS expression. Importantly we have demonstrated in two separate

patient cohorts that NIS expression is associated with poor outcome, it is unlikely that NIS is an oncogenic protein given its normal function, we hypothesise that enhanced NIS expression is an 'effect' of enhanced cell signalling pathway activation involved in tumour aggression, and may serve as a useful marker of aggression and in ER+ breast cancer it is a marker of upregulation of non-genomic ER signalling particularly p44/42 MAPK and PI3K/Akt signalling.

In conclusion, this study was undertaken to probe the relationship between the ER and NIS expression and function. Our results support that the ER is important for NIS expression and function and suggest that NIS regulation in ER+ breast cancer is regulated by cell signalling pathways resulting from non-genomic activation of the ER.

6 Src kinase in ER+ Breast Cancer: a pilot study for novel therapeutic targets

6.1 Introduction

Adjuvant hormone therapy results in substantial improvements in disease free and overall survival for woman with early hormone receptor positive breast cancer[69]. Despite these benefits, a substantial proportion of patients will develop de novo or acquired resistance to hormone therapy and this presents a significant clinical problem.

The precise molecular mechanisms involved in breast cancer cell resistance to endocrine therapy is poorly understood but there is strong evidence suggesting it involves crosstalk between the ER, growth factor receptors and other downstream cellular signalling pathways [189] resulting in ligand-independent activation of the ER and tumour cell growth. Indeed, we previously demonstrated that HER 1-3 expression is significantly associated with early relapse in an ER+, tamoxifen treated cohort [172]. Evidence is now emerging that endocrine resistance not only results in oestrogen independent growth but is also associated with altered cell-cell and cell-matrix adhesive interactions, promoting a more invasive phenotype[190].

c-Src non receptor tyrosine kinase is over expressed and activated in a large number of human malignancies and the relationship between activation and cancer progression appears significant[191] . The precise mechanism of its action has not been fully elucidated, but c-Src is known to interact with a diverse array of molecules, including growth factor receptors and cell-cell adhesion receptors, integrins and steroid receptors including the ER[192, 193] promoting tumour cell proliferation, survival, differentiation, migration and invasion[194, 195]. Recent in vitro studies have demonstrated the over expression and over activity of Src during the acquisition of tamoxifen resistance in ER positive cell lines [190, 196]. Src

inhibition was seen to significantly reduce the invasive behaviour of these cells. In addition, inhibition of c-Src has been shown to reduce the incidence of breast cancer metastases and increases survival in mice. Progress in knowledge of c-Src in tumour genesis and has resulted in Src Kinase inhibition being investigated as a therapeutic target for anti invasive therapies in breast cancer [197, 198].

This study, using a large cohort of ER+ tamoxifen treated patients, was undertaken to examine if c-Src expression is involved in de novo resistance to tamoxifen treatment. We examine the role of c-Src expression in human ER+ breast cancers, to determine if in vivo c-Src expression, activation or cellular location is associated with response to tamoxifen therapy and patient survival.

6.2 Material & Methods

6.2.1 Patients and tissues

The local ethics committee granted ethical approval for this study to utilise a database that details the outcome of ER positive patients diagnosed with primary operable breast cancer between 1980 and 1999 treated with adjuvant tamoxifen. Within this cohort all patients received adjuvant tamoxifen (mean time 4.8 years), 26 % of patients received adjuvant chemotherapy and 18% received adjuvant radiotherapy. Formalin fixed paraffin embedded tissue, taken at time of surgical resection, was used for tissue microarray (TMA) construction, as described previously [172].

6.2.2 Immunohistochemistry

Immunohistochemistry was performed on 10 normal breast sections and 10 prostate cancer samples, in addition to the 262 ER positive breast cancer specimens. Full activation of c-Src requires phosphorylation at tyrosine (Tyr) 419 in addition to the absence of phosphorylation at tyrosine 519. A phosphospecific antibody (Cell Signalling, Technology) raised in rabbit to

phosphorylated Y416, SrcpY^{416} which corresponds to human Tyr 419 was used, as described in the literature[196] . In addition an antibody recognising Total Src (36D10, Cell Signalling Technology) was used. Prior to performing IHC, antibody specificity was confirmed by western blotting (figure 6-1). As expected, activated c-Src, SrcpY^{416}, was detected as a single 60kDa band and decreased in response to the Src kinase inhibitor dasatinib. Titration of the optimal antibody dilution was undertaken in breast tumour specimens prior to the procedure.

Tissue sections were dewaxed and rehydrated through graded alcohols and then subject to heat induced antigen retrieval by pressure steaming in preheated 10mM citrate buffer for 5 mins. Immunostaining was then performed; sections were first treated with hydrogen peroxide and then blocked using horse serum, followed by incubation in primary antibody (1: 50 dilution, SrcpY^{416} overnight) (1: 200 for Src36D10, 1 hour). DakoCytomation EnVision was applied for 30 mins and sections incubated with DAB (1:50 dilution). Finally, sections were counterstained, dehydrated and mounted. Positive and negative (isotype matched antibody) control slides were incorporated in each run.

Tissue staining intensity was scored blind by 2 independent observers using a weighted histoscore method [176] also known as the Hscore system[177]. Histoscores were calculated from the sum of (1 x % cells staining weakly positive) + (2 x % cell staining moderately positive) + (3 x % cells staining strongly positive) with a maximum of 300. Each cellular location was separately assessed with a weighted histoscore assigned to any membrane, cytoplasm and nucleus staining. The histoscores for each core were then averaged. Where one core was missing the remaining core(s) scores were used. To determine high and low expression the median value for all scores was used. The inter-class correlation coefficient (ICCC) for each protein was calculated to confirm consistency between observers.

6.2.3 Western blot analysis

MCF-7 cells treated with varying concentrations of dasatinib were lysed in RIPA buffer (50 mM Tris pH7.6, 150 mM sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 1 mM sodium ortho-vanadate and 1:100 Calbiochem protease inhibitor cocktail set 1) and centrifuged at 12 000 rpm for 10 min, the supernatant removed and protein concentration determined using BCA/$CuSO_4$ assay. 40 µg of protein per well was resolved by 4-12% gradient Bis-Tris gel electrophoresis (Invitrogen, UK); proteins were transferred to nitrocellulose membranes (Millipore, UK), which were blocked for 1 hour in 5% BSA and probed with primary antibodies: anti-phospho $SrcY^{419}$ (1:10000) and anti-total Src (1:10000 Cell Signaling Technologies, UK) at 4^0C overnight. Membranes were then incubated with secondary antibodies (anti-rabbit 1:5000 or anti-mouse 1:5000, Cell Signalling Technologies) and visualized with ECL kit (Amersham, UK). Where necessary, the membranes were stripped by incubating with Re-Blot Plus stripping buffer (Chemicon, UK) before re-probing with other antibodies including anti-αTubulin (1:8000 Santa Cruz, USA) to confirm equal protein loading.

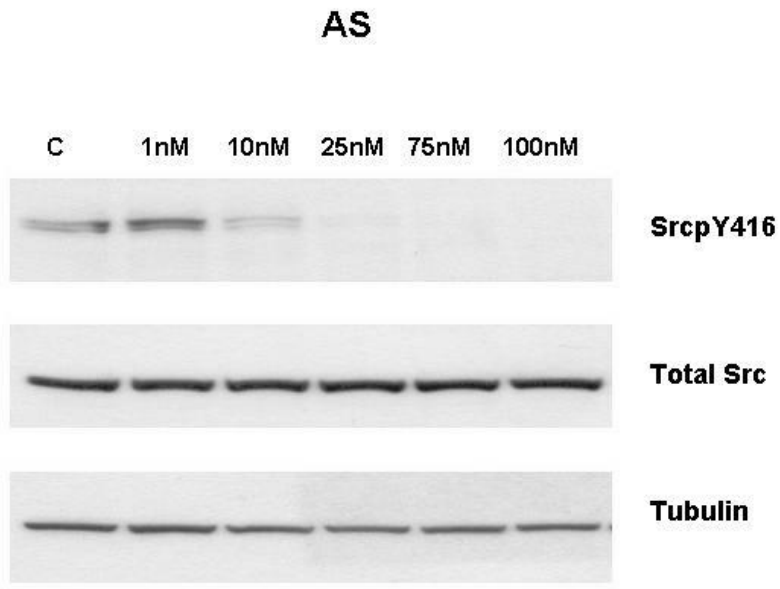


Figure 6-1 Western Blot

Phosphospecific antibody recognising activated c-Src ($SrcpY^{416}$) is demonstrated as a single 60kDa band (lane 1- control, C). In addition phosphorylation of c-Src was observed to fall following treatment with increasing concentrations of the Src kinase inhibitor dasatinib (lanes2-6) and total Src were not affected by this. Tubulin was used as a control

6.2.4 Statistical analysis

The statistical software package SPSS version 11.5 was used for all analysis. Interclass correlation coefficient was employed to confirm consistency between observers. Protein expression data were not normally distributed and are shown as median and inter quartile ranges. Pearson Chi Square test was employed to assess association between staining intensity and known clinical parameters and survival analyses were conducted using Kaplan-Meier method, curves were compared with the log-rank test. Hazard ratios (HR) were calculated using Cox Regression analysis.

6.3 Results

6.3.1 Clinical & pathological characteristics

Clinical and pathological characteristics for all patients (n=262), including age, grade, nodal status, histology, size and Nottingham Prognostic Index are detailed in Table 6-1. The mean duration of tamoxifen therapy was 4.82 years. 55 patients (21%) had breast cancer specific deaths, 77 patients (29.4%) had breast cancer relapse, 60 of these patients while receiving tamoxifen therapy.

	Number	Valid %
Grade		
1	60	23%
2	124	48%
3	73	28%
unknown	5	
Nodal Status		
0	128	53%
1-3	72	30%
4+	40	17%
unknown	22	
Histological type		
ductal	218	83%
lobular	20	8%
other	24	9%
Size (mm)		
T1(<20)	89	36.3%
T2 (20-50)	149	59%
T3 (>50)	14	6%
unknown	10	
NPI		
<3.5	66	35.5%
3.5-5.5	66	35.5%
>5.5	94	50.5%
unknown	16	14
Age (years)		
</=50	42	16%
50+	220	84%
Chemotherapy		
yes	68	26%
no	194	74%
PgR status		
PgR+	165	63%
PgR-	93	35%
unknown	4	1.5%

Table 6-1: Patient and tumour characteristics

Note: Grade= Bloom and Richardson grade. Nodal status= number of positive nodes, Histological type: ductal, invasive ductal carcinoma; lobular, invasive lobular carcinoma; other includes mucinous, mucoid etc.

Abbreviation: NPI, Nottingham Prognostic Index (grade+ nodal status+ 0.02x size in mm)

6.3.2 Localisation of total Src and activated c-Src

Localisation of Total Src and activated c-Src in normal breast

Ten normal breast sections were stained for total Src and activated c-Src. Low expression of total Src was observed in the cytoplasm of 60% and nucleus of 40%, however no activated c-Src expression was observed at any location.

Localisation of activated c-Src expression in ER positive breast cancer tissue

A total of 262 ER positive tumour samples were analysed for activated c-Src expression. 57.3% (150/262) of tumours expressed activated Src in the cytoplasm; median histoscore 20 (interquartile range, IQ 0-61.5). 58.4% (153/262) of tumours expressed activated c-Src in the nucleus; median histoscore 10 (interquartile range 0-45). High levels (greater than the median value) of activated c-Src expression in the cytoplasm or nucleus was therefore detectable in over 50% (n=153) of all ER positive breast tumours analysed. 2.7% (7/260, 2 samples missing) of tumours expressed activated Src in the membrane, median histoscore 0. Due to the low rate of membrane expression observed it was not deemed appropriate to apply these results to further statistical tests. In order to confirm that the antibody was able to detect membrane staining 10 prostate tumours were also stained for activated c-Src. Activated c-Src was much more commonly located to the cell membrane of prostate cells compared to the ER positive breast carcinomas. Figure 6-2 illustrates the staining patterns observed in the ER positive breast cancer specimens compared to prostate cancer specimens.

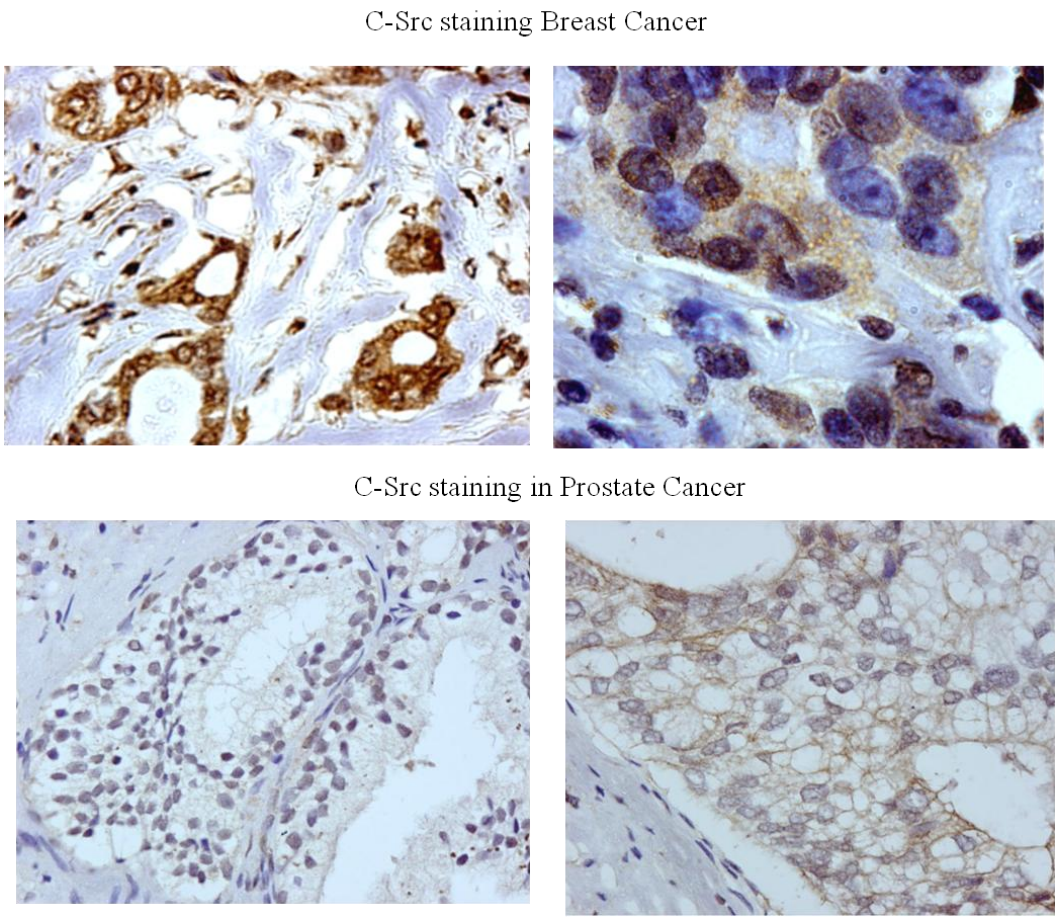


Figure 6-2 Immunohistochemical staining patterns in Breast and Prostate Cancer

The different localisations of activated c-Src, SrcpY^{416} in prostate and breast tumour samples. In breast tumours activated c-Src was most commonly present in the cell cytoplasm and cell nucleus. In the prostate cancer the majority of staining observed for phosphorylated c-Src was located to the membrane and nuclear expression was rarely observed.

6.3.3 Activated c-Src and patient outcome

High expression level (above the median value) of activated c-Src within the nucleus of tumour cells was significantly associated with improved overall survival (p=0.047)

and decreased recurrence in tamoxifen treated patients (p=0.02), figure 6-3A-B. On Cox regression analysis this was not demonstrated to be independent for survival or recurrence. The location of activated c-Src around the nucleus was also significant, tumours with uniform staining had improved outcome in comparison to patients with only perinuclear staining

(figure 6-3C, p=0.0153). Activated c-Src within the cell cytoplasm was not significantly associated with patient outcome.

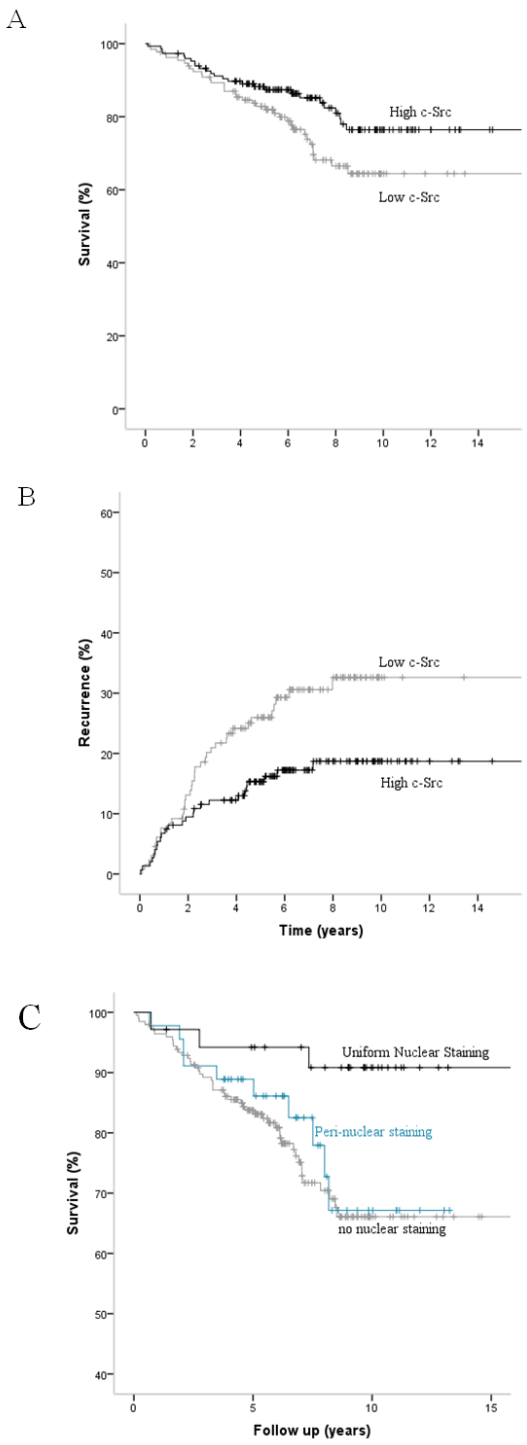


Figure 6-3 c-Src and patient Survival

(A) Overall survival difference between ER+ patients with high and low expression of activated nuclear c-Src. p=0.047; B) disease recurrence in ER+ patients with high and low expression of activated nuclear c-Src. p=0.02; C) Overall survival differences between activated c-Src depending on pattern of nuclear staining. Uniform nuclear staining was significantly associated with improved survival compared to no nuclear staining or only perinuclear. p=0.0153.

6.3.4 Activated c-Src and prognostic indices

Activated c-Src within the nucleus was associated with node negativity and low NPI (Pearson-Chi Square, p=0.03 and p=0.046 respectively). Activated c-Src within the cytoplasm of cells was not associated with nodal status, NPI, tumour grade or size. No significant correlation was found with Ki67 (proliferative index). In contrast when the cohort was subdivided by Progesterone receptor (PgR) status (histoscore >10), activate c-Src nuclear expression remained highly significant in the ER+ /PgR + subgroup (n=165, p=0.004). However in the ER+/ PgR negative subgroup significance was lost (n=93, p=0.56). PgR status was not available for 4 tumours from our cohort of 262 patients. The cohort was not stratified for HER2 status as only 4 tumours were found to be positive using the Herceptest.

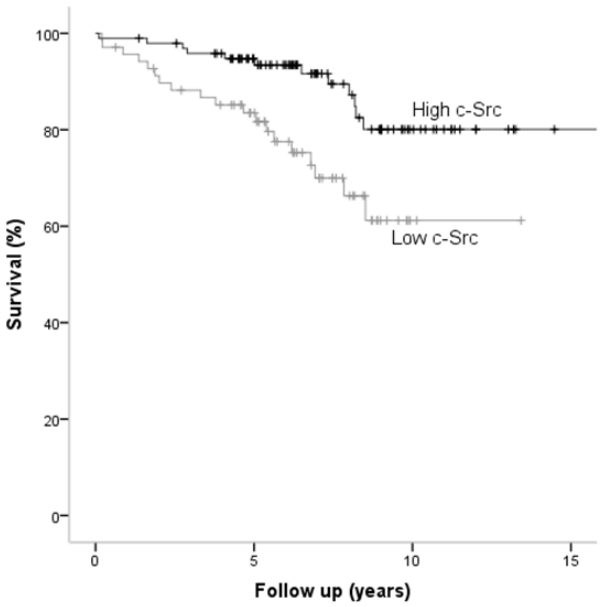


Figure 6-4 c-Src and survival in ER+/PgR+ breast cancer

6.3.5 Total Src expression in ER positive breast cancer

Of the 262 patients only 231 tumour samples were scored for total Src expression. 95.8% (220/231) of tumours expressed total Src in the cytoplasm, median histoscore 97

(interquartile range 40-150). 70.6% (153/230) of tumours expressed total Src in the cell membrane, median histoscore 26 (interquartile range 0-95). No total Src was seen within the cell nucleus. Total Src expression (at any location) was not significantly associated with any clinical parameters or patient outcome.

6.4 Discussion

Although cell line studies strongly support the role of c-Src in endocrine resistant breast cancer progression, translational studies investigating human breast tumour expression, activation and correlation with clinical parameters are surprisingly limited. Using a large cohort of ER positive breast cancer patients treated with adjuvant tamoxifen we have shown that high levels of activated c-Src are present in over 50% of tumour specimens and we also demonstrate that nuclear c-src activation is significantly associated with improved overall and disease free survival. Subgroup analysis demonstrates that this benefit is only seen in ER+/PgR+ patients and not within ER+/PgR negative group.

As c-Src is a non receptor tyrosine kinase that is localized to the intracellular membranes and cytoplasm of the cell[199] it was surprisingly that in the current study we rarely observed activated c-Src in the cell membrane. However antibody specificity was confirmed by western blotting. A single 60kDa band suggesting that phosphorylated Src kinase was detected. In addition, phosphorylation of c-Src (but not total c-Src) was observed to fall following treatment with increasing concentrations of the Src kinase inhibitor dasatinib confirming that the antibody detected phosphorylated Src only (figure 6-1). Although these experiments confirmed that the antibody used in the study was specific for phosphorylated Src kinase, it did not answer the question about the location of phosphorylated Src observed in this cohort. We therefore stained prostate tumours to assess the localisation of activated Src in a different tumour type. In prostate cancer the majority of staining observed for phosphorylated Src was located to the membrane and nuclear expression was rarely observed.

These results suggest that the lack of membrane staining and high level of nuclear staining observed in the current study was associated with our ER positive breast cancer cohort, and was not a characteristic of the antibody used. However the Y^{416} sequence is highly conserved amongst the src kinases so this does not exclude detection of other src family kinases along with c-Src using this antibody. Our detection of nuclear c-src expression and activation is in line with recent literature as c-Src has been reported both within the nucleus and nucleolus [200, 201] of other solid tumours. Previous IHC work demonstrated that in non malignant breast cells c-Src is distributed within the cytoplasm, whereas in malignant breast cells the majority of c-Src appears concentrated around the nucleus [202].

In this present study we found that high levels of activated c-Src was present in over 50% tumour specimens analysed and nuclear activated c-Src was significantly associated with improved overall survival and decreased recurrence. Ito *et al* [131] also found that elevated activated cSrc was inversely correlated with biological aggressiveness in 73 breast cancer specimens and suggested that c-Src may have an important role in malignant transformation of breast cells rather than malignant progression. Madan *et al* [203] subsequently demonstrated that c-Src activation did not correlate with the development of invasive tumour properties but correlated with malignant transformation. In ductal carcinoma *in situ,* activated c-Src was found to correlate with high tumour grade, high proliferation and HER 2 positivity, suggesting that high cSrc activity may identify a subset of DCIS at risk of disease progression to invasive carcinoma[204].

The body of evidence does, however, still support a role for c-Src in malignant progression. Compared with adjacent normal tissues, elevated Src expression and/or activity has been reported in a wide range of tumour types, including breast cancer [202] and in many of these tissues, an increase in Src activity correlates with disease stage or malignant potential [205,

206]. Tumour cell lines possessing elevated Src activity are often highly metastatic, displaying an increased capacity for migration and invasion in vitro [207-209] .

Recent in vitro breast cell line work, demonstrate over expression and over activity of Src during the acquisition of tamoxifen resistance in ER+ cell line[190, 196] . Src inhibition was seen to significantly reduce the invasive behaviour of cells. Hiscox et al found elevated Src kinase activity in endocrine resistance models was independent of Src gene or protein level. Tamoxifen resistance may be either de novo (present before tamoxifen treatment) or "acquired". In this present study all analysis was performed on tumour samples taken prior to tamoxifen treatment and whilst we do not find that active c-Src correlates with *de novo* endocrine resistance it is interesting that within our cohort the survival benefit was only in ER+/PgR+ patients and not in the ER+/PgR negative group. PgR expression is a marker of a functional ER and a number of laboratory studies have demonstrated the importance of molecular characteristics such as PgR and HER2 in predicting tumour response to endocrine therapy. We have previously reported that ER+/PgR negative tumours are more likely to relapse on tamoxifen [172] and a number of other laboratory studies report a reduction in PgR expression in ER+ cells is consistent with acquired tamoxifen resistance[210]. It is therefore possible that in tumours with a functioning ER (ER+/PgR+) "active" cSrc is in the nucleus and not able to perform its role in promoting tumour progression. Tumours acquiring tamoxifen resistance over time have an adaptive change in growth factor signalling (such as a reduction in PgR expression, increased EGFR expression), therefore Src kinase being downstream of such signalling networks may not become fully active until later during the development of tamoxifen resistance. High levels of activated cSrc expression in the cell cytoplasm have been reported in recurrent breast carcinoma samples [196], although expression was not compared with the primary tumour sample. Comparison of primary breast tumour cSrc expression with expression in recurrent or metastatic tumours following

endocrine resistance would be a preferable model. Within our laboratory we have examined this in prostate cancer specimens. In hormone sensitive prostate cancer active cSrc was associated with improved survival but after development of hormone therapy resistance, active cSrc was associated with reduced time to death (unpublished data).

It is also likely that our patient cohort represents a good prognostic group and that the aggressive phenotype associated with Src kinase is limited to poor prognostic cancers. Indeed Finn et al [197] recently reported a highly significant relationship between breast cancer cell line sub type based on gene expression of cytokeratins and sensitivity to src kinase inhibition, suggesting that the "triple negative" breast cancers were most likely to benefit from Src inhibition. ER negative tumours correlate with poor tumour differentiation, high proliferation rate and other unfavourable characteristics, and are in general considered a more aggressive breast carcinoma. An inverse correlation between Src and ER levels has been reported, ER negative primary breast cancer and cell lines showed increased Src levels and/ or activity compared to ER + cancers [211] .

In conclusion, we found elevated levels of activated cSrc within the cell nucleus of ER+ breast cancer was associated with improved patient outcome in a large cohort of Tamoxifen treated ER positive patients. Although we are unable to substantiate the in vitro studies suggesting a role for c-Src in tamoxifen resistance we feel that further clarification defining the role of cSrc in the different subtypes of breast cancer, particularly in ER negative breast cancer and recurrent tumours, is warranted as this likely represents the group in which targeted Src Kinase inhibition may be beneficial to patient outcome.

7 Closing Discussion and Conclusion

The focus of this research was ER+ breast cancer and targeting patient therapy in this heterogeneous group. This work attempts to translate the biology of the ER and cell signalling interactions to aid the correct identification of patients for both current therapy and more novel therapeutic approaches.

This work supports the hypothesis proposed by Cui et al [45] that PgR down regulation reflects enhanced growth factor signalling pathways. In ER+ patients high NIS expression demonstrated in two separate cohorts has a negative impact on patient outcome. NIS expression in ER+/PgR+ was not associated with poor outcome, but highly significant in ER+/PgR-, in addition NIS expression was found to significantly correlate with MAPK and PI3K pathways, both signalling pathways up regulated in carcinogenesis and implicated in endocrine resistance in ER+ breast cancer. We anticipate that reporting of these findings may give insight into NIS regulation in ER+ breast cancer and give further clues to cellular events that may be important to cell trafficking and function of NIS in breast cancer.

This research proposes that the combined endocrine receptor (CER) may give insight into the function of the ER and ER signalling pathways. It utilises IHC expression level of both the ER and PgR and is based on the hypothesis that high PgR expression in ER+ tumours represents an intact/ functional (genomic) ER signalling pathway. Reduced expression of either the ER or PgR is a marker of up-regulation of cell signalling pathways involved in carcinogenesis which also influence ER expression and function via non-genomic actions. Translating this hypothesis in terms of endocrine response- tumours with a high level of ER genomic function derive maximal inhibition (and benefit) from therapies targeting this, such as ER-antagonist tamoxifen or via deprivation of its primary ligand oestrogen as they have little in the way of escape pathways. In contrast tumours with impaired response still have a

functioning ER and derive benefit from endocrine therapy, but they also have basally active growth factor receptor or other cell signalling pathways that via bidirectional crosstalk and following endocrine therapy can be upregulated and may eventually offer an escape mechanism. Potential escape mechanisms include- downregulation of the ER so it is no longer available (loss of ER expression) or via alterations to the ER rendering it un-inhibitable/ 'blocked' whilst still being detectable in the cell, or perhaps the up-regulation of these pathways, which also promote survival, proliferation and metastasis, as a result of endocrine therapy pushes the balance towards a tumour cell that is so virulent that inhibition of the ER is no longer sufficient to adequately restrain its replication. There is a wealth of ongoing research examining endocrine response / resistance. The development of large-scale computational and genetic approaches offer a potential means of identifying key mediators [70]. The CER appears to give a better indication of endocrine response than either the ER or PgR independently in this cohort, therefore, imminently more important than hypothesising why this may be at the cellular level, will be retesting and validation in another cohort and reporting of results.

For the foreseeable future IHC is likely to remain the primary method of hormone receptor testing and the aim was to develop a score that represents tumour biology and may differentiate levels of risk in early ER+/HER2 with low tumour burden , a group of patients that pose a real challenge to clinicians. The Clinical Outcome Score (COS) is intended to represent a pragmatic equivalent of gene prognostic signatures in which oestrogen receptor signalling pathway, proliferation and HER2/ growth factor receptor activation pathways are indicated to be highly represented in. COS needs to be validated, preferably in combination with a gene prognostic profile analysis if funding where available.

Whilst optimistic regarding these exploratory results we have to remain open minded regarding how they will be received/ criticised for lack of a mathematical model in the

original planning and prepare an answer for why it is any surprise that applying a combination of prognostic factors is highly significant. However COS is based on three key pathways heavily implicated in gene prognostic profiles, and our exploratory results defend themselves- the clinical outcome score appears to be an excellent method of discriminating early ER+/HER2 negative tumours who are at risk as a result of tumour biology. It is anticipated that this when used in combination with tumour burden may help identify risk in this heterogeneous group and guide adjuvant therapy.

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